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IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE CARBOHYDRATE RECOGNITION MOLECULES IN THE LEECH NERVOUS SYSTEM

presented by

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IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE CARBOHYDRATE RECOGNITION MOLECULES IN THE LEECH NERVOUS SYSTEM

By

Robert Norman Cole

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE CARBOHYDRATE RECOGNITION MOLECULES IN THE LEECH NERVOUS SYSTEM

By

Robert Norman Cole

Carbohydrate recognition is involved in patterning of the leech nervous system. We have characterized several potential carbohydrate recognition molecules in the leech. This thesis describes two of these molecules, a 130 kD glycoprotein expressed on glial cells and a lactose-binding protein expressed on a subset of neuronal and epithelial cells.

In the first project, the glial-specific 130 kD glycoprotein was used as a marker to show that glial processes project along key sites of morphogenic movement and neuronal differentiation during leech neurogenesis.

While carbohydrate recognition involves a glycoconjugate binding to a carbohydrate-binding protein (lectin), there is no information on leech lectins. Therefore, the second project focuses on the isolation of two leech lectins from leech membranes that bind lactose, have molecular weights of 35 kD and 63 kD, and are termed LL35 and LL63. LL35 and LL63 are related immunologically, but have different sugar affinities and tissue distributions. Because the nervous system contains LL35, but not LL63, I concentrated on characterizing LL35. LL35's saccharide binding activity was calcium-independent, active over a wide pH range, and sensitive only

to galactose derivatives, with strong preferences for beta-galactosides. Detergent and reducing agents were necessary to insure complete extraction and retention of LL35 binding activity. These characteristics are similar to the L30 class of calcium-independent galactose-binding lectins first described in vertebrates, but which recently have been described also in the invertebrate class Nematoda.

LL35 is developmentally regulated. It is most prominent on a small subset of neurons in the embryo. In the adult, LL35 is most prominent on epithelial cells. In the adult, LL35 also is expressed on muscle cells present in the central nervous system (CNS). Muscle cells in various peripheral organs are devoid of LL35. Because LL35 is selectively expressed on a neuronal subset, it may mediate axonal projections during development. LL35 presence solely on CNS muscle but not on peripheral muscles supports the suggestion that LL35 is involved with nervous system-specific functions. Because up to 100 ug of LL35 can be isolated from one leech, the function of this L30-like, lactose-binding lectin can be readily studied in this classical neurobiological system.

DEDICATION:

For Father. I wish you were here.

ACKNOWLEDGEMENTS

It may sound like a cliché, but it is true. I can not begin to express my immense joy, excitement, gratitude, and even relief in finally completing my Ph.D. If not for Dr. Birgit Zipser, my mentor, I would never had achieve it. She rescued me from certain academic death at the University of Maryland. She gave me a new opportunity, unyielding support and encouragement to excel, and a new direction in research. I am forever thankful.

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I owe a special thank you to Dr. John Wang, in effect my second mentor and sixth committee member. His many hours of technical advice and guidance made my thesis project possible. His knowledge and enthusiasm for carbohydrate-binding proteins sparked my curiosity in and pursuit of postdoctoral training in the field of glycobiology.

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I especially want to thank Michelle Erhart for her heroic help in pulling all the pieces together in the final throws of assembling my thesis and for not making me sing karaoke. Shi, shi.

And finally I want to thank my mother, sisters, brothers, nieces, and grandfather for their love and constant moral support during my quest for a Ph.D., even though it did not take an eternity.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2Me	2-mercaptoethanol
aMeGal	alpha-methyl-galactose
aMeMan	alpha-methyl-mannose
ASF	asialofetuin
bMeGal	beta-methyl-galactose
CNS	central nervous system
cpm	counts per min
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(beta-aminoethyl ether)
FCS	fetal calf serum
Gal	galactose
GalNAc	N-acetyl-galactosamine
GalNH2	galactosamine
Glc	glucose
kD	kilodalton
Lac	lactose
LacNAc	N-acetyl-lactosamine
LL16	Leech Lectin 16

LL35	Leech Lectin 35
LL63	Leech Lectin 63
mAb	monoclonal antibody
Man	mannose
MOPS	3-[N-morpholin]propane sulfonic acid
Mr	apparent molecular weight
MW	molecular weight
PAGE	polyacrylamide electrophoresis
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
TX-100	Triton X-100

INTRODUCTION

Interactions between carbohydrate moieties of proteins or lipids (glycoconjugates) and carbohydrate-binding proteins (lectins) have been demonstrated to mediate many non-neuronal recognition events. For example, the hepatic clearance of serum asialoglycoproteins (Ashwell and Harford, 1982; Ashwell and Morell, 1974) the binding of sperm to oocytes (Shur, 1989; Wassarman, 1989), the compaction and uterine implantation of developing embryos (Bayna et al., 1988; Dutt et al., 1987), the attachment of lymphocytes to postcapillary venules in select organs [lymphocyte homing; (Stoolman et al., 1987; Yednock and Rosen, 1989)] and the initiation of bacterial infection or phagocytosis (Ofek and Sharon, 1988; Sharon, 1987) involve carbohydrate recognition. Surface glycoconjugates on glial, nerve and muscle cells also have been implicated in mediating neuronal recognition events during the formation of the nervous system. This suggestion originates from the finding that glycosyltransferases on the surface of chick retinal cells can behave as receptors for oligosaccharides (Roseman, 1970; Roth et al., 1971). Since then, exogenous plant lectins, with affinities for specific carbohydrate structures, and antibodies, generated against glycoconjugates or previously identified lectins in non-neuronal systems, have been used to demonstrate the restricted expression of cell surface glycoconjugates and their potential

carbohydrate-binding proteins on subsets of neurons (Bastiani et a 1987; Regan et al., 1986; Scott et al., 1990; Stewart and Touloukia 1990), growth cones (Dodd et al., 1984; Pfenninger et al., 1984; Reichert and Meier, 1990), and at synaptic sites (Groswald and Kel 1984; Grunwald et al., 1985; Sanes and Cheney, 1982; Scott et al., 1990; Scott et al., 1988). These observations imply a role for surface glycoconjugates and carbohydrate-binding proteins in neuronal patterning. Temporal correlations between the developmental periods of synapse formation and axonal growth and the expression of glycoconjugates or putative carbohydrate-binding proteins also have been used as evidence for their involvement in synapse formation (Bezamahouta et al., 1988; Denburg, 1989; Dontenwill et al., 1985; Zanetta et al., 1978). In addition, neuronal migration and axonal growth on glycoconjugate substrates of laminin and fibronectin *in vitro* can be mediated by glycosyltransferase activity (Runyan et al., 1986; Runyan et al., 1988) and inhibited by lectins (DeGeorge and Carbonetto, 1986). These observations constitute strong, although indirect, evidence for the involvement of carbohydrate interactions during the development of the nervous system.

In contrast, the leech embryonic nervous system presents a model wherein the role of carbohydrate recognition during neuronal pattern formation can be directly examined. The leech embryo provides a serial collection of developmental time points due to the anterior to posterior developmental gradient of its 32 segmentally reiterative ganglia (Fernandez and Stent, 1982; Weisblat et al., 1980). As in vertebrate systems, the leech nervous system

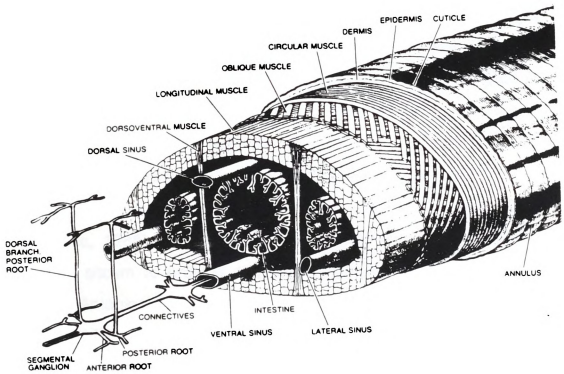
expresses cell-type specific surface glycoproteins that define subsets of processes (Hockfield and McKay, 1983; McKay et al., 1983; Peinado et al., 1987a; Peinado et al., 1990; Zipser and McKay, 1981) distinguished by their carbohydrate epitopes (Bajt et al., 1990). The developing leech nervous system in cultured embryos recently has been demonstrated to be readily permeable to macromolecules (Zipser and Cole, 1991; Zipser et al., 1989). Thus, using the embryonic leech nervous system, carbohydrate recognition can be directly investigated through macromolecular manipulation (e.g. antibodies, lectins, enzymes) of a virtually intact nervous system in its normal microenvironment. At present, this is not possible in most vertebrate model systems.

The leech nerve cord represents the central nervous system of the leech. It is enclosed within the blood sinus that lies ventral to the alimentary canal (Figure 1) and is composed of a chain of ganglia containing nerve, glia, and muscle cells suspended in a dense network of connective tissue. The entire nerve cord is covered by a continuous layer of squamous epithelia cells (Coggeshall and Fawcett, 1964). We currently are characterizing several potential carbohydrate recognition molecules in the leech. My thesis describes two of these molecules, a 130 kD glycoprotein expressed on glial cells and a lactose-binding lectin expressed on a subset of neuronal and epithelial cells.

I. The Glial Glycoprotein.

The glial 130 kD glycoprotein is a member of a family of 130 kD surface glycoproteins that are specific to different cell types

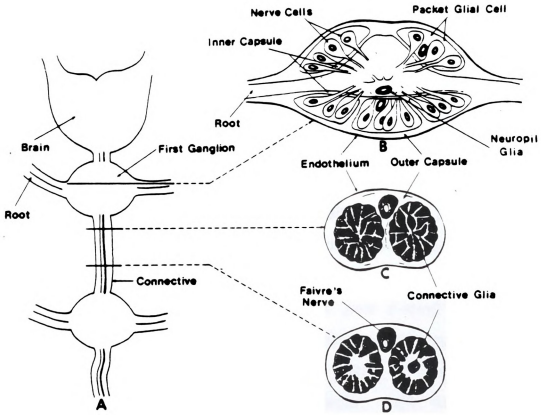
Figure 1. Diagrammatic representation of the relative position of the leech nerve cord within the blood sinus lying ventral to the intestine. Dorsal is up. (from Nicholls and Van Essen, 1974. Copyright (1974) by Scientific American, Inc.).


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

within the leech nervous system. The observation that these glycoproteins can be distinguished by their carbohydrate moieties suggests that the functional regions of these molecules are located in their glycans. In the adult leech, the 130 kD glial glycoprotein identified by monoclonal antibody (mAb) Laz6-297 is regionally restricted to glial processes associated with axon tracts (Flaster and Zipser, 1987). It is not expressed by glial cell bodies or by the packet glial cells, which envelope only the cell bodies of ganglionic neurons and lack processes (Figure 2). These observations suggest that the glial glycoprotein may be involved in axon tract formation. Glial cells are known to direct cellular differentiation and morphogenic movement during neurogenesis (Hatten and Mason, 1986; Rakic, 1985). Their surface glycoproteins and gangliosides have been shown to provide adhesive substrates for neuronal migration (Antonicek et al., 1987; Edmondson and Hatten, 1987; Lindner et al., 1986; Mendez-Otero et al., 1988) and differentiation (Denis-Donini et al., 1984; Mudge, 1984) and they may guide axons by promoting neurite extension (Fallon, 1985; Schreyer and Jones, 1987; Silver and Rutishauser, 1984; Tomaselli et al., 1986) and axonal branching (Chamak et al., 1987), or by inhibiting neurite growth (Caroni and Schwab, 1988).

If the leech glial glycoprotein is involved in axon tract formation, it must be expressed in the embryo during neurogenesis. The first part of my thesis (Chapter 1) describes the onset of expression of the glial glycoprotein at key sites of morphogenic movement and neuronal differentiation during the development of

Figure 2. Diagrammatic representation of the leech nerve cord illustrating the general organization of the leech central nervous system. (A) The head ganglion (brain) and the first two segmental ganglion are shown. Axons project between adjacent ganglia through the connectives and between a ganglion and the peripheral tissues via the roots. (B-D) Sections of the nerve cord show the glial cells in relation to other structures. The glial nuclei are shown only for two packet glial cells, one neuropile glial cell, and one connective glial cell. The root glial cell is not indicated. Dorsal side is up in B, but down in C and D. (from Kuffler and Potter, 1964. Copyright (1964) by The American Physiological Society.)

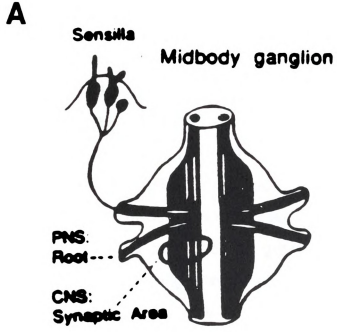

Nerve Cells
Packet Glial Cell
Inner Capsule
Root
Brain
First Ganglion
B
Neuropil Glia
Endothelium
Outer Capsule
Root
C
Connective
Faivre's Nerve
Connective Glia
A
D

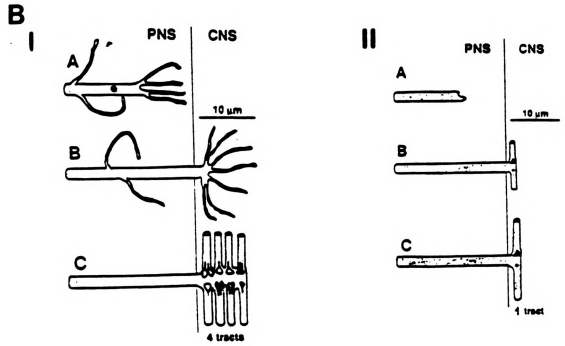
the nervous system in the leech embryo and has already been published (Cole et al., 1989).

II. Leech Lectins.

A functional group of neurons, the sensory afferents, are identified by their expression of a mannose-containing epitope identified by the mAb Lan3-2 (Hockfield and McKay, 1983; McKay et al., 1983; Peinado et al., 1990; Zipser and McKay, 1981). Other mAbs, generated against leech carbohydrate structures, reveal subsets of these neurons that are chemically-coded with different carbohydrate epitopes (Bajt et al., 1990; Peinado et al., 1987a; Peinado et al., 1990). Cell bodies of the sensory afferents are located either singly or clustered into sensillae in the annuli of each body segment or in the intestinal tract endothelium (Hogg et al., 1983; Phillips and Friesen, 1982; Stewart et al., 1985). During development, axons from these cell bodies grow through the peripheral tissues in a fasciculated tract towards the ganglia of the ventral nerve cord (Figure 3A) (Hogg et al., 1983; Johansen et al., 1985; McKay et al., 1983; Phillips and Friesen, 1982; Stewart et al., 1985; Zipser et al., 1989). A fine spray of neurites surround the growing tip, apparently sampling the environment as elongation progresses (Figure 3BI) (Zipser et al., 1989). Upon entering a ganglion of the central nervous system, the bundle of sensory afferent axons defasciculate, send branches into four separate sensory domains within the synaptic region of the central nervous system, and then regroup and exit anteriorly and posteriorly from the ganglion as a fasciculated tract in the ventro-lateral region

Figure 3. Diagrammatic representation of the growth of sensory afferent axons into the leech ganglion under normal and experimental conditions. (A) The cell bodies of sensory afferents are located either singlely or clustered into sensillae in the body wall (or gut) of the leech. These sensory afferents project axons into the segmental ganglia (CNS) through the peripheral roots. (from Zipser and Cole, 1991. Copyright (1991) by Society for Neuroscience.) (BI) Normally, the sensory afferent axons grow in the periphery (PNS) as a fasciculated tract surrounded by a fine spray of neurites. Upon entering the synaptic region of the ganglion (CNS) the sensory afferents defasciculate and form four distinct synaptic fields extending anteriorly and posteriorly through the ganglion. (BII) In the presence of monoclonal antibody Lan3-2 Fab fragments the sensory afferent axons still grow as fasciculated tract in the PNS, but with the absence of the spray of neurites. Upon entering the CNS, the axons fail to defasciculate and instead form a single anterior to posteriorly directed tract through the ganglion. (redrawn from Zipser et al., 1989. Copyright (1989) by Cell Press.)


A
Sensilla
Midbody ganglion
PNS:
Root
CNS:
Synaptic Area


B
I
PNS
CNS
A
10 µm
B
C
4 tracts
II
PNS
CNS
A
10 µm
B
C
1 tract

interganglionic connectives (Fernandez, 1978; McKay et al., 1983; Zipser et al., 1989). Following a nerve crush injury, regenerating sensory afferents preferentially grow along tracts expressing the Lan3-2 mannose-containing epitope (Peinado et al., 1987b). Exposing the growing sensory afferents to Fab fragments of Lan3-2 inhibits the defasciculation of the sensory afferents within the central nervous system (Figure 3BII). The sensory afferent axons instead remain tightly bundled and continue to grow as a single tract anteriorly and posteriorly through the ganglion (Zipser et al., 1989). Similar results are obtained by exposing growing sensory afferents to N-glycanase, thereby cleaving the N-linked carbohydrate moieties from surface proteins in embryos, or to mannose that was covalently linked to BSA, thereby competing with the Lan3-2 mannose-containing epitope for a putative carbohydrate-binding protein in the embryo (Zipser and Cole, 1991).

These results constitute strong evidence for a carbohydrate recognition event mediating projection of sensory afferents in the leech central nervous system. These data also suggest that the leech nervous system expresses a carbohydrate-binding protein, or lectin. There are no reports on the lectins in the leech. I attempted, therefore, to isolate leech lectins. The second part of my thesis describes the identification and characterization (Chapter 2) of two leech lactose-binding proteins, which have apparent molecular weights of 35 and 63 kD, termed Leech Lectin 35 (LL35) and Leech Lectin 63 (LL63), respectively. Because the nervous system contains LL35, but not LL63, I focused on characterizing LL35 and describing its immunocytochemical distribution in the leech (Chapter 3).

CHAPTER 1. GLIAL PROCESSES, IDENTIFIED THROUGH THEIR GLIAL-SPECIFIC 130 kD SURFACE GLYCOPROTEIN, ARE JUXTAPOSED TO SITES OF NEUROGENESIS IN THE LEECH GERMINAL PLATE

INTRODUCTION

Glial cells can direct cellular differentiation and morphogenic movement during neurogenesis (reviewed in Hatten and Mason, 1986; Rakic 1985). Candidate molecules mediating these glial functions are surface molecules - glycoproteins and gangliosides - or factors secreted into the extracellular space (reviewed in Dodd and Jessell, 1988; Jessell, 1988). Soluble factors secreted by glial cells promote neuronal survival (Barde et al., 1980; Brenneman et al., 1987; Lindsay, 1979; Tanaka and Obata, 1982) and neurite extension (Guenther et al., 1985; Monard et al., 1983; Rudge et al., 1985). They may guide axons by chemotaxis (Gundersen and Barrett, 1980; Letourneau, 1978) or by selectively removing or redirecting neuronal processes (Lefebvre et al., 1987). Surface glycoproteins and gangliosides on glia provide adhesive substrates for neuronal migration (Antonicek et al., 1987; Edmondson and Hatten, 1987; Lindner et al., 1986; Mendez-Otero et al., 1988) and differentiation (Denis-Donini et al., 1984; Mudge, 1984). They also may guide axons by promoting neurite extension (Fallon, 1985; Schreyer and Jones, 1987; Silver and Rutishauser, 1984; Tomaselli et al., 1986) and

axonal branching (Chamak et al., 1987) or by inhibiting neurite growth (Caroni and Schwab, 1988; reviewed in Patterson, 1988).

Studying the developmental function of glia in the leech takes advantage of the glial properties which led to the first electrophysiological description of neuron-glia interactions (Kuffler and Nicholls, 1966; Kuffler and Potter, 1964). The four macroglial cell types of the leech nervous system are large and easily identifiable by their morphological properties at the light and electron microscopic levels (Gray and Guillery, 1963; Coggeshall and Fawcett, 1964; Morrissey and McGlade-McCulloh, 1988). In the segmental chain of 32 ganglia, the glial cells assume stereotypical positions. Fiber tracts extending out of the leech ganglion are enveloped by the connective and the root macroglia. A pair of connective macroglia, wrapping the three axon tracts of the connective extending between ganglia, can be identified by their prominent glial fibrils (Gray and Guillery, 1963) and their internal 77 kD presumptive cytoskeletal protein (Flaster and Zipser, 1987). Root macroglial cells, whose cell bodies occur near the ganglion, extend out the anterior and posterior roots into peripheral nerves. Both the root and the connective glia share an unidentified internal antigen recognized by monoclonal antibody Lan3-5 (Macagno et al., 1983). Three pairs of packet glia envelop the 400 neuronal cell bodies in the cortex of a standard midbody ganglion (Macagno, 1980). Finally, in the core of the ganglion, the single pair of neuropile glia envelops the intraganglionic segment of the connective, the

transverse commissures, and the central synaptic area (Coggeshall and Fawcett, 1964).

Of interest to this study is a 130 kD glial antigen, recognized by monoclonal antibody Laz6-297, that is spatially regulated on leech macroglial cells. This antigen is abundant on all processes of the connective and the root glia, but is not detected on their cell bodies. In the case of the neuropile glial cells, the antigen is abundant only on processes that extend into the intraganglionic connectives and commissures, and along the perimeter of the neuropile, but is not detected on the glial processes that reach into the central synaptic area (Flaster and Zipser, 1987). The glial antigen is absent altogether from the packet macroglia, which lack processes. Thus, this glial antigen is expressed on macroglial processes that are associated with axon tracts. The Laz6-297 glial antigen is one of several 130 kD glycoproteins expressed by different cell types in the leech nervous system. Since the glial antigen is found on macroglial processes of the root, neuropile, and connective, I suggest naming it the RNC protein.

Here, I report the appearance of previously undocumented primordial glial processes identified through their expression of the RNC protein. These glial processes are juxtaposed to sites of early and late neuronal differentiation, morphogenic movement, and axon tract formation.

MATERIALS AND METHODS

Leech embryos and adult nerve cords

Mud leeches, *Haemopis marmorata*, were obtained from Rochester Hollow Grinder (NY), bred at room temperature in aerated aquaria containing dilute artificial seawater (Forty Fathoms; 0.5 gm/liter distilled water), and fed chicken liver three times a week. After four to six weeks, gravid leeches were placed in plastic boxes containing moist sphagnum moss. Boxes were checked daily for cocoons. Once found (day 0), the cocoon was placed in a 120 ml cup with moist sphagnum moss and maintained in an incubator at 20°C ± 1°C.

Monoclonal antibody (mAb) Laz6-297

Laz6-297 was derived by immunizing mice with the 130 kD region of nerve cord extracts excised from polyacrylamide gels (Flaster et al., 1983).

Immunocytochemistry

Embryos of a desired age were removed from their cocoons, opened dorsally to remove yolk, pinned out in a Sylgard-coated Petri dish, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. After fixation, embryos were post-fixed with absolute methanol for 30 min and then rinsed with 0.9% NaCl, 50 mM phosphate buffered saline (PBS). Adult nerve cords used for controls were similarly fixed, then dehydrated and

xylene-extracted. Preserved embryos were incubated overnight in the glial monoclonal antibody (mAb) Laz6-297 supernatant containing 10% fetal calf serum and 0.5% Triton X-100. The staining pattern was visualized using the horseradish peroxidase-conjugated second antibody method (Cappel or Dako Laboratories) or the peroxidase-anti-peroxidase method (PAP, Dako Laboratories). Stained embryos were mounted in Permount (Fisher Scientific Co.). Adult ganglia were incubated with antibodies in the presence of 2% Triton X-100 and then similarly stained and mounted.

Live embryos were stained in the absence of Triton X-100. Freshly dissected embryos were immediately placed on ice and incubated in the Laz6-297 supernatant for 4 h, rinsed with PBS, then placed in the horseradish peroxidase-conjugated second antibody solution for 2 h. After visualization of staining with the diaminobenzidine reaction, embryos were fixed and mounted in Permount.

For immunoblot analysis, nerve cords from adults or embryos were removed from 10% ethanol-anesthetized leeches and extracted by heating ganglia at 100°C from 4 min in sample buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 80 mM Tris, pH 6.8). Extracts were separated on 7.5% acrylamide mini-gels and electroblotted onto nitrocellulose as previously described (Flaster and Zipser, 1987). Immunoblots were probed for the glial 130 kD antigen using Laz6-297 supernatant, iodinated second antibody (goat anti-mouse IgG [ICN]), diluted to 150,000 cpm/ml, and visualized by autoradiography.

Staging embryos

Embryos were staged according to the number of distinguishable neuromeres. Accuracy in counting neuromeres was checked by staining sibling embryos with a mAb against neurons (Lan3-8) (Zipser and Mckay, 1981) or muscle (Lan3-14 and Laz10-1) (Thorey and Zipser, 1991; Zipser and Mckay, 1981). An error of $\pm$ 2 neuromeres was estimated.

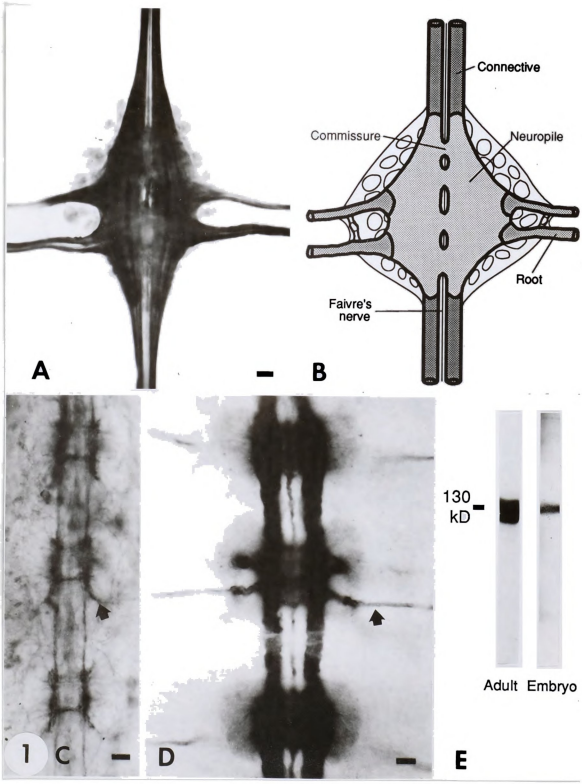
RESULTS

Distribution of the RNC protein on adult and embryonic glial processes

The distribution of the RNC protein was visualized through staining with mAb Laz6-297. Staining an adult ganglion as a wholemount revealed a continuous matrix of glial processes, extending from the ganglionic core into the central and peripheral axon tracts (Figure 4A). As schematically represented in Figure 4B, this matrix is composed of processes from three types of paired macroglial cells. The ganglionic core contains antibody-stained processes of the neuropile glia. Outside the ganglion, the three axonal tracts of the connective are enfolded by the connective glia, and the anterior and posterior ganglionic roots are wrapped by the root macroglia. The packet glia, which envelops neuronal cell bodies in the ganglionic cortex, is unstained. Thus, processes of the root, neuropile, and connective glia express the RNC protein recognized by Laz6-297.

The surface location of the RNC protein is demonstrated by staining glial processes in the live germinal plate of an 8-day embryo, where antibodies have access only to the cell surface and to the extracellular space. Figure 4C shows three successive segments with three primordial ganglia or neuromeres still abutting on one another. A chain of anterio-posteriorly directed glial processes, surface-stained with mAb Laz6-297, links the three successive

Figure 4. The 130 kD glial-specific surface glycoprotein, termed the RNC protein, is expressed on the processes of the root, neuropile, and connective macroglial cells in both adult and embryonic leech ganglia. (A) Staining a whole mount of an adult *Haemopis marmorata* ganglion with mAb Laz6-297 reveals a continuous matrix of stained glial processes surrounded by unstained cell bodies in the ganglionic cortex. Bar represents 50 μm. (B) A schematic representation of the adult ganglion illustrates the distribution of the root, neuropile, and connective macroglial processes in the overall matrix. (C) The surface location of the RNC protein is demonstrated by staining a live 8-day embryo with mAb Laz6-297. Three successive neuromeres show surface-stained root (arrow), neuropile, commissure, and connective processes. Bar represents 10 μm. (D) A miniaturized version of the adult RNC protein distribution in a chain of three ganglia from a 9-day embryo (32 neuromere stage) stained with mAb Laz6-297. Commissures are visible transversing the neuromere between the large bilateral tracts. The dorsal branch of the posterior root is indicated by arrow. Bar represents 10 μm. (E) Immunoblots prepared from adult and embryonic (11-day) ganglia, stained with mAb Laz6-297 and visualized with iodinated secondary antibody, both show a distinct band with an apparent molecular weight of 130 kD. (from Cole et al., 1989. Copyright (1989) by Alan R. Liss, Inc.)


A
B
Connective
Commissure
Neuropile
Root
Faivre's nerve
C
D
E
130 kD
Adult
Embryo

neuromeres. Inside the neuromeres, glial processes form transverse commissures. Other processes are already extending laterally away from the ganglia into peripheral roots. In this germinal plate wholemount, intraganglionic axonal tracts are clearly visible; they are obscured in wholemounts of adult ganglia by the glial processes outlining the perimeter of the central neuropile. Thus, glial processes can be detected in developing axon tracts of the leech nervous system through their RNC surface protein.

Staining the germinal plate of a 9-day embryo (32 neuromere stage) with Laz6-297 (Figure 4D) reveals a miniaturized version of glial processes that is representative of the adult leech nervous system (Figure 4A). A chain of segmental ganglia is linked by the three fiber tracts of the connective. Prominently stained glial processes envelop the large bilateral tracts and the medial tract (Faivre's nerve) of the connective along its inter- and intraganglionic course. Two antibody-stained peripheral roots emerge from each side of the ganglion and branch into several peripheral nerves en route to the segmental organs. Only the dorsal branch of the posterior root (arrow) is shown in this focal plane. The absence of any other staining with Laz6-297 in this wholemount of a germinal plate demonstrates the unique distribution of the RNC protein on glial processes.

The adult and the embryonic RNC proteins have similar molecular weights as well as distributions. On immunoblots, the RNC protein extracted from adult and embryonic (11-day, 32 neuromere stage) ganglia occurs in a broad band, with the apparent

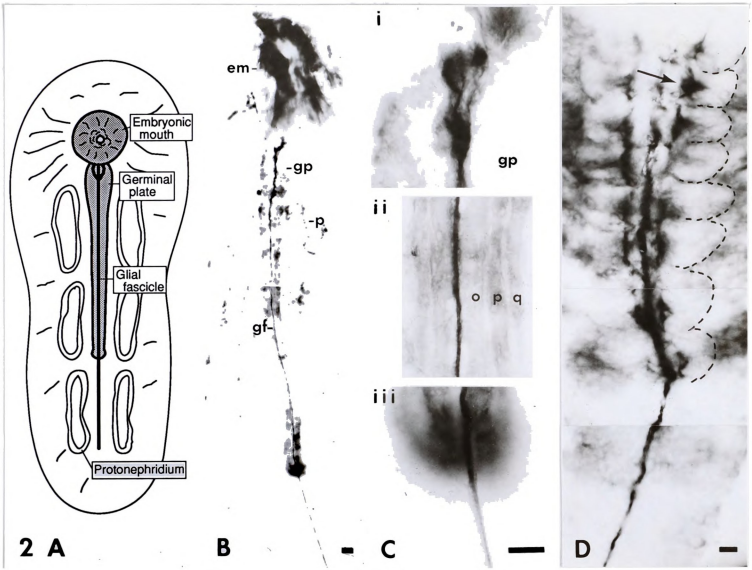
molecular weight of 130 kD (Figure 4E). I used this RNC protein as a developmental marker to identify glial processes in the leech germinal plate.

Primordial glial fascicle along the embryonic midline

Staining a 5-day-old embryo (0 neuromere stage) with Laz6-297 led to the detection of a previously undocumented primordial glial fascicle along the embryonic midline, illustrated in a diagram (Figure 5A) and in a low power micrograph (Figure 5B). It extends from the embryonic mouth through the ventral germinal plate and out onto the embryonic envelope, as far as the posterior border of the last pair of protonephridia. Higher magnification (Figure 5C) shows that just below the embryonic mouth the glial fascicle has separated into three separate strands joined at the anterior edge of the germinal plate. In this 5-day embryo, the germinal plate consists of five pairs of bandlets, generated by the unequal cell divisions of five pairs of teloblasts, symmetrical to the glial fascicle (Figures 5A and 9). As the teloblasts are adding stem cells to the bandlet, they appear to be sliding posteriorly with respect to the glial fascicle. The N teloblast and its n-bandlet, which produces the majority of the central ganglionic neurons (Weisblat et al., 1984), straddle the midline (Fernandez and Stent, 1982) demarcated by the glial fascicle, while the other four bandlets lie laterally (Figures 5C and 9).

Staining an 8-day embryo (8 neuromere stage) with Laz6-297 demonstrates a novel spatial relationship between the primordial

Figure 5. Prior to neurogenesis, a primordial glial tract expresses the RNC protein along the ventral midline of the embryo. This fascicle separates into three columns outlining the future neuropile during neuromere formation. (A) A schematic representation of the 5-day (0 neuromere stage) embryo, presented in B, illustrates the relative position of the midline glial fascicle stained with mAb Laz6-297. (Redrawn from Fernandez and Stent, 1982). (B) Midline glial fascicle in 5-day (0 neuromere stage) embryo. em, embryonic mouth; gf, glial fascicle; gp, germinal plate; p, protonephridia. Bar represents 20 μm. (C) The midline glial fascicle is shown at higher magnification in the (i) anterior, (ii) medial, and (iii) posterior regions of the germinal plate from the same embryo in Fig. 5B. (i) Anteriorly, the midline glial fascicle has begun to separate into three processes just below the embryonic mouth. (ii) It is fasciculated along the rest of the germinal plate. Three of the five pairs of germinal bandlets (o, p, q) forming the germinal plate are shown. The n-bandlet straddles the midline and lies dorsal to the o-, p-, and q-bandlets. (iii) Posteriorly, the fascicle extends out past the teloblasts (not in focus) onto the embryonic envelope. Bar represents 10 μm. (D) An 8-day embryo (8 neuromere stage), stained with mAb Laz6-297, shows the spreading of the midline glial fascicle into three processes during aggregation of neuroblasts into neuromeres. The right side of the eight neuromeres is outlined. The glial processes thicken and are in contact with the medial aspects (arrow) of each neuromere and outline the future neuropile. Where the glial processes remain fasciculated, neuromeres have not formed yet. Bar represents 10 μm. (from Cole et al., 1989.

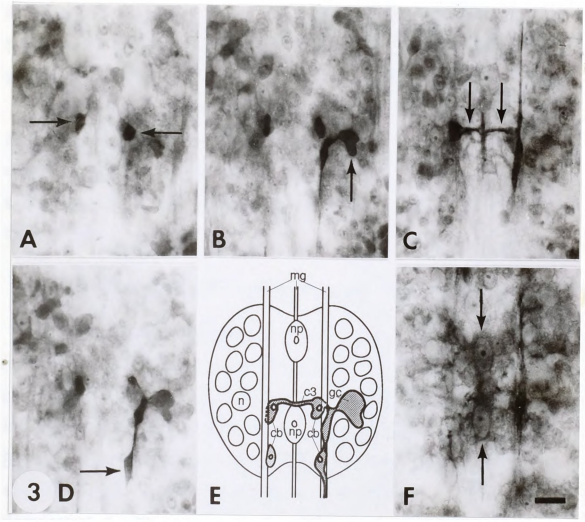

Embryonic mouth
Germinal plate
Glial fascicle
Protonephridium
2 A
em
gp
p
gf
B
i
gp
ii
o p q
iii
C
D

glial fascicle and the developing, segmentally-reiterated neuromeres (Fig 5D). Eight neuromeres are apparent through the aggregation of neuroblast stem cells. As far down as the eighth neuromere, the glial fascicle has separated into three separate processes. One process remains at the midline, where it broadens. The two other tracts spread apart and contact the medial aspects of the primordial hemiganglia. These processes outline the future neuropile, into which the cortically-arranged cell bodies will project their primary axons.

Novel primordial glial cells projecting along the future axon tracts

As development proceeds, other primordial glial cells express the RNC protein at low levels on their processes and cell bodies. This distinguishes them from the adult macroglial cells, which lack the RNC protein on their cell bodies (Figure 4A and B). The growth of these primordial glial processes coincides with the formation of the future central and peripheral axon tracts. In neuromere 15 of a 9-day embryo (20 neuromere stage), a pair of cell bodies lies ventral and medial to the lateral glial tracts (Figure 6A and D). A broad, paddle-like growth cone is present, searching towards the periphery along the presumptive future peripheral root (Figure 6B). Other, thinner glial processes already have elongated inside the central tracts. For example, these paired cell bodies also send processes, first dorsally, then medially, joining at the midline to form the third commissure (Figure 6C). A second pair of glial cell bodies resides dorso-medially to the lateral glial tracts in the posterior half of the

Figure 6. Novel primordial glial cells express the RNC protein on both their cell bodies and processes, and project broad veils along the future axon tracts. Micrographs are of a 9-day embryo (20 neuromere stage) stained with mAb Laz6-297. A-D are different focal planes of neuromere 15. E is a composite drawing of A-D. F is neuromere 11 from the same embryo. (A) Cell bodies (arrows) of primordial glia are located ventral and medial to the lateral glial tracts. (B) In a more dorsal plane of the same neuromere, a broad, paddle-like growth cone (arrow) from a primordial glia searches toward the periphery. (C) In the dorsal-most plane of this neuromere, other processes from the primordial glia, depicted in A, join at the midline to form the third commissure (arrows). (D) Another primordial glial cell body (arrow) is expressing low levels of the RNC protein, and is located dorsal and medial to lateral glial tracts. Its processes project anteriorly and posteriorly along the right lateral glial tract. (E) A composite drawing of three primordial glial cells and their processes in neuromere 15. c3, commissure 3; cb, cell body; gc, growth cone; n, neuroblast; np, presumptive neuropile glia. (F) Unstained cell bodies of the presumptive neuropile marcoglia (arrows). Bar represents 10 μm. (from Cole et al., 1989. Copyright (1989) by Alan R. Liss, Inc.)


A
B
C
3 D
mg
np
n
c3
gc
cb
np
cb
E
F

neuromere; only one of the pair is shown (Figure 6D and E). They project anterio-posteriorly, following the lateral processes of the original primordial fascicle. Thus, the temporo-spatial appearanceof primordial glia, detected by the RNC protein, illustrates the sequence of axonal tract maturation. The central tracts, both longitudinal and commissural, are initiated prior to the peripheral tracts.

Framed by primordial glial processes are two large unstained oval cells (Figure 6F, neuromere 11), the presumptive neuropile macroglial cells. The subsequent maturation of adult macroglial cell processes in commissures and peripheral roots is revealed in older embryos.

The temporo-spatial formation of the adult commissures

Adult macroglial processes with the RNC protein are detected in the 9-day embryo (27 neuromere stage; Figure 8A). The proliferation of these adult neuropile glial processes was an indicator for the maturation of the intraganglionic commissures [described by Fernandez (1978)]. The four adult glial commissures express the RNC protein and arise in a temporal sequence, demonstrated with respect to the different developmental ages of neuromeres 18, 20, and 24 (Figures 7A and 8A). Glial commissure three appeared first in the posterior, developmentally youngest, neuromere 24. In neuromere 20, glial commissure two has formed, and glial commissure one is just beginning to appear. Glial commissure three has enlarged further. Glial commissure four is

Figure 7. A schematic representation of glial processes forming the commissures and roots is shown. In this and the following figure, spatial relationships are preserved, with the youngest neuromere most posterior. (A) The developmental sequence of the glial commissural processes. (iii) Commissure 3 is the first to appear. (ii) Next, commissure 2 develops, followed by commissure 1. (i) In the most mature neuromere, all four commissures are present. (B) The formation of the anterior root. (ii) Initially, three embryonic roots emerge from the neuromere. (i) As the neuromere matures, the first two embryonic roots merge at the lateral border of the neuromere, then separate into the anterior branch and the medial branch. AA, anterior branch of the anterior root; AR, anterior root; DP, dorsal branch of the posterior root; PP, posterior branch of the posterior root; PR, posterior root. (from Cole et al., 1989.

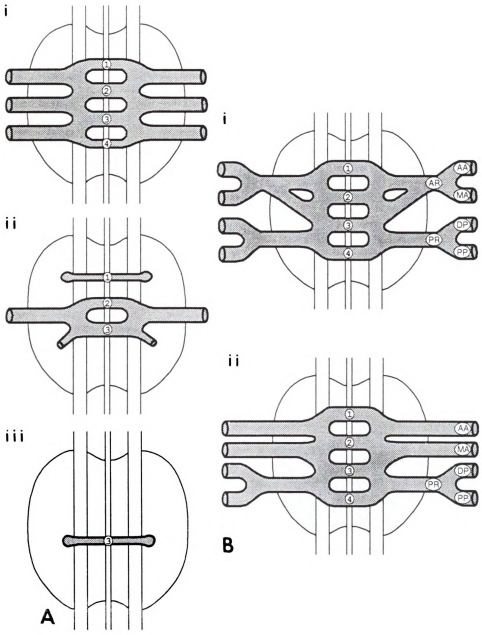
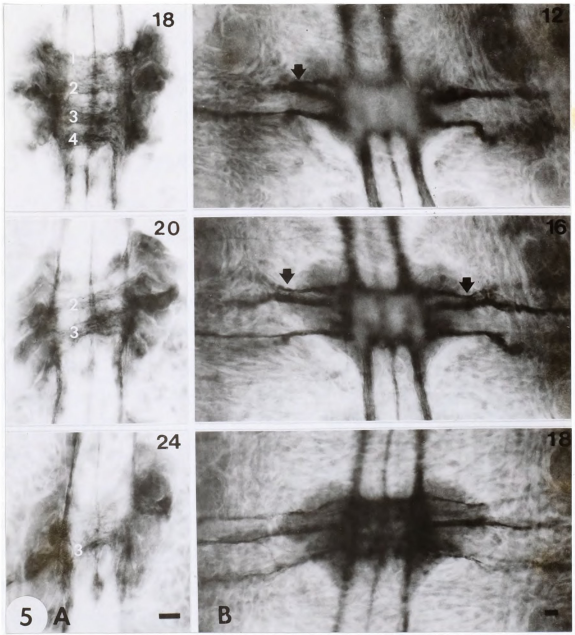

i
ii
iii
A
i
ii
B
AA
AR
MA
MP
PR
PP

Figure 8. The temporo-spatial formation of glial commissural and root tracts. (A) The maturation of the glial commissures (numbered) represented in three neuromeres (18, 20, 24) of a 9-day embryo (32 neuromere stage) stained with mAb Laz6-297. (B) The formation of the anterior root shown in three neuromeres (12, 16, 18) of an 11-day embryo (32 neuromere stage) stained with mAb Laz6-297. The arrows mark the merging point of the anterior and medial branches of the root glia. Bars represent 10 μm. (from Cole et al., 1989.)


18
1
2
3
4
12
20
2
3
16
24
3
18
5 A
B

the last to develop; it is seen only in neuromere 18, the most anterior and mature of the three neuromeres depicted. As the glial commissures thicken, they meet laterally and extend towards the periphery as three peripheral roots. Glial commissures one and two are continuous with an anterior root, glial commissures two and three enter a medial root, and glial commissures three and four merge to form the posterior root (Figures 7 and 8).

Formation of the adult anterior root

The restructuring of the embryonic roots was followed by staining glial processes with Laz6-297 in three neuromeres of different developmental ages (Figures 7B and 8B: 11-day embryo, 32 neuromere stage). In the youngest neuromere, neuromere 18, three pairs of roots exit into the periphery (Figure 8B, neuromere 18). In neuromere 16, the anterior and medial roots begin to merge at the lateral border of the neuromere (Figure 8B, arrows). By neuromere 12, the anterior and the medial roots have joined into one anterior root.

Laz6-297 staining reveals the peripheral divisions of the anterior and the posterior roots depicted diagrammatically in Figure 7B. The adult posterior root splits in the periphery into a dorsal posterior and a posterior posterior branch and is already formed in the youngest neuromere shown, neuromere 18 (Figure 8B). The two embryonic roots, merging into the anterior root (neuromere 12 in Figure 8B), separate again in the periphery into an anterior anterior branch and a medial anterior branch (not in focal plane).

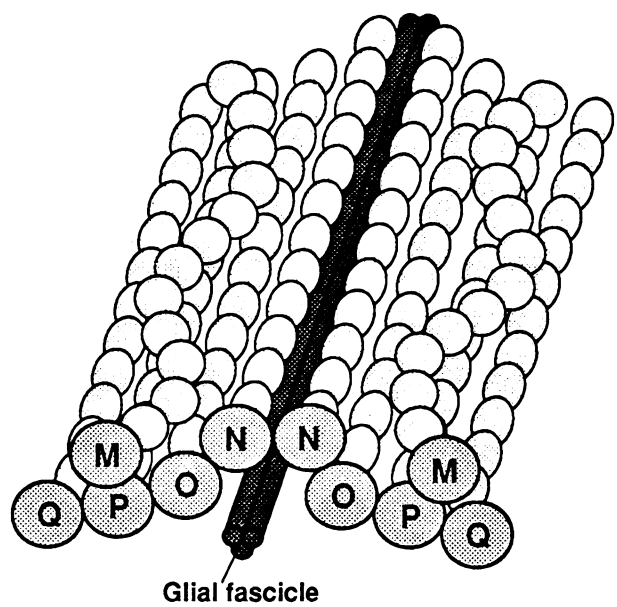
DISCUSSION

Embryonic glial processes were detected by their expression of the RNC surface protein. I used this RNC protein, a glial specific 130 kD glycoprotein, as a developmental marker to map the spatio-temporal appearance of glial processes in the leech germinal plate. I report that embryonic glial processes are associated with teloblast movement, neuronal differentiation, and axon tract formation. The RNC surface protein allows the identification of glial processes prior to the proliferation of adult macroglia, which express previously reported internal antigens only after axonal tracts have formed (Macagno et al., 1983; Morrissey and McGlade-McCulloh, 1988). The early expression of the RNC surface protein suggests its participation in neurogenesis.

Position of the primordial glial fascicle during the teloblast/bandlet stage

Prior to neurogenesis, a glial fascicle appears along the primary axis of symmetry in the embryo. It is present during the morphogenic movement of the five pairs of teloblasts that create the germinal plate. The glial fascicle is centered symmetrically between two bilateral rows of teloblasts, each producing a cohesive bandlet of stem cells through unequal divisions (Figure 9). Since the embryonic mouth and the glial fascicle present an anterior and a midline boundary to the growing bandlets, the teloblasts are displaced posteriorly each time they divide. The

Figure 9. A schematic representation of the formation of the germinal plate from five bilateral pairs of teloblasts (M-Q) in relation to the midline glial fascicle. Each teloblast produces a bandlet of stem cells by unequal cleavage. The n-bandlet straddles the midline and is dorsal to the o-, p-, and q-bandlets. The m-bandlet has a zig-zag arrangement due to spiral divisions of the M-teloblast and lies dorsal to the o-, p-, and q-bandlets (Fernandez and Stent, 1982). The midline glial fascicle may guide teloblast movement, as the teloblasts produce stem cell bandlets. Since the closer a bandlet is to the glial fascicle, the greater the number of central neurons it produces, the glial fascicle also may release a factor to induce neuroblast development. (from Cole et al., 1989.


M
N
N
M
Q
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O
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Q
Glial fascicle

glial fascicle may facilitate the posterior movement of the dividing teloblasts.

Neuroblast formation takes place in juxtaposition to glial processes. The n-bandlet, overlying the glial fascicle, produces the majority of central neurons. The farther away a bandlet is from the glial fascicle, the fewer central neurons it produces. As measured in glossiphoniid leeches, the n-, o-, p-, q- and m-bandlets contribute approximately 180, 120, 24, 20, and 10 neurons, respectively, to the adult ganglion (Weisblat et al., 1984). The n-bandlet, closest to the glial fascicle, may be stimulated to produce most of the central neurons by a factor released from the glia. Other position-dependent effects on bandlet differentiation have been demonstrated in glossiphoniid embryos, through ablation studies of the provisional ectoderm (Ho and Weisblat, 1987). In *Drosophila*, the molecular mechanism underlying position-dependent differentiation of ectodermal cells into neuronal precursors is thought to involve cell-cell communication mediated by the Notch locus gene, which encodes for a transmembrane protein containing extracellular epidermal growth factor-like repeats (Wharton et al., 1985). The RNC protein may mediate a similar neuronal induction in the leech.

Separation of the primordial glial fascicle during aggregation of neuroblasts

Glial processes also are present while ganglionic precursors aggregate into primordial hemiganglia. The aggregation of neuroblasts into segmentally-reiterated hemiganglia coincides

with the separation of the primordial glial fascicle. One process stays at the ventral midline, while the other two move apart to cover the the medio-dorsal aspect of the aggregating hemiganglion. The glial processes expand to outline the future neuropile, restricting the ganglionic neurons to the cortex of the neuromere. The separation and expansion of these glial processes may result in response to the aggregating neuroblasts. In *Manduca sexta*, sensory axons entering the antennal lobe initiate stereotypic changes in the shape and position of neuropile-associated glial cells (reviewed in Tolbert and Oland, 1989).

Differentiating neurons will initiate a primary axon medially towards these glial processes outlining the neuropile (Fernandez, 1978). A trophic factor may be released from the glial processes, stimulating axonal initiation, thereby directing the polarity of the central primary axons. In dissociated cerebellar tissue culture, the granule cell neurites extend towards the advancing lamella of astrocytes (Mason et al., 1988).

Thus, the original midline position of the glial fascicle is optimal for acting on teloblast movement and neuroblast induction. The subsequent position of the glial processes outlining the future neuropile is optimal for determining the polarity of axonal initiation.

Glial processes projecting along future axon tracts

Embryonic glial processes are associated with the formation of the central and the peripheral axon tracts. During the initial

formation of axon tracts, only primordial glia were detected. These primordial glial cells may become the macroglia seen in the adult, or they may be transient cells. Other transient cells, postulated to play a role in axon tract formation, are documented in the leech [e.g., bipolar cells (Stewart et al., 1987) and midline cells (McGlade-McCulloh et al., 1989)]. Short glial processes with wide, paddle-like growth cones appear to explore the terrain and then elongate in the direction of future axonal tracts. Initially, these glial processes are detected along central tracts; later, they appear in peripheral tracts. As these central and peripheral tracts expand, adult macroglial processes are detected through Laz6-297 staining.

Primordial glia are known to prefigure the future axon tract pattern during the development of grasshoppers (Bastiani and Goodman, 1986), newts (Singer et al., 1979), chicks (Krayanek and Goldberg, 1981), and mice (Silver and Sidman, 1980; Silver et al., 1982). Similarly, primordial glial processes in the leech germinal plate are shown here to establish the orthogonal pattern of the future axon tracts. It remains to be determined what role the RNC protein, expressed on the surface of these processes, plays in axon tract formation.

CHAPTER 2. CARBOHYDRATE-BINDING PROTEINS IN THE LEECH.

I. ISOLATION AND CHARACTERIZATION OF LACTOSE-BINDING PROTEINS.

INTRODUCTION

Carbohydrate recognition in the nervous system is postulated to mediate many aspects of neurogenesis including neuronal migration, axonal growth, and synapse formation (reviewed in Jessell et al., 1990). In many sensory systems the neuronal projections, pathways and targets express restricted distributions of glycoconjugates and lectins, implying a biological role in the formation or function of these systems. Restricted distributions of glycoconjugates containing galactose or its derivatives appears to be a common theme, although limited distributions of other saccharide structures (e.g. mannose, heparin sulfate) have been reported (Barakat et al., 1989; Pfenninger et al., 1984; Wang and Denburg, 1992). Functional subsets of rat and chick dorsal root ganglion neurons, which transmit peripheral sensory information to highly restricted laminae within the dorsal horn of the spinal cord, express galactose (Alvarez et al., 1989a; Alvarez et al., 1989b; Pfenninger et al., 1984), N-acetyl-galactosamine (GalNAc) (Dodd et al., 1984; Scott et al., 1990), and N-acetyl-lactosamine (LacNAc) (a disaccharide of galactose beta(1-4) linked to N-acetyl-glucosamine) (Dodd and Jessell, 1985; Jessell and Dodd, 1985) containing glycoconjugates on their cell bodies, growth cones and at synaptic sites. Likewise, plant lectins that detect galactose or GalNAc

demonstrate a restricted retinal staining pattern (Blanks and Johnson, 1984; Kivelä, 1992). In contrast, plant lectins that bind to mannose, glucose, N-acetyl-glucosamine or sialic acid nonspecifically label most retinal layers (Kivelä and Tarkkanen, 1987; Söderström, 1988). Additionally, during the development of the chick retino-tectal system, retinal neurons are presented with a dorso-ventral gradient of GalNAc in the tectum (Marchase, 1977).

A developmentally regulated gradient of GalNAc-containing glycoconjugates also occurs in the rat accessory olfactory system (Schwarting et al., 1992a). Embryonically, this GalNAc glycoconjugate occurs throughout the system. Postnatally, the GalNAc glycoconjugate is expressed on the central cell bodies, rostral half of the nerves and in their target areas. In other regions of the olfactory system, an alpha-galactosyl glycoconjugate defines a dorso-medial projection of axons between the olfactory epithelium and the olfactory bulb. This gradient is established in the rat embryo and maintained in the adult. A beta-galactose-terminating glycoconjugate, on the other hand, appears to indicate an intermediate stage in the maturation of the neuronal connections between the olfactory epithelium and the olfactory bulb (Schwarting et al., 1992b).

Recently, patterns of glycoconjugates containing galactosides have been found on inner ear hair cells and afferent neurons innervating rat whiskers. The probing of bullfrog and guinea pig vestibular organs with plant lectins has indicated different distributions of glycoconjugates containing galactose or GalNAc on inner ear hair cells within the utriculus (vertical orientation) and

sacculus (horizontal orientation) (Baird et al., 1991). A transiently expressed LacNAc glycoconjugate occupies a spatial pattern within the rat trigeminal pathway that appears to result from the interaction between afferent neurons of vibrissae (whiskers) and their targets (Christensen et al., 1991).

A restricted distribution of galactose-containing glycoconjugates implies a restricted distribution of galactose-binding lectins. Using complementary DNAs and antibodies that recognize rat lung lectins, restricted distributions of potential complimentary galactose-binding lectins have been identified on cell bodies and synaptic terminals of subsets of sensory neurons and on most motoneurons in the brain stem, spinal cord and dorsal root ganglion (Hynes et al., 1990; Regan et al., 1986). Likewise, in the cerebral cortex, where concentrations of galactose-containing glycoconjugates rapidly increase during developmental periods of dendrite arborization, axon outgrowth and synaptogenesis (Svennerholm et al., 1989), galactose-binding lectins are localized to postsynaptic structures within layer I of the cortex (Joubert et al., 1989). Galactose-binding lectins also are localized to parallel fibers in the molecular layer of the developing cerebellum (Kuchler et al., 1989). These studies imply that galactose-binding lectins are involved in neuronal development. However, the function(s) of these lectins remain unknown.

Animal lectins can be classified into two groups based on the structure of their carbohydrate-recognition domains and their requirement of calcium to maintain carbohydrate-binding activity (reviewed in Drickamer, 1988; Kasai, 1990; Wang et al., 1991).

Calcium-dependent lectins as a group recognize many different specific saccharide structures and are widely distributed in vertebrates and invertebrates. In contrast, most calcium-independent lectins have carbohydrate specificities for beta-galactosides and were thought to be present only in vertebrates. Recently, however, a calcium-independent, beta-galactose-specific lectin with significant sequence homologies to vertebrate lectins was found in the nematode (Hirabayashi et al., 1992). This observation demonstrated that are these lectins present also in invertebrates and that they have fundamental roles that are not restricted to vertebrates. The preferential localization of calcium-independent galactose-binding proteins to subsets of sensory neurons in the dorsal root ganglia of embryonic rats implicate these lectins in the development and function of primary sensory neurons in the dorsal root ganglion (Regan et al., 1986).

Understanding the function of endogenous animal lectins in the nervous system would be facilitated by a model system that was less complex than the vertebrate nervous system and that was readily amenable to experimental manipulation. The leech nervous system provides such a system. It is a well-characterized classical neurobiological model system. Its sensory afferent system is chemically coded with restricted distributions of carbohydrate epitopes (Bajt et al., 1990) and the projection of the sensory afferents into the synaptic region of the leech is mediated by a carbohydrate recognition event (Zipser and Cole, 1991; Zipser et al., 1989). However, currently there is no information on presence or function of the lectins in the leech. I attempted, therefore, to

isolate such carbohydrate-binding proteins from the leech. I have identified and am characterizing two leech lactose-binding proteins with apparent molecular weights of 35 and 63 kD, termed Leech Lectin 35 (LL35) and Leech Lectin 63 (LL63), respectively. I have focused most on LL35, which is present in the leech nerve cord. I propose that LL35 is a member of the L30 calcium-independent galactose-binding lectin group.

MATERIALS AND METHODS

Leech nerve cords

Mud leeches, *Haemopis marmorata*, were obtained from Rochester Hollow Grinder (NY) and maintained at 5°C in aerated aquaria containing dilute artificial seawater (Forty Fathoms; 0.5 gm/liter distilled water). Leeches were fed chicken liver or frozen brine shrimp three times a week.

Leech nerve cords (CNS), consisting of the supra- and subesophageal ganglia and the segmental ganglia, were dissected from anesthetized leeches and stored at -70°C.

ASF affinity chromatography

Asialofetuin (ASF) was prepared from fetuin according to (deWaard et al., 1976). Briefly, 500 mg of fetuin (Gibco-BRL, Sprio method) was resuspended in distilled water. The pH of this solution was adjusted to pH 2 slowly by adding a few of drops of 3 N HCl. The fetuin solution was incubated at 80°C for 1 h, then immediately cooled on ice. The pH of the solution was adjusted to pH 7 slowly by adding a few drops of 1 N NaOH. The pH adjusted solution was dialyzed against 200 mM $NaHCO_3$, pH 8, for 1 h at 4°C, followed by three changes of coupling buffer over a 24 h period at 4°C. 20 ul of ASF solution was removed for estimation of protein concentration.

Coupling of ASF to Affigel-15 agarose beads (Biorad) was performed according to Biorad specifications. Care was taken to insure that the time from thawing Affigel-15 beads to the addition

of ASF solution took no longer than 15-20 min. Affigel-15 beads from two 25 ml bottles were resuspended in the supplied solution and poured into a fritted glass Buchner funnel. Beads were washed with 3 l of cold distilled water aided by vacuum suction. The washed moist cake of beads was resuspended with 20 ml of cold distilled water and divided into two 50 ml conical tubes. Excess water was removed by low speed centrifugation (20 xg, 1 min, 4°C). Half of the ASF solution was added to each tube of Affigel-15 beads. ASF was coupled to the Affigel-15 beads overnight at 4°C with agitation. The ASF-Affigel-15 coupled beads were poured into a fritted glass Buchner funnel and washed with 200 ml of cold Coupling buffer. The effluent was saved for estimation of protein concentration. ASF-coupled beads were then washed with 100 ml cold of 3N GuHCl, followed by 2 l of cold distilled water. ASF-Affigel-15 beads were store in 0.2% NaN_3 at 4°C. The efficiency of coupling of ASF to Affigel-15 beads was calculated from determination of the total amount of protein in the ASF solution prior to coupling and of the protein in the coupling buffer wash after the coupling reaction.

Isolating lactose-binding proteins from leech nerve cords

The following buffers were used in the isolation of lactose-binding proteins: Buffer 1 (150 mM NaCl, 10 mM Tris base, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% NaN_3, 1 mM PMSF) and Buffer 2 (Buffer 1 containing 1 mM mercaptoethanol). Crude membrane fractions were prepared by homogenizing 100 leech nerve cords with a glass tissue grinder (Thomas Scientific) in 1 ml of Buffer 2 at 4°C. The

homogenate was centrifuged at 82 xg for 5 min at 4°C to remove nuclei and large cellular debris. The resulting supernatant was centrifuged at 104,000 xg for 60 min at 4°C. The 104,000 xg supernatant was removed and the pellet was resuspended in 1 ml of Buffer 2. Membrane proteins were extracted by addition of 50 ul of 20% Triton X-100 to the resuspended pellet and incubation at 4°C for 1 h on a rotorary shaker. The Triton X-100 extract was centrifuged at 16,000 xg for 1 min in an Eppendorf microcentrifuge. The supernatant was passed several times through a 3 ml syringe containing 0.9 ml of ASF-Affigel beads in that had been washed with 5 ml 25 mM lactose and equilibrated with 20 ml 1% TX-100 in Buffer 2. After loading the sample onto the ASF column, the column was washed with Buffer 1, followed by various sugars dissolved in Buffer 1. Proteins eluting from the column were monitored at 280 nm and concentrated by ethanol precipitation.

Isolating lactose-binding proteins from whole leech

Crude membrane fractions were prepared from 30 leeches. After anesthetization in cold 10% ethanol, leech digestive tracts were opened and flushed with phosphate-buffered saline (PBS: 0.9% NaCl, 50 mM sodium phosphate, pH 7.4). Then, whole leeches were homogenized in 50 ml of Buffer 2 for 3 min at 4°C with a polytron homogenizer. The homogenate was centrifuged at 104,000 xg for 60 min at 4°C. The supernatant was removed and the pellet was resuspended in 30 ml of Buffer 2. Proteins were extracted by adding 1.75 ml of 20% Triton X-100 and incubating on ice for 1 h. The Triton X-100 extracts were passed through a 20 ml syringe

containing 12 ml of ASF-Affigel-15 beads that had been washed with 20 ml of 25 mM lactose and equilibrated with 1% Triton X-100 in Buffer 2. After washing with 100 ml Buffer 1, the column was washed with various sugars in Buffer 1. Proteins eluting from the column were monitored at 280 nm. Fractions containing lactose-binding proteins were pooled and concentrated by centrifugation using a Centricon-10 filtering apparatus (Amicon). A sample was removed for estimation of protein concentration, while the rest was aliquoted and stored at -70°C.

Protein assay

Protein concentration of samples are determined according to the Bradford (1976) method or to the Peterson (1977) modification of the Lowry et al. (1951) method for estimation of total protein.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein samples were heated at 100°C in sample buffer for 4 min and electrophoretically separated on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels (Laemmli, 1970). Gels were either silver stained (Biorad) for protein or used to prepare immunoblots. Immunoblots were prepared by electrophoretically transferring separated proteins from SDS-polyacrylamide gels to nitrocellulose paper (Schleicher and Schuell, pore size 0.45 um) with applied voltage of 90 V (Garrels, 1979; Towbin et al., 1979). Unbound sites on the nitrocellulose were blocked by incubating immunoblots with either 10% powdered milk, 0.05% Tween 20, and 0.2% NaN_3 in PBS (milk blocking solution) or 3% BSA, 0.1% Tween 20,

and 0.2% NaN_3 (BSA blocking solution) in PBS overnight at 4°C. Blocked immunoblots were incubated for 2-4 h with a primary antibody (monoclonal or polyclonal, anti-LL35D at 1/5000 dilution and anti-LL35D-35 at 1/10 to 1/100 dilution), rinsed, incubated again in the blocking solution, and incubated for 1-2 h with HRP-conjugated goat-anti-mouse IgG (1/250 dilution) (Cappel) or HRP-conjugated goat-anti-rabbit IgG (1/250 dilution) (Biorad). After rinsing the immunoblot free of unbound antibody, antibody binding was visualized by the diaminobenzidine (DAB) reaction. The apparent molecular weights of the proteins in gels were estimated by comparing their migration with those of a set of low molecular weight standards (Biorad). On immunoblots, molecular weights were estimated by applying to the gels prestained high molecular weight standards (BRL).

Radioiodination of lactose-binding proteins

Lactose-binding proteins were radioiodinated by the Chloramine-T method (Langone, 1980). 1.5 ul of 1 mCi $Na^{125}I$ (>350 mCi/ml; New England Nuclear) was added to 100 ul samples of 5-40 ug/ul lactose-binding proteins in Buffer 1 containing 25 mM alpha-methyl-galactose (aMeGal) or lactose. 5 ul of Chloramine T (1 mg/ml Buffer 1 plus 25 mM aMeGal or lactose) was added with constant agitation at room temperature to start the reaction. After 30 sec, the reaction was stopped by adding 10 ul of sodium metabisulfite (1 mg/ml Buffer 1 plus 25 mM aMeGal or lactose). After adding 100 ul of 2% BSA, the radioactive mixture was applied to a Sephadex G-25 (medium) column (0.7 x 25 cm) to remove free

^{125}I. Radiolabeled lactose-binding proteins in the void volume were rebound to 1 ml of ASF-Affigel-15 beads in a 3 ml syringe. Radiolabeled proteins were eluted with 25 mM aMeGal or lactose from the ASF affinity column. An aliquot of the radiolabeled aMeGal eluted proteins was removed for analysis by SDS-PAGE and autoradiography. BSA was added to a final concentration of 1% to the rest of the radiolabeled sample prior to dialyzing the sample against Buffer 1 (at least 3 changes at 4°C over 24 h period) to remove the sugar.

Binding specificity of lactose-binding proteins:

The binding specificity of the lactose-binding proteins was assayed according to Ho et al. (1990). 100 ul of a competing reagent was added to a reaction mixture containing 200 ul of a 1:1 (v/v) suspension of ASF-Affigel-15 beads in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris, 0.2% NaN_3, pH 7.4) and 1 ml of ^{125}I-labeled LL35 (5000 cpm) or ^{125}I-labeled LL63 (4000 cpm). Samples were incubated by rapid shaking at room temperature for 4 h, then washed three times with 2 ml of TBS by centrifugation and resuspension. Radioactivity associated with the beads was quantified by gamma counting.

To investigate the effects of pH on LL35 binding, binding was assayed in the following buffers, each containing 150 mM NaCl: 10 mM glycine-HCl (pH 5); 10 mM Tris-base (pH 7.5); 10 mM sodium bicarbonate (pH 10). To investigate the effects of chelating agents on LL35 binding, LL35 binding to ASF beads was assayed in TBS containing 10 mM EDTA or EGTA with or without 25 mM lactose.

Polyclonal antibodies

Lactose-binding proteins were isolated from Triton X-100 extracts of crude membranes prepared from whole leech homogenates. After loading the ASF column with the Triton X-100 extract, lactose-binding proteins were eluted from the ASF column with 25 mM lactose. Fractions containing the lactose-binding proteins were concentrated by centrifugation using a Centrion-10 filter (Amicon) prior the separating the proteins on a 12.5% SDS-polyacrylamide gel. Polyclonal antiserum, anti-LL35D (anti-Leech Lectin 35, Denatured form), was derived by immunizing anesthetized rabbits with the proteins excised from the 35 kD region from an SDS-polyacrylamide gel of leech lactose-binding proteins. A rabbit was immunized initially with approximately 50 ug of protein by intramuscular injection. After 143 days the rabbit was immunized a second time with approximately 500 ug of protein. Fourteen days later 25 ml of blood was collected through the ear vein of the anesthetized rabbit. Blood was incubated at 37^{o}C for 1 h, then allowed to stand overnight at 4^{o}C. Serum was separated from the blood clot by centrifugation and stored at -70^{o}C.

Affinity purification of polyclonal antibodies

Polyclonal antiserum, anti-LL35D-35, was obtained by affinity purifying anti-LL35D against the 35 kD protein bands on western blots prepared from leech nerve cord proteins according to Smith and Fisher (1984). Briefly, 30 leech nerve cords were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and transferred

to nitrocellulose. The protein blot was blocked with 3% BSA, 0.3% Tween 20, 0.2% NaN_3 in PBS overnight at 4°C. The blocked blot was incubated with a 1:500 dilution of anti-LL35D with 3% BSA in PBS overnight at room temperature. The following day the right and left edges were cut from the blot and primary antibody binding was visualized by incubating the strips with HRP-conjugated goat anti-rabbit IgGs (Biorad) for 1 h and developing with the DAB reaction. The region of the 35 kD band was identified on the undeveloped blot and cut out. This band was cut into small strips and placed into an 1.5 ml microfuge tube. The strips were washed for 15 min with three 5 min washes of 0.5% Tween 20 in PBS and bound antibodies were eluted from strips with three 30 sec washes of 200 ul 0.1M glycine-HCl in PBS, pH 2.3. The pH of the glycine-HCl elutant was immediately adjusted to pH 7 with 2 M Tris-base buffer, pH 11. The strips were washed again with 0.5% Tween 20 in PBS. A 1/500 dilution of polyclonal antiserum was reapplied to the washed strips overnight at room temperature. The cycle of binding and elution of polyclonal antibodies to specific bands on the strips of nitrocellulose was repeated three more times.

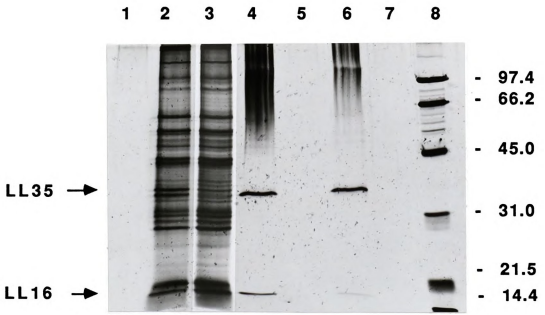
Eluted antibodies were dialyzed twice against cold distilled water and once against PBS over a 24 h period. The dialyzate was concentrated by lyophylization and resuspended in a minimal volume of PBS containing 0.2% NaN_3 and stored at 4°C.

RESULTS

Two lactose-binding proteins, LL35 and LL16, are present in leech nerve cords

The leech nerve cord represents the central nervous system of the leech. It is enclosed within a blood sinus that lies ventral to the alimentary canal. It is composed of a chain of ganglia consisting of nerve, glia, and muscle cells suspended in a dense feltwork of connective tissue and is covered by a continuous layer of squamous epithelia cells (Coggeshall and Fawcett, 1964). Crude membranes, prepared from leech nerve cords of the mud leech *Haemopis maramota*, were solubilized in a non-ionic detergent in the presence of a reducing agent and fractionated by affinity chromatography on a column containing asialofetuin-agarose beads (ASF column). The majority of the proteins in the extracts, as monitored by SDS-PAGE analysis, did not bind to the column (Figure 10, lanes 2 and 3). After extensive washing to remove the detergent and the reducing agent, the ASF column was washed with various sugars. Washing the ASF column with 100 mM xylose or alpha-methyl mannose did not elute any bound proteins from the ASF column (data not shown). In contrast, washing the column with 25 mM lactose reproducibly eluted two proteins. SDS-PAGE analysis of the lactose fractions revealed a prominent protein band at an apparent molecular weight (Mr) of 35.2 $\pm$ 2.4 kD and a minor band at a Mr of 16.2 $\pm$ 1.5 kD (Figure 10, lane 4). Neither of these bands was detected in cytosolic

Figure 10. The leech nerve cord contains two lactose-binding proteins, LL35 and LL16. *Haemopis maramota* proteins in nerve cords before and after fractionation on an asialofetuin (ASF) affinity column were analyzed by SDS-PAGE. Crude membrane fractions prepared from 200 leech ventral nerve cords were solubilized with 1% Triton X-100 in the presence of 1 mM 2-mercaptoethanol. This extract was divided equally into two samples. Each sample was passed through an ASF affinity column (0.8 x 4 cm) equilibrated with Buffer 1 containing detergent and reducing agent. After extensive washing with plain Buffer 1, proteins that bound to the column were eluted with various sugars. Proteins in 300 ul samples of sugar elutions were concentrated by ethanol precipitation prior to separating by SDS-PAGE and visualizing by silver staining. Lanes: (1) column prewashed with 25 mM lactose; (2) 5 ug sample of total extract applied to column; (3) 5 ug sample of proteins not binding to the column; (4) 25 mM lactose elution of sample 1; (5) 25 mM aMeGal elution sample 1 after lactose elution; (6) 25 mM aMeGal elution of sample 2; (7) 25 mM lactose elution of sample 2 after aMeGal elution; (8) molecular weight standards. Arrows on left mark the positions of LL35 and LL16.


1 2 3 4 5 6 7 8
LL35
LL16
97.4
66.2
45.0
31.0
21.5
14.4

fractions of nerve cords. Washing the ASF column with 25 mM lactose prior to applying the extract did not elute any proteins previously bound to the column (Figure 10, lane 1), demonstrating that the 35 and 16 kD lactose-binding proteins, termed Leech Lectin 35 (LL35) and Leech Lectin 16 (LL16), respectively, were isolated from extracts of nerve cords.

Bands corresponding to LL35 and LL16 were distinguishable in samples of proteins loaded onto the ASF column (Figure 10, lane 2). LL35 was not a major protein in nerve cord extracts. All LL35 was removed from the extracts by passage though the ASF column (Figure 10, lanes 2 and 3) and enriched in the lactose elution (Figure 10, lane 4). LL16, however, is a member of a large population of 16 kD proteins in nerve cord extracts, most of which did not bind to the ASF column (Figure 10, lanes 2 and 3). Passing the flow-through volume over a second ASF column did not isolate additional LL16 (or LL35) from the extracts. The majority of 16 kD proteins in leech nerve cord extracts were not lactose-binding proteins.

LL35 and LL16 also could be eluted with alpha-methyl-galactose (aMeGal). Reloading the ASF column with CNS extract (not shown) and washing it with 25 mM aMeGal eluted both LL35 and LL16 (Figure 10, lane 6). In sequential applications of the two sugars, lactose followed by aMeGal (Figure 10, lanes 4 and 5) or lactose followed by aMeGal (Figure 10, lane 6 and 7), the second sugar did not elute any additional proteins. Thus, in terms of eluting the lactose-binding proteins isolated from the nerve cords from the ASF column, lactose and aMeGal were equivalent. This finding is significant because the use of aMeGal allowed us to separate LL35

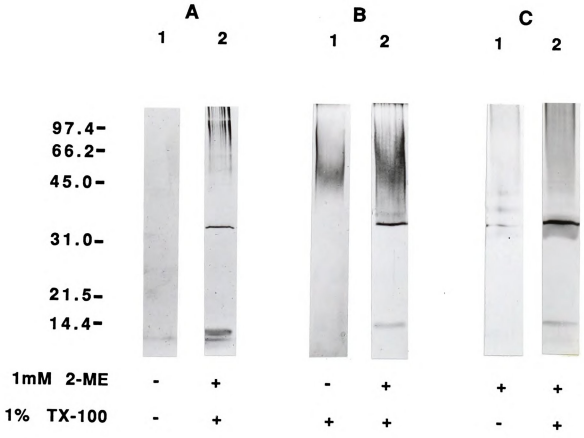
and LL16 from additional lactose-binding proteins that are found in peripheral tissues of the leech (see Figures 12 and 13).

Extraction of LL35 and LL16 from leech nerve cords requires the presence of both detergent and reducing agent

To investigate the requirement for detergent or a reducing agent in the extraction of LL35 and LL16 from membrane fractions of leech nerve cords, LL35 and LL16 were isolated in the presence or absence of 2-mercaptoethanol (2ME) or Triton X-100 (TX-100) (Figure 11). Leech lectins were extracted from paired samples of the crude membrane preparations of nerve cords. From each pair of samples, proteins in the control sample were extracted in the presence of both 1 mM 2ME and 1% TX-100 (Figure 11, lanes 2). Proteins from the experimental sample were extracted without detergent and reducing agent, or in the presence of only detergent or only reducing agent (Figure 11 lanes 1). Each extract individually was passed over an ASF column and the lectins were eluted. Proteins in fractions containing LL35 and LL16 were analyzed by SDS-PAGE. The silver stain smear extending from the top of the gel to approximately 45 kD may have originated from a contaminant in the detergent, because it is only seen when detergent is used in the extraction.

A small fraction of the total extractable LL35 was isolated in the presence of the reducing agent alone (Figure 11C), but there was no discernible LL16. In the presence of only detergent (Figure 11B) or in the absence of both detergent and reducing agent (Figure 11A),

Figure 11. Detergent and reducing agents are necessary for extraction and recovery of LL35 by ASF affinity chromatography. (A-C) Each set of lanes represents three different preparations of crude membranes from 200 nerve cords that were split into two equal samples. Proteins were extracted from one of the two paired samples in the presence or absence of 1 mM 2-mercaptoethanol (2-ME) or 1% Triton X-100 (TX-100) as indicated in the chart below. Each extract was passed individually through an ASF affinity column. Proteins in the aMeGal elutions were separated by SDS-PAGE and visualized by silver staining. Numbers in kD of MW standards are indicated on the left.


A
1
2
B
1
2
C
1
2
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
1mM 2-ME
- + - + + +
1% TX-100
- + + + - +

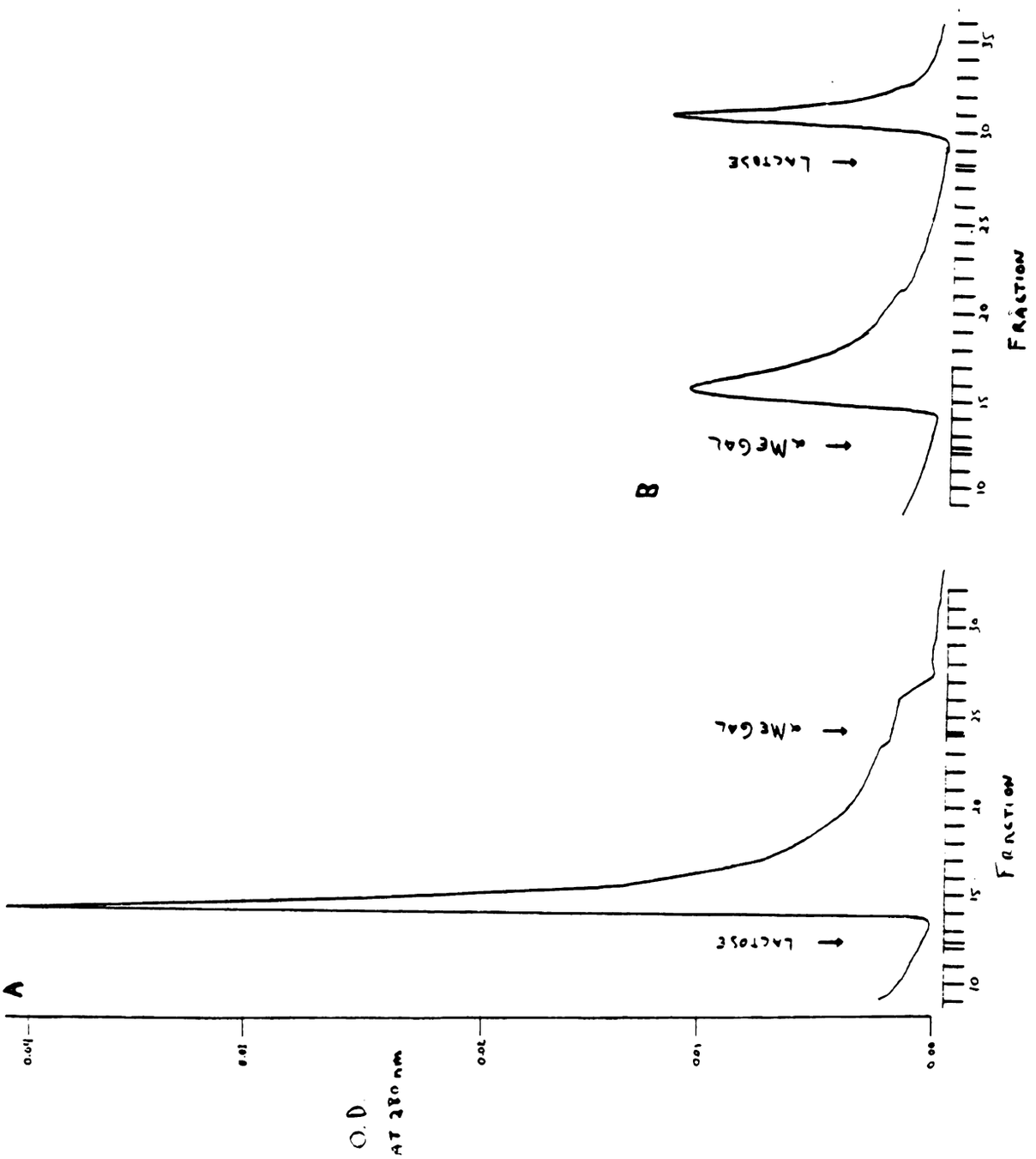
neither LL35 or LL16 were isolated. Thus, both 2ME and TX-100 were necessary for the isolation and retention of LL35 and LL16 binding to the ASF column during their extraction from the leech nerve cord.

Isolation of lactose-binding proteins from the whole leech

To optimize the yield of LL35 and LL16 from the leech, we isolated the lactose-binding proteins not just from the laboriously dissected central nerve cords, but from the whole leech, thus taking advantage of the leech's extensive peripheral nervous system as an additional source of material. Homogenates of whole leech were extracted with detergent and reducing agent and isolated by ASF affinity chromatography. The elution of proteins from the ASF column was monitored by absorbance at 280 nm during the sequential applications of lactose and aMeGal (Figure 12). Eluting the column first with lactose resulted in a large peak and the subsequent application of aMeGal did not elute any additional material (Figure 12A). However, applying aMeGal first resulted in a small peak and the subsequent application of lactose also resulted in a second small peak with the same amplitude (Figure 12B). Thus, the lactose-binding proteins derived from whole leech had differential sensitivities to lactose and aMeGal, contrasting with the equivalent sensitivity for lactose and aMeGal exhibited by the lactose-binding proteins derived from the nerve cord.

The proteins eluting from the ASF column by the sequential application of lactose and aMeGal were analyzed by SDS-PAGE

Figure 12. Differential fractionation by asialofetuin affinity chromatography of carbohydrate-binding proteins in crude membrane fractions prepared from whole leeches. Crude membrane fractions prepared from 5 leeches were solubilized with 1% Triton X-100 in the presence of 1mM 2-mercaptoethanol. This extract was divided equally into two samples. Each sample was passed through an ASF affinity column (0.8 x 4 cm) equilibrated with Buffer 1 containing detergent and reducing agent. After extensive washing with plain Buffer 1, proteins that bound to the column were eluted with 25 mM lactose followed by 25 mM aMeGal (A) or 25 mM aMeGal followed by 25 mM lactose (B). Elution of proteins from the ASF column were monitored at 280 nm. Arrows indicate when sugar solution reach top of the column.


A
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LACTOSE
αMEGAL
FRACTION
10
15
20
25
30
35

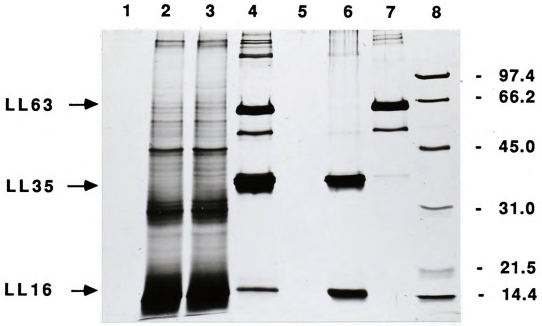
analysis (Figure 13). Washing the column first with lactose eluted LL35 and LL16 (Figure 13, Lane 4). Significantly larger amounts of LL35 and LL16 could be isolated from the whole leech than from just the nerve cord (compare Figures 10 and 13). However, additional species of lactose-binding proteins that were not detected in the nerve cord were now present. The most prominent species was a 63 ± 1.7 kD protein, termed Leech Lectin 63 (LL63) (Figure 13, lane 4). Less abundant protein species migrated in a band at 50.5 ± 3.2 kD and in three bands above 150 kD. SDS-PAGE analysis of the aMeGal wash following the lactose elution confirmed that no proteins eluted (Figure 13, lane 5). Thus, all galactose-binding proteins were eluted by lactose.

Analyzing the proteins eluting from the ASF column by applying aMeGal prior to lactose revealed that LL35 and LL16 were the only major proteins present (Figure 13, Lane 6). The subsequent lactose wash eluted the other lactose-binding proteins, primarily LL63 (Figure 13, Lane 7). Thus, LL35 and LL16 differ from the other lactose-binding proteins in their affinity for aMeGal. This affinity of LL35 and LL16 for aMeGal enabled the isolation of these two lactose-binding proteins from the other lactose-binding proteins that were present in the whole leech but absent from the central nerve cord.

Saccharide-binding specificities of LL35

A high yield of LL35 was obtained from whole leeches by ASF affinity chromatography and elution with 25 mM aMeGal. The aMeGal

Figure 13. The three lactose-binding proteins, LL63, LL35, and LL16, present in the whole leech can be separated based on their affinity for aMeGal. Proteins in 300 ul samples of sugar elution peaks from the elution profile shown in Figure 12 were concentrated by ethanol precipitation prior to separation by SDS-PAGE and visualized by silver staining. Lanes: (1) Lactose pre-wash of ASF affinity column; (2) 5 ug sample of total extract applied to column; (3) 5 ug sample of proteins not binding to the column; (4) lactose peak (Fig. 3A, fraction 14); (6) aMeGal peak (Fig. 3A, fraction 27); (7) aMeGal peak (Fig. 3B, fraction 15); (8) lactose peak (Fig. 3B, fraction 30); (9) molecular weight standards. Arrows on left mark LL63, LL35 and LL16.


1
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LL63
LL35
LL16
97.4
66.2
45.0
31.0
21.5
14.4

fractions containing LL35 were pooled and concentrated by filtration using a Centricon-10 microconcentrator, which has a cutoff range of 10 kD. Much of LL16 was lost during this procedure. Between 25 to 100 ug of LL35 per leech was recovered by this procedure, as estimated from assays for total protein. Isolated LL35 was radioiodinated with ^{125}I, re-isolated on an ASF column and eluted with 25 mM aMeGal. SDS-PAGE and autoradiography of the radioiodinated proteins in the aMeGal fraction revealed that the majority of the radioactivity was incorporated into LL35, with a minute amount in a band at 16 kD (Figure 14). Re-isolation of radiolabeled LL35 by ASF affinity chromatography demonstrated that its binding activity was retained after radioiodination.

The saccharide binding specificity of LL35 was determined by assaying the ability of various sugars to block the binding of ^{125}I-LL35 to ASF-beads (Table 1). In the absence of sugar, 82% of the total radioactivity added to the reaction mixture was recovered bound to the ASF beads. At 25 mM concentrations, mannose and glucose derivatives had no effect on LL35 binding. Corresponding galactose derivatives, however, blocked the binding of ^{125}I-LL35 to ASF beads, demonstrating that LL35 binding required an axial bond at carbon 4 (C4). Comparing the effect of the derivatives of galactose on LL35 binding indicates that the beta anomer of galactose (beta-methyl-galactose) was a 10% more effective inhibitor of LL35 binding than the alpha anomer (aMeGal). This implies that LL35 has some discrimination of the bonds surrounding the anomeric (or reducing) carbon (C1). LL35 binding was sensitive to substitution at the C2 carbon, since galactosamine inhibited LL35 binding by only

Figure 14. Radioiodination of LL35. LL35 isolated from extracts of whole leech crude membranes by ASF affinity chromatography was eluted with 25 mM aMeGal, concentrated by filtration and radioiodinated with ^{125}I by the chloramine T method (Langorn, 1978). Radioiodinated proteins were re-isolated on an ASF affinity column and eluted with 25 mM aMeGal. Lanes: (1) Silver stained SDS polyacrylamide gel of radioiodinated proteins (500 cpm) in the aMeGal fraction; (2) Autoradiogram of gel in lane 1 developed after 3 days with enhancers. Arrows on left mark LL35 and LL16. Numbers in kD of MW standards are indicated on right.

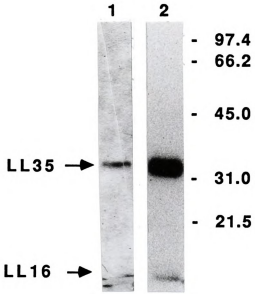

1
2
97.4
66.2
45.0
LL35
31.0
21.5
LL16

Table 1. EFFECTS OF SACCHARIDES ON LEECH LECTIN 35 BINDING*

Saccharide	CPM bound	% Inhibition
None	4114 ± 57	0
Galactose	335 ± 27	92
Glucose	4065 ± 47	1
Mannose	4054 ± 43	1
a-Methyl-Galactose	718 ± 27	83
b-Methyl-Galactose	276 ± 10	93
a-Methyl-Mannose	4104 ± 67	0
b-Methyl-Glucose	4153 ± 37	-1
Galactosamine	2648 ± 47	36
Glucosamine	4068 ± 90	1
Mannosamine	4002 ± 35	3
N-Acetyl-Galactosamine	1276 ± 24	69
N-Acetyl-Glucosamine	4194 ± 40	-2
N-Acetyl-Mannosamine	4199 ± 89	-2
Lactose	156 ± 7	96
Sucrose	4183 ± 95	-2

*0.2 ml aliquots of ASF-affigel bead suspension (1 vol: 1 vol) were added to 1 ml of ^{125}I-labeled LL35 (5000 cpm) and incubated with 0.1 ml of 300 mM of various sugars or TBS. Final concentration of sugars was 25 mM. Data represents the means of three determinations ± SD.

36%. Neutralizing the amine group, however, by adding an acetyl group, as in N-acetyl-galactosamine, recovered 30% inhibiting capacity of the galactose derivative. The most potent inhibitors of LL35 binding were lactose (which is galactose in a beta(1-4)-linkage to glucose), followed by beta-methyl-galactose and galactose.

The effect of chelating agents and pH on LL35 binding

A possible requirement for divalent cations for LL35 binding was determined by assaying the ability of ^{125}I-labeled LL35 to bind to ASF beads in the presence of 10 mM EDTA or EGTA (Table 2). LL35 binding did not appear to be dependent on the presence of divalent cations. While both chelators somewhat reduced the ability of LL35 to bind to ASF-affigel beads, 91 or 75% of LL35 binding activity was retained in the presence of 10 mM EDTA or EGTA, respectively. However, the addition of 25 mM lactose in the presence of either chelator completely blocked the binding of LL35.

The effect of pH on the binding of ^{125}I-LL35 to ASF beads also was determined (Table 2). LL35 binding of greater than 50% occurred over a wide pH range from 5 to 10. The gradual decrease in LL35 binding was slightly greater as the pH was raised above 7.5 than when the pH was lowered below 7.5. The reduction in LL35 binding in the presence of EGTA was probably due to the drop in pH caused by the addition of 10 mM EGTA to the Tris buffer. At this concentration of EGTA the pH of the Tris buffer dropped from 7.5 to 5.

Table 2. EFFECTS OF CHELATORS AND pH ON LEECH LECTIN 35 BINDING*

Treatment	CPM bound	% Inhibition
None	4048 ± 70	0
Chelating Agents:		
EDTA	3699 ± 535	9
EDTA + Lactose	188 ± 14	95
EGTA	3021 ± 213	25
EGTA + Lactose	251 ± 76	94
pH:		
pH 5: Glycine-HCl buffer	3213 ± 439	21
pH 7.5: Tris-Base buffer	3770 ± 383	7
pH 10: Na_2CO_3 buffer	2227 ± 576	45

*0.2 ml aliquots of ASF-affigel bead suspension (1 vol: 1 vol) were added to 350 ul of ^{125}I-labeled LL35 (5000 cpm) and incubated with 750 ul of 16 mM chelating agents (EDTA or EGTA) or buffers (glycine-HCl, Tris-Base, or Na2CO3) in the presence or absence of lactose. Final concentrations of chelating agents, buffers and lactose were 10 mM, 10 mM and 25 mM, respectively. Data represents the means of three determinations ± SD.

Saccharide-binding specificities of LL63

A high yield of LL63 was obtained from whole leeches by ASF affinity chromatography following by sequential elution with aMeGal and lactose. Lactose-binding proteins were isolated from whole leeches by ASF affinity chromatography, concentrated by filtration through a Centricon-10 microconcentrator and radioiodinated with ^{125}I. After radioiodination the lactose-binding proteins were passed through a second ASF column and fractionated, making use of their affinities for aMeGal and lactose. First, LL35 was removed by eluting with aMeGal and then the remainder of the lactose-binding proteins were harvested by lactose elution. SDS-PAGE and autoradiography of the radioiodinated proteins in the lactose elution demonstrated that radioactivity was incorporated into a single band at 63 kD (Figure 15). Thus, when LL63 was highly purified and iodinated, it retained its binding activity.

The saccharide binding specificity of LL63 was more sensitive to substituted derivatives of galactose than LL35, as determined by assaying the ability of various sugars to block the binding of ^{125}I-LL63 to ASF-beads (Table 3; Figure 16). Again, 81% of the total of the ^{125}I-LL63 radioactivity added to the reaction mixture was recovered bound to the ASF beads in the absence of sugars. At 25 mM concentrations, glucose and mannose derivatives were poor or ineffective inhibitors of LL63 binding. Galactose and its derivatives, however, strongly inhibited LL63 binding. As with LL35, an axial bond at C4 was required for LL63 binding. In contrast to

Figure 15 Radioiodination of LL63. LL63 isolated from extracts of whole leech crude membranes by ASF affinity chromatography was eluted with 25 mM lactose, concentrated by filtration, and radioiodinated with ^{125}I by the chloramine T method (Langorn, 1978). Radioiodinated proteins were re-isolated on an ASF affinity column and eluted with 25 mM aMeGal followed by 25 mM lactose. Lanes: (1) Silver stained SDS polyacrylamide gel of radioiodinated proteins (1000 cpm) in the lactose fraction; (2) Autoradiogram of gel in lane 1 developed after 1 day with enhancers. Arrows on left mark LL63. Numbers in kD of MW standards are indicated on right.

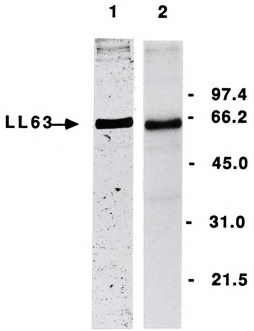

1
2
LL63
97.4
66.2
45.0
31.0
21.5

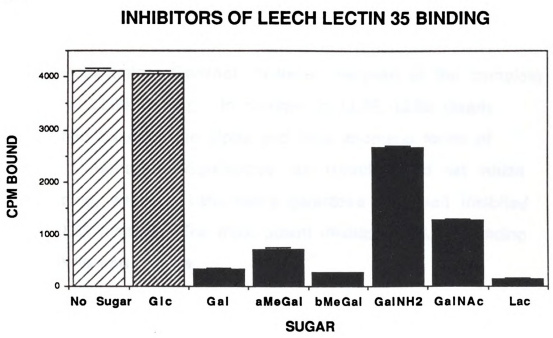
Table 3. EFFECTS OF SACCHARIDES ON LEECH LECTIN 63 BINDING*

Saccharide	CPM bound	% Inhibition
None	3247 ± 88	0
Galactose	801 ± 65	75
Glucose	3298 ± 211	-2
Mannose	2671 ± 347	18
a-Methyl-Galactose	3138 ± 229	3
b-Methyl-Galactose	720 ± 27	78
a-Methyl-Mannose	3379 ± 316	-4
b-Methyl-Glucose	3541 ± 148	-9
Galactosamine	3393 ± 228	-4
Mannosamine	3104 ± 118	4
N-Acetyl-Galactosamine	270 ± 14	92
N-Acetyl-Mannosamine	3370 ± 99	-4
Lactose	256 ± 19	92

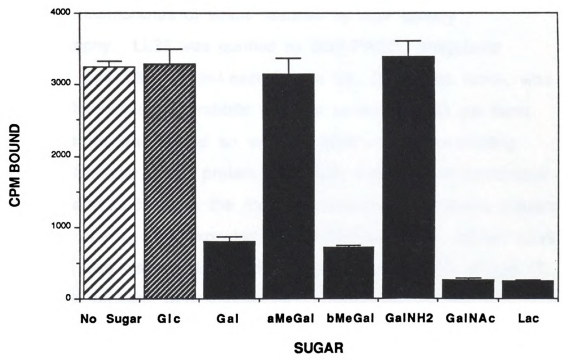
*0.2 ml aliquots of ASF-affigel bead suspension (1 vol: 1 vol) were added to 1 ml of ^{125}I-labeled LL63 (4000 cpm) and incubated with 0.1 ml of 300 mM of various sugars or TBS. Final concentration of sugars was 25 mM. Data represents the means of three determinations + SD, except for N-acetyl-b-mannosamine where the mean is calculated from two determinations.

Figure 16. Comparison of the effect of galactose derivatives on LL35 (A) and LL63 (B) binding to ASF-affigel beads in the presence or absence of various saccharides at 25 mM concentrations. Glucose (Glc), galactose (Gal), alpha-methyl-galactose (aMeGal), beta-methyl-galactose (bMeGal), galactosamine (GalNH2), N-acetyl-galactosamine (GalNAc), lactose (Lac).

A


INHIBITORS OF LEECH LECTIN 35 BINDING
CPM BOUND
4000
3000
2000
1000
0
No Sugar
Glc
Gal
aMeGal
bMeGal
GalNH2
GalNAc
Lac
SUGAR

B

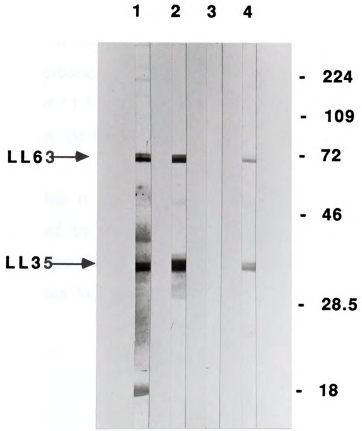

INHIBITORS OF LEECH LECTIN 63 BINDING
CPM BOUND
4000
3000
2000
1000
0
No Sugar
Glc
Gal
aMeGal
bMeGal
GalNH2
GalNAc
Lac
SUGAR

LL35, LL63 was highly sensitive to substitution at the C2 carbon. The absence of a charged group at C2 was extremely important to LL63 binding, because galactosamine (GalNH2) did not inhibit LL63 binding. Neutralizing this charged amine with an acetyl group (N-acetyl-galactosamine, GalNAc), however, resulted in the complete inhibition of LL63 binding. In contrast to LL35, LL63 clearly distinguished between the alpha and beta anomeric forms of galactose. Alpha-methyl-galactose, as expected, did not inhibit LL63 binding, whereas beta-methyl-galactose (bMeGal) inhibited 78% of LL63 binding. The most potent inhibitors of LL63 binding were GalNAc and lactose.

LL35 and LL63 are immunologically related

Polyclonal antibodies were generated against purified and denatured LL35 (Figure 17). Lactose-binding proteins were isolated from crude membranes of whole leeches by ASF affinity chromatography. LL35 was purified by SDS-PAGE. Polyclonal antiserum, anti-LL35D (anti-Leech Lectin 35, Denatured form), was generated by immunizing rabbits with the excised 35 kD gel band. This antiserum was tested on western blots of lactose-binding proteins. Staining these protein blots with India ink demonstrated that LL35 and LL63 were the major lactose-binding proteins present (Figure 17 lane 1). As expected, anti-LL35D antiserum stained LL35. However, it also stained LL63 and a minor band at 50 kD (Figure 17, lane 2). No proteins were stained by the preimmune serum in parallel analysis (Figure 17, lane 3).

Figure 17. LL35 and LL63 are immunologically related. Polyclonal antiserum, anti-LL35D, was generated by immunizing rabbits with the LL35 SDS-polyacrylamide gel band. Lactose-eluted proteins isolated from crude membranes of whole leeches by ASF affinity chromatography were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. Proteins on strips of nitrocellulose were visualized by staining strips with india ink (lane 1) or by indirect immunocytochemistry (lanes 2-4). Lanes: (1) India ink; (2) polyclonal antisera, anti-LL35D; (3) preimmune sera; (4) polyclonal antisera, anti-LL35D-35, (anti-LL35D antiserum affinity purified against LL35 from the CNS). Arrows on left mark LL63 and LL35 bands. Numbers in kD of MW standards are indicated on right.


1
2
3
4
LL63
LL35
224
109
72
46
28.5
18

The cross reactivity between LL35 and LL63 was not abolished by affinity purifying the antiserum. Anti-LL35D antibodies were affinity purified against the LL35 band on protein blots of nerve cord proteins. These affinity purified antibodies were designated anti-LL35D-35. Anti-LL35D-35 still bound to both the LL35 and LL63 bands (Figure 17, lane 4), indicating that LL35 and LL63 share common epitopes. Preliminary peptide mapping studies of LL35 and LL63, however, indicate that LL35 and LL63 are distinct proteins. Using the Cleveland et al. (1977) method for analyzing protein fragments, *Staphylococcus aureus* V8 protease digested LL35 from 35 kD into 23 and 11.5 kD fragments. Limited V8 protease digests of LL63 have not yet demonstrated common peptide fragments with those of LL35.

Antiserum that is specific for LL35 and does not recognize LL63 was obtained by immunizing rabbits with native lactose-binding proteins (see Chapter 3) suggesting that denatured LL35 and LL63 share epitopes in common while native LL35 has its own distinct epitopes.

DISCUSSION

The leech contains two prominent lactose-binding proteins with molecular weights of 35 and 63 kD, which I have termed Leech Lectin 35 (LL35) and Leech Lectin 63 (LL63). Using a simple one step affinity chromatography procedure, both LL35 and LL63 were isolated from crude membrane fractions of the whole leech. LL35 and LL63, however, have different tissue distributions since LL35, but not LL63, is located also in membrane fractions prepared from the leech nerve cord. Because I am interested in isolating lectins from the leech nervous system, I focused my attention on LL35.

LL35 appears to share characteristics with the family of calcium-independent vertebrate lectins (reviewed in Barondes, 1984; Drickamer, 1988; Kasai, 1990; Wang et al., 1991). This lectin family is characterized by their carbohydrate-binding activity being independent on the presence of calcium, being specific for beta-galactosides, and being dependent on the presence of reducing agents to protect against air oxidation. Based on their molecular weights, this calcium-independent family of lectins can be subdivided into a L14 (10-16 kD) group and a L30 (29-35 kD) group, both of which are readily solubilized by lactose in the absence of detergent. Calcium chelating agents had little effect on the carbohydrate-binding activity of LL35 indicating that LL35 is a calcium-independent lectin. LL35 had a clear preference for galactosides, with a slightly higher specificity for beta-galactosides. At 25 mM concentrations, beta-methyl-galactose and lactose completely inhibited LL35

binding. Other derivatives of galactose were less effective in inhibiting LL35 binding and corresponding mannose or glucose epimers the galactose derivatives did not affect LL35 binding.

Consistently greater amounts of LL35 were isolated from leech nerve cords when the extraction buffer contained both detergent and a reducing agent. The requirement for the reducing agent was only necessary during extraction. After extraction and in the absence of detergent and a reducing agent, LL35 retained its carbohydrate-binding activity. Ca-independent lectins may require reducing agents to retain their carbohydrate-binding activity only during their isolation. The rat lung and brain lectin, RL-29 (29 kD), can be stored for several days in the absence of reducing agents without losing binding activity (Cerra et al., 1985) and the recombinant form of the IgE-binding lectin, εBP (31 kD), is resistant to air oxidation (Frigeri, 1990). Likewise, detergent was only necessary during extraction of LL35. Although LL35 may bind detergent, the retention of carbohydrate-binding activity after the removal of detergent, as well as the ability to isolate some LL35 in the absence of detergent, implies that LL35 is a soluble lectin associated with membranes through interactions with carbohydrates moieties of membrane glycoconjugates. Detergent was included in the extraction buffer to insure complete extraction of LL35.

Although sequencing data is not yet available, based on the LL35 binding characteristics, I propose that LL35 is a member of the vertebrate L30 group of calcium-independent beta-galactose-binding lectins. The calcium-independent galactose-binding proteins were previously found only in vertebrates. Recently, the nematode was

demonstrated unequivocally to possess a 32 kD galactose-binding protein homologous to the vertebrate calcium-independent lectins (Hirabayashi et al., 1992). Interestingly, the nematode lectin is a tandem repeat of the L14 group. The function of these calcium-independent lectins is still unknown. However, it is now clear that this family of galactose-binding proteins is not unique to vertebrates, but appears to play fundamental roles in most animals. The discovery of lectins in less complex organisms, such as the nematode and the leech, will aid in elucidating the functions of these lectins.

Interestingly, a 16 kD lactose-binding protein (LL16) co-fractionated with LL35 from the leech nerve cord. LL16 does not appear to be a breakdown product of LL35 because preliminary peptide mapping of limited digests of LL35 produced fragments of approximately 23 and 11.4 kD, not 16 kD. It will be interesting to determine whether LL16 is a member of the L14 group of galactose-binding proteins.

The other major leech lactose-binding lectin, LL63, was isolated from the whole leech but was not found in the nerve cord. Because LL63 is not present in the nerve cord, it is likely that LL63 is not a precursor of LL35. LL63 probably is involved in some process other than the development or function of neurons in general. The finding that LL63 does not bind aMeGal was fortuitous. Such clear discrimination of the lectin for galactose based on the orientation of the anomeric carbon allowed LL63 to be efficiently separated from LL35 and LL16. It also provided additional evidence that LL63 was not a precursor of LL35. The increased yield of LL35

when isolated from whole leech was not due to protein degradation of LL63 during isolation. If degradation of LL63 to LL35 had occurred, then a significant amount of LL35 would be expected to co-isolate with LL63. LL35 was clearly separated from LL63 by washing the ASF affinity column with aMeGal prior to lactose elution of LL63. Both LL35 and LL63 were able to distinguish between galactose and GalNAc, although in an opposite fashion. LL35 had a higher affinity for galactose than GalNAc, a property similar to that of the rhizobium bacterial lectin, BJ38 (Ho et al., 1990), and the plant lectin, ricin (Nicolson et al., 1974). In contrast, LL63 binds to GalNAc with a higher affinity than to galactose, a property similar to that of the slime mold lectin, discodin (Cooper et al., 1983), and the plant lectin, soybean agglutinin (Pereira et al., 1974). Therefore, I concluded that LL63 and LL35 were distinct proteins based on their different tissue distributions, different sugar specificities, and on preliminary peptide mapping studies indicating that LL35 and LL63 do not share peptide fragments.

LL35 and LL63, however, are immunologically related. Polyclonal antibodies raised against the LL35 gel band (anti-LL35D) and subsequently affinity purified against the LL35 band (anti-LL35D-35) cross-reacted with LL63. This suggests that LL63 and LL35 share common epitopes. A similar relationship was found between the L14 and L30 lectins and the 67 kD elastin or laminin receptor (Castronovo et al., 1992; Hinek et al., 1988). Thus LL16, LL35, and LL63 appear to constitute a family of distinct but related lactose-binding proteins with many similarities to the L14, L30, and 67 kD laminin receptor family of related cell surface multi-

functional beta-galactose-binding proteins. The functions of these lectins are as of yet unknown. The ability to isolate as much as 100 ug of LL35 or LL63 from one leech provides a source for large quantities of these lectins that will be useful in elucidating their functions.

CHAPTER 3. CARBOHYDRATE-BINDING PROTEINS IN THE LEECH. II. LACTOSE-BINDING PROTEIN, LL35, IS LOCATED IN NEURONAL AND EPITHELIAL TISSUES.

INTRODUCTION

The binding of carbohydrate ligands to complementary carbohydrate-binding proteins, or lectins, on cellular membranes has been demonstrated to mediate gamete recognition, clearance of serum glycoproteins, lymphocyte homing, and phagocytosis (reviewed in Jessell et al., 1990; Sharon and Lis, 1989). The restricted distribution of lectins and carbohydrate structures to subsets of neurons (Bastiani et al., 1987; Regan et al., 1986; Scott et al., 1990; Stewart and Touloukian, 1990), growth cones (Dodd et al., 1984; Pfenninger et al., 1984; Reichert and Meier, 1990), and at synaptic sites (Groswald and Kelly, 1984; Grunwald et al., 1985; Sanes and Cheney, 1982; Scott et al., 1990; Scott et al., 1988) during periods of synapse formation and axonal growth (Bezamahouta et al., 1988; Denburg, 1989; Dontenwill et al., 1985; Zanetta et al., 1978) suggests that lectins may mediate many aspects of neuronal development or function.

A group of mannose-binding lectins (Dontenwill et al., 1985; Kuchler et al., 1992; Zanetta et al., 1985) and galactose-binding lectins (Hynes et al., 1990; Joubert et al., 1989; Kuchler et al., 1989; Regan et al., 1986) in the nervous system have received the most attention. The galactose-binding proteins are members of the calcium-independent lectins which, based on their molecular

weights, can be subdivided into an L14 (10-16 kD) group and an L30 (29-35 kD) group (reviewed in Kasai, 1990; Wang et al., 1991). The L14 group can be isolated from most vertebrate organs, whereas the L30 group has been isolated so far only from the lung (Crittenden et al., 1984; Sparrow et al., 1987), leukocytes (Clement et al., 1990; Ho and Springer, 1982; Lui et al., 1985), fibroblasts (Raz et al., 1988; Roff and Wang, 1983), melanomas (Raz et al., 1989), and neurons (Regan et al., 1986). Until recently these galactose-binding proteins were thought to be present only in vertebrates. However, a calcium-independent, beta-galactose-specific lectin with significant sequence homologies to vertebrate lectins recently was found in the nematode (Hirabayashi et al., 1992), indicating that these lectins have fundamental roles that are not restricted to vertebrates. The function of these lectins in any of these tissues is not known.

Understanding the function of endogenous calcium-independent lectins in the nervous system would be greatly facilitated by a model system that is less complex than the vertebrate nervous system and that is readily amenable to experimental manipulation. The leech nervous system provides such a model system. It is a well-characterized classical neurobiological model system. Its sensory afferent system is chemically coded with restricted distributions of carbohydrate epitopes (Bajt et al., 1990) and the projection of the sensory afferents into the synaptic region of the leech is mediated by a carbohydrate recognition event (Zipser and Cole, 1991; Zipser et al., 1989).

In Chapter 2, I reported the isolation and characterization of the leech lectin LL35. This calcium-independent lactose-binding

protein, with a molecular weight of 35 kD, was initially isolated from the membranes of the leech nerve cord, which represents the central nervous system of the leech. Because LL35 could be isolated in large amounts from the whole leech (25-100 ug per leech), it was necessary to determine the tissue distribution of LL35. Determined immunocytochemically, LL35 is restricted to a few cell types. Among neurons, LL35 is expressed by a small subset of sensory afferents. Among muscle cells, LL35 is only expressed by the CNS muscle but not by any other peripheral muscle. And, LL35 is expressed by all epithelial cells.

MATERIALS AND METHODS

Leech embryos and adult nerve cords

Mud leeches, *Haemopis marmorata*, were obtained from Rochester Hollow Grinder (NY) and either bred at room temperature or maintained at 5°C in aerated aquaria containing dilute artificial seawater (Forty Fathoms; 0.5 gm/liter distilled water). Breeding leeches were fed chicken liver or frozen brine shrimp three times a week. After four to six weeks, gravid leeches were placed in plastic boxes containing moist sphagnum moss. Boxes were checked daily for cocoons. Once found (day 0), the cocoon was placed in a 120 ml cup with moist sphagnum moss and maintained in the incubator at 20°C ± 1°C.

Leech nerve cords (CNS), consisting of the supra- and subesophageal ganglia and the segmental ganglia, were dissected from anesthetized non-breeding leeches and stored at -70°C.

Cyrosections of whole leeches

Leeches were anesthetized by immersion in 10% cold ethanol and pinned to a piece of cardboard covered with parafilm. The pinned leech was placed in a cooler and quickly frozen by surrounding it with dry ice. The frozen leech was cut into sections and stored at -70°C. Forty micron sections of the leech clitellum region were cut at -25°C on a cyrostat microtome and mounted onto polylysine-gelatin coated slides. Sections, stored at -70°C, were fixed in 4% paraformaldehyde in 50 mM phosphate, pH 7.4, for 30 min, washed in

phosphate-buffered saline (PBS: 0.9% NaCl, 50 mM phosphate, pH 7.4) for 5 min, dipped once in distilled water, and either stained immediately or stored at -20°C.

Isolation of lactose-binding proteins from whole leech

The following buffers were used in the isolation of lactose-binding proteins: Buffer 1 (150 mM NaCl, 10 mM Tris base, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2% NaN_3, 1 mM PMSF) and Buffer 2 (Buffer 1 containing 1 mM mercaptoethanol). Crude membrane fractions were prepared from 30 leeches. After anesthetizing in cold 10% ethanol, their digestive tracts were opened and flushed with PBS. They were then homogenized in 50 ml of Buffer 2 for 3 min at 4°C with a polytron homogenizer. The homogenate was centrifuged at 104,000 xg for 60 min at 4°C. The supernatant was removed and the pellet was resuspended in 30 ml of Buffer 2. Proteins were extracted by adding 1.75 ml of 20% Triton X-100 and incubating on ice for 1 h. The Triton X-100 extracts were passed through a 20 ml syringe containing 12 ml of ASF-Affigel-15 beads that had been washed with 20 ml of 25 mM lactose and equilibrated with 1% Triton X-100 in Buffer 2. After washing the with 100 ml Buffer 1, the column was washed with 25 mM lactose in Buffer 1. Proteins eluting from the column were monitored at 280 nm. Fractions containing lactose-binding proteins were pooled and concentrated by centrifugation using a Centricon-10 filtering apparatus (Amicon). A sample of the lactose-binding proteins was removed for protein estimation and the rest was aliquoted and stored at -70°C.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein samples were heated at 100°C in sample buffer for 4 min and electrophoretically separated on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels (Laemmli, 1970). Immunoblots were prepared by electrophoretically transferring separated proteins from SDS-polyacrylamide gels to nitrocellulose paper (Schleicher and Schuell, pore size 0.45 um) with applied voltage of 90 V (Garrels, 1979; Towbin et al., 1979). Unbound sites on the nitrocellulose were blocked by incubating immunoblots with either 10% powdered milk, 0.05% Tween 20, and 0.2% NaN_3 in PBS (milk blocking solution) or 3% BSA, 0.1% Tween 20, and 0.2% NaN_3 (BSA blocking solution) in PBS overnight at 4°C. Blocked immunoblots were incubated for 2-4 h with primary antibody (monoclonal or polyclonal, anti-LL at 1/1000 to 1/5000 dilution and anti-LL35D-35 at 1/10 to 1/100 dilution), rinsed, incubated again in the blocking solution, and incubated for 1-2 h with HRP-conjugated goat-anti-mouse IgG (1/250 dilution) (Cappel Laboratories) or HRP-conjugated goat-anti-rabbit IgG (1/250 dilution) (Biorad). After rinsing the immunoblot free of unbound antibody, antibody binding was visualized by the diaminobenzidine (DAB) reaction reaction. The apparent molecular weights of the proteins in immunoblots were estimated by applying to the gels prestained high molecular weight standards (BRL).

Monoclonal antibodies (mAb)

Monoclonal antibody Lan3-2 was derived by immunizing mice with homogenates of leech nerve cords (Zipser and McKay, 1981).

Monoclonal antibodies Laz6-297 (Flaster et al., 1983), Lan9-84 (Bajt et al., 1990) and Lan10-1 (Thorey and Zipser, 1991) were derived by immunizing mice with the proteins excised from the 130 kD region of a SDS-polyacrylamide gel of leech nerve cords.

Polyclonal antibodies

Polyclonal antiserum, anti-LL (anti-Leech Lectins), was derived by immunizing rabbits with native lactose-binding proteins. The lactose-binding lectins were isolated by passing Triton X-100 extracts of crude membranes prepared from whole leech homogenates through an ASF affinity column, eluting the lactose-binding proteins from the column with 25 mM alpha-methyl-galactose, and concentrating them by centrifugation using a Centrion-10 filter (Amicon). The concentrated lactose-binding proteins were washed with PBS to remove NaN_3 before immunizing the rabbits with the proteins. An anesthetized rabbit was immunized initially with approximately 100 ug of protein by intramuscular injection. After 29 days the rabbit was immunized a second time with approximately 250 ug of protein. Thirteen (9-10-91 bleed) and sixteen (9-13-91 bleed) days later 10 and 20 ml of blood, respectively, was collected through the ear vein of the anesthetized rabbit. Blood was incubated at 37°C for 1 h, then allowed to stand overnight at 4°C. Serum was separated from the blood clot by centrifugation and stored at -70°C. Both bleeds had high titers of antibodies, however, only the antiserum from the 9-10-91 bleed was used in the experiments describe in this chapter.

Affinity purification of polyclonal antibodies

Polyclonal antiserum, anti-LL35N (anti-Leech Lectin 35, Native form), was obtained by affinity purifying anti-LL antiserum against the 35 kD protein bands on western blots prepared from leech nerve cord proteins according to Smith and Fisher (1984). Thirty leech nerve cords were separated by electrophoresis on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The protein blot was blocked with 3% BSA, 0.3% Tween 20, 0.2% NaN_3 in PBS overnight at 5°C. The blocked blot was incubated with a 1:500 dilution of anti-LL with 3% BSA in PBS overnight at room temperature. The following day, the right and left edges were cut from the blot and primary antibody binding was visualized by incubating the strips with HRP-conjugated goat anti-rabbit IgGs (Biorad) for 1 h and developing with the DAB reaction. The region of the 35 kD bands was identified on the undeveloped blot and cut out. This band was cut into small strips and placed into an 1.5 ml microcentrifuge tube. The strips were washed for 15 min with three, 5 min washes of 0.5% Tween 20 in PBS and bound antibodies were eluted from strips with three, 30 sec washes of 200 ul of 0.1 M glycine-HCl in PBS, pH 2.3. The pH of the glycine-HCl elutant was immediately adjusted to pH 7 with 2 M Tris-base buffer, pH 11. The strips were washed again with 0.5% Tween 20 in PBS. A 1/500 dilution of polyclonal antiserum was reapplied to the washed strips overnight at room temperature. The cycle of binding and elution of polyclonal antibodies to specific bands on the strips of nitrocellulose was repeated three more times.

Eluted antibodies were dialyzed twice against cold distilled water and once against PBS over a 24 h period. The dialysate was concentrated by lyophylization and resuspended in a minimal volume of PBS containing 0.2% NaN_3 and stored at 4°C.

Immunocytochemistry

Embryos of a desired age were removed from their cocoons, opened dorsally to remove yolk, pinned out in a Sylgard-coated Petri dish, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. After fixation, embryos were post-fixed with absolute methanol for 30 min to remove endogenous peroxidase activity and then rinsed with PBS. Preserved embryos were incubated overnight in the monoclonal or polyclonal antibodies in 10% fetal calf serum, 0.2% Triton X-100, PBS, pH 7.4. Anti-LL was diluted to 1/5000. Anti-LL35N was used at dilutions ranging from 1/1 to 1/10. The primary antibody was removed by three, 5 min rinses with 0.5% Triton X-100 in PBS. Embryos were then incubated with biotinylated goat-anti mouse or rabbit Fab fragments (dilution 1/800) (Dako) in 10% fetal calf serum, 0.2% Triton X-100, PBS, pH 7.4 for 2-4 h. Embryos were again washed three times with 0.5% Triton X-100 in PBS and then incubated with horseradish peroxidase conjugated to avidin for 1-2 h. Visualization of the antibody binding to leech tissue was enhanced using the nickel-enhanced DAB reaction according to Lee et al. (1990). Briefly, after primary and secondary antibodies had been applied, the tissue was rinsed in 175 mM sodium acetate. Next, the tissue was incubated for 10 min in a fresh solution of 0.25 mg/ml of DAB and

2.5% nickel sulfate in 175 mM sodium acetate. The chromagen reaction was started with a few drops of 0.3% H_2O_2 and terminated by several washes of PBS. Stained embryos were mounted in Permount (Fisher Scientific Co.).

Adult nerve cords also were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature, but instead of post-fixing in methanol, nerve cords were dehydrated sequentially with 70, 95 and 100% ethanol, extracted with xylene, and returned to PBS through decreasing concentrations of ethanol. Rehydrated adult ganglia were incubated with antibodies in the presence of 2% Triton X-100 and then stained and mounted as described for staining embryos.

Fixed cyrosections were surrounded with three layers of rubber cement prior to applying primary antibodies. Primary antibodies were diluted in 10% FCS, 0.2% Triton X-100, and 0.2% NaN_3 in PBS and incubated on sections in a moist chamber at room temperature overnight. Sections were rinsed briefly with 0.5% Triton X-100 in PBS, stained and mounted as described for staining embryos.

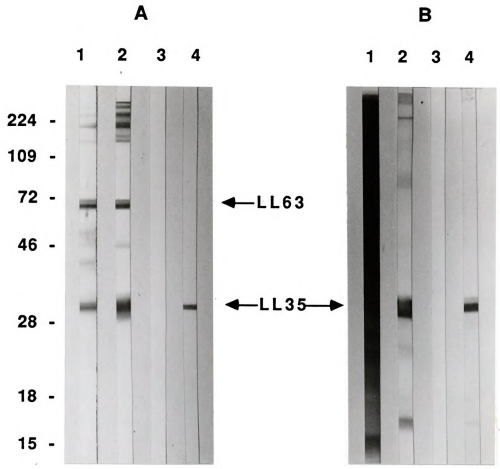
RESULTS

Affinity purification of polyclonal antibodies to LL35

Lactose-binding proteins were isolated from whole leeches using asialofetuin (ASF) affinity chromatography. In the presence of alpha-methyl-galactose (aMeGal), LL35 (a lactose-binding leech lectin with a molecular weight of 35 kD) plus small amounts of other proteins eluted from the ASF column (see Chapter 2). Immunization of rabbits with aMeGal-eluted leech lectins generated the polyclonal antiserum, anti-LL (anti-Leech Lectins). The binding specificity of anti-LL antiserum was tested on protein blots of the lactose-binding proteins eluted from an ASF column by lactose (Figure 18A and 18B). India ink staining of these protein blots revealed the positions of the two major lactose-binding proteins in the leech, LL35 and LL63 (Figure 18A, lane 1). Anti-LL antiserum bound to LL35, LL63, a group of high molecular weight proteins, and a minor band at 50 kD (Figure 18A, lane 2). The preimmune rabbit serum, in parallel analysis, did not stain any of the lactose-binding proteins (Figure 18A, lane 3). Because a small amount of LL63 contaminating the original immunization is unlikely, these data support the finding that LL35 and LL63 are immunologically related (Chapter 2).

Protein blots of nerve cord proteins were stained with anti-LL to test whether anti-LL antiserum also recognizes LL35 in the nerve cord (Figure 18B). India ink staining of protein blots of nerve cords

Figure 18. Binding specificities of anti-LL and anti-LL35N. Lactose-binding proteins from the whole leech (A) or proteins from leech nerve cords (B) were electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. Strips of these protein blots were stained for total protein or for LL35 with polyclonal antisera. Lanes: (1) India ink; (2) anti-LL, polyclonal antiserum generated by immunizing rabbits with native lactose-binding lectins (see Material and Methods); (3) rabbit preimmune antiserum; (4) anti-LL35N (anti-LL antiserum affinity purified against nerve cord LL35. Arrows on right mark LL35 and LL63 bands. Numbers in kD of MW standards are indicated on left.


A
B
1 2 3 4
1 2 3 4
224 -
109 -
72 -
46 -
28 -
18 -
15 -
LL63
LL35

showed a complex mixture of proteins with various molecular weights (Figure 18B, lane 1). Staining nerve cord blots with anti-LL antiserum revealed primarily LL35 (Figure 18B, lane 2), although two high molecular weight bands and a low molecular weight band at approximately 16 kD also were identified. On this blot, the low molecular weight band ran at 16 kD, however, this band also has been seen at 18 kD. It is not clear yet whether this low molecular weight band represents LL16 described previously (see Chapter 2). Again, the rabbit preimmune serum in parallel analysis did not bind to any of the nerve cord proteins (Figure 18B, lane 3). The absence of staining of a 63 kD band on blots of nerve cord proteins confirmed the previous observation that LL63 was not located in the nerve cord (see Chapter 2).

To isolate antibodies in the anti-LL antiserum that specifically recognize LL35 in leech nerve cords, the anti-LL antibodies were affinity purified against the LL35 band on protein blots of leech nerve cord proteins. These affinity purified antibodies were designated anti-LL35N (anti-Leech Lectin 35, Native form). Anti-LL35N only bound to LL35 on protein blots of leech lactose-binding proteins (Figure 18A, lane 4) or of nerve cord proteins (Figure 18B, lane 4). These polyclonal antibodies, anti-LL and anti-LL-35N, were used to examine the tissue distribution of LL35 in embryonic and adult ganglia.

LL35 is present in 14 day embryonic nervous system

Proteins of 14 day embryonic ganglia were immunoblotted with anti-LL antiserum to determine the presence of LL35. Comparing anti-LL immunostaining of adult and embryonic nerve cord proteins demonstrated that LL35 was present in 14 day embryonic ganglia (Figure 19). In blots of adult leech nerve cord proteins, 18 and 16 kD bands also were identified by anti-LL (Figure 19, lane 1). In contrast, only LL35 was recognized by anti-LL in 14 day old embryonic ganglia (Figure 19, lane 2). Presumably, the 16-18 kD bands are expressed latter in development. LL63, as expected, was not present in the developing nerve cord.

Distribution of LL35 in the embryonic and adult leech nervous system

Halfway through embryonic development (14 day embryos) the lactose-binding lectin, LL35, is expressed on a small subset of neuronal cell bodies and axons which appear to belong to peripheral sensory neurons based on their distribution pattern. Anti-LL antiserum immunocytochemically stained neuronal cell bodies that are segmentally reiterated in the sensory organs, called sensillae, of the leech skin (Figure 20). These cell bodies project axons centripetally through peripheral nerves and enter the ganglia in single tracts via the anterior and posterior roots (Figures 21 and 22A). The axons innervated the neuropile, located in the center of the ganglia, in a strictly ipsilateral manner; axons from the skin on

Figure 19. LL35 is present in ganglia from 14 day old embryos. Proteins in 50 ug of adult leech nerve cord homogenate (lane 1) or in 9 embryonic ganglia (lane 2) were electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose. The protein blot was stained with anti-LL. Arrow on right marks the LL35 band. Numbers in kD of MW standards are indicated on left.

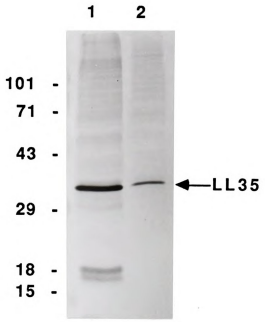
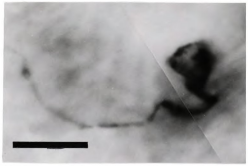

1
2
101
71
43
29
18
15
LL35

Figure 20 A neuronal cell body in the skin expresses LL35. Anti-LL bound to a neuronal cell body in the leech skin. The stained process of this neuron projected through peripheral nerves and entered the ganglion in a single tract via the posterior roots. It innervated the neuropile, located in the central of the ganglia, in a strictly ipsilateral manner (see Figs. 21 and 22). Bar represents 10 um



the one side of the leech projected into the neuropile in the same side of the ganglion. Axons did not terminate in the neuropile, rather they continued to grow into the interganglion axon tracts, termed connectives, which link the segmentally reiterated ganglia of the nerve cord. As a result, axons expressing LL35 extended as a bilateral tract system through the entire leech nerve cord, which represents the central nervous system (CNS) of the leech. Because none of the cell bodies in the CNS ganglia expressed the lactose-binding lectin, it is likely that all of the axons in the nerve cord which express LL35 originated from the cell bodies in the periphery. At this developmental stage, the only other cell types expressing this lactose-binding lectin in the nerve cord were epithelial cells (see also below, Figure 26) which covers the ganglia, connectives and roots (Coggeshall and Fawcett, 1964).

To verify that the species of lactose-binding lectin expressed by sensory afferents was LL35, the embryonic nervous system also was stained with the affinity purified polyclonal antibodies, anti-LL35N (Figure 22B). Anti-LL35N clearly stained the same axon tract pattern as anti-LL, although the staining was somewhat weaker. Interestingly, neither anti-LL or anti-LL35N stained axons in a smooth, uniform manner. Instead, staining appeared to have a punctate or dotted pattern along the axon tracts. Preimmune serum in parallel analysis did not show any staining in ganglia from 14 day old sibling embryos (Figure 22C).

To show that only a small subset of sensory afferents expressed the lactose-binding lectin, the entire population of sensory afferents was visualized by its mannose-containing Lan3-2

Figure 21. Schematic representation of the embryonic ganglia pictured in Figure 22. A chain of ganglia, joined by interganglionic connectives, form the nerve cord. The nerve cord is enclosed within the a blood sinus that lies ventral to the alimentary canal. The nerve cord consists of nerve, glia, and muscle cells and processes that are suspended in a dense feltwork of connective tissue and covered by a continuous layer of squamous epithelia cells. The bilateral longitudinal axon tracts stained by anti-LL and anti-LL35N are shown. The axons entered the ganglion from the periphery as shown and join the ipsilateral axon tract. Anterior is up. Bar equals 20 um.

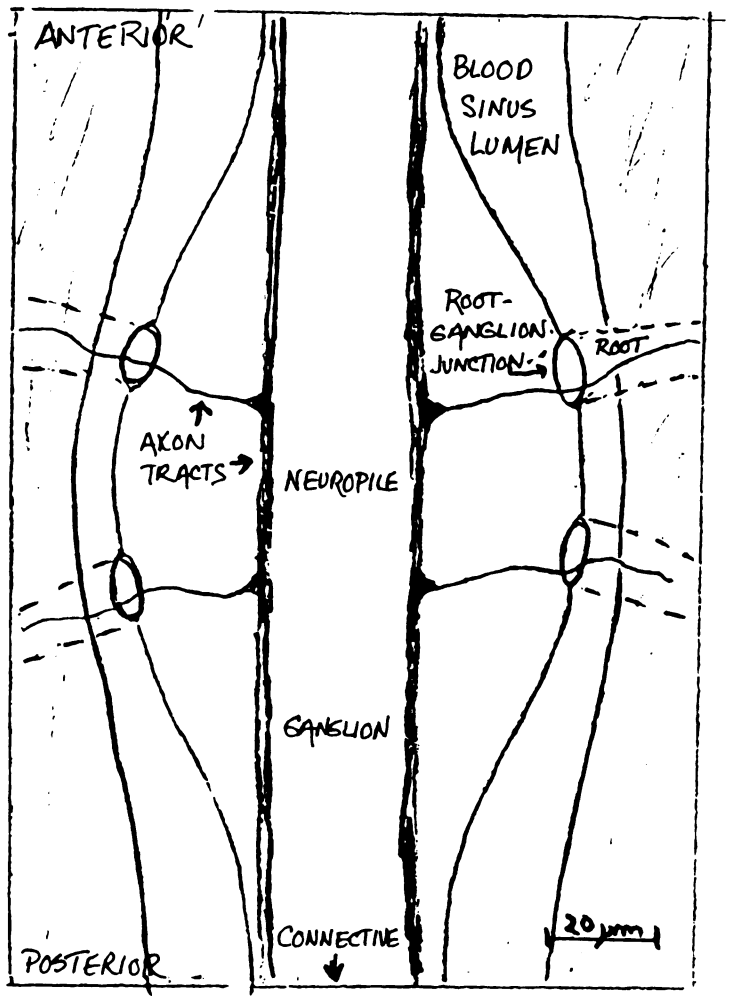
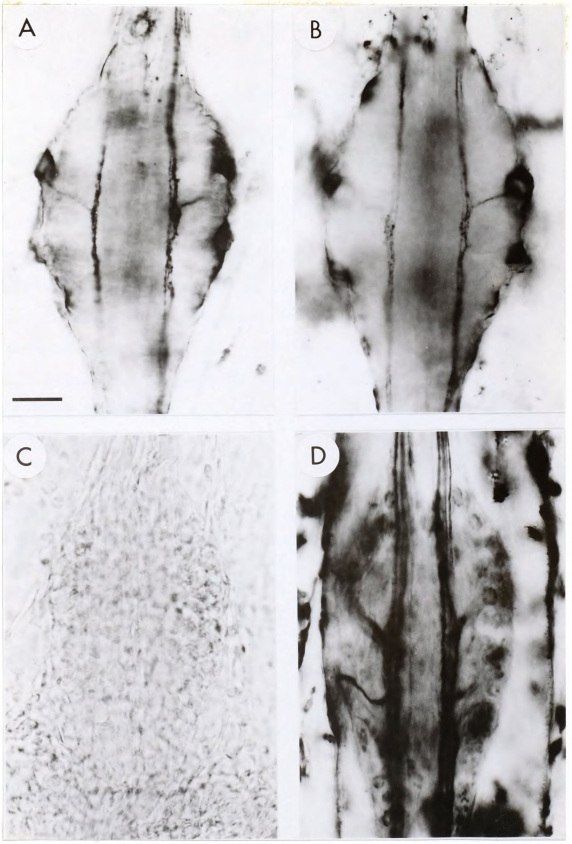

ANTERIOR
BLOOD SINUS LUMEN
ROOT-GANGLION JUNCTION
ROOT
AXON TRACTS
NEUROPILE
GANGLION
CONNECTIVE
20 μm
POSTERIOR

Figure 22. LL35 is expressed by a subset of axons in 14 day embryonic ganglia. Ganglia from sibling 14 day embryos at the same developmental stage were immunocytochemically stained with anti-LL (A), anti-LL35N (B), rabbit preimmune serum (C) or mAb Lan3-2 (D). Lan3-2 stains a functional set of axons, called the sensory afferents. Anti-LL and anti-LL35N stain a smaller set of axons than Lan3-2. Anterior is up. Bar equals 20 um.


A
B
C
D

epitope in a sibling 14 day embryo. This population of sensory afferents forms a considerably larger tract system, occupying a neuropile domain with a medial-lateral width of 8.5 um. In contrast, the small subset of sensory afferents which expresses LL35 only extends over a width of 2.8 um. It remains to be seen whether this small subset of sensory afferents which expresses this lactose-binding lectin corresponds to a previously studied subset of sensory afferent neurons. Four subsets of sensory afferent neurons that selectively express four different carbohydrate epitopes have been identified by monclonal antibody staining (Bajt et al., 1990; Johansen et al., 1992; Peinado et al., 1987; Peinado et al., 1990). The carbohydrate epitope of one of these subsets apparently expresses galactose-containing structures, because its respective mAb is blocked by galactose (Cole and Zipser, 1991; Song, unpublished data).

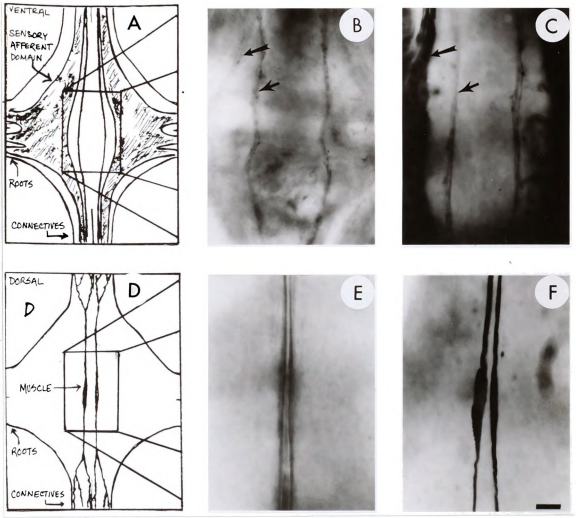
The onset of expression of the galactose-binding protein was examined during development. Sensory afferents present early in development did not possess detectable levels of lactose-binding lectin. At day 9, sensory afferents growing into the CNS ganglia can be visualized by their mannose-containing Lan3-2 epitope (McGlade-McCulloh, 1990; Zipser et al, 1989). However, even after an additional 2 days of development (11 day embryos), LL35 positive sensory afferents were not detected (data not shown). In 14 day embryos, LL35 positive sensory afferents were observed (Figures 20-22). The lactose-binding lectin was expressed more strongly in the anterior, more mature embryonic ganglia than in the less mature posterior ganglia. It is not known whether the subset of sensory

afferents that expresses the lactose-binding lectin differentiated late or whether LL35 appears with a delay onset on already existing sensory afferents. Just as the axonal subset expressing lactose-binding lectin appeared late in development, so do other subsets of sensory afferent axons [Laz2-369, Laz7-79, Lan2-3 (Johansen et al., 1992; Song and Zipser, 1992)] that are identified by the expression of carbohydrate epitopes.

LL35 persists on sensory afferents in the adult ganglion (Figure 23B), although in low abundance, as evidenced by the low intensity of antibody staining. As in the embryo, only a small subset of sensory afferents expressed LL35; the majority of the sensory afferents, again identified by their Lan3-2 mannose-epitope, did not express this lactose-binding lectin. In the adult ganglia, anti-LL35N stained sensory afferent axons which run in a medial tract. The medial tract had a similar diameter when its sensory afferent axons were visualized by their mannose-containing Lan3-2 epitope (Figure 23C), suggesting that most, if not all, of the afferents in this tract express the lactose-binding lectin.

In adult CNS ganglia, the lactose-binding lectin also is expressed on the muscle cells (Figure 23E). In each CNS ganglia, a single pair of muscle cells extended their processes anteriorly and posteriorly along either side of the midline on the dorsal surface of the neuropile and eventually branch into several smaller processes surrounding the two large bundles of axons in the connectives (Tulsi and Coggeshall, 1971) (Figure 23D). Anti-LL35N bound to these longitudinal muscles (Figure 23E). Previously, these muscle cells have been extensively studied with respect to their developmental

Figure 23. LL35 expression on a subset of axons and on the ganglionic muscles in adult ganglia. (A) Diagrammatic representation of the sensory afferent domains in the synaptic neuropile. Inset represents the neuropile region shown in B and C. (B) Anti-LL35N positive axons in the neuropile. (C) Lan3-2 positive sensory afferent axons in the neuropile. In B and C, the long arrow indicates the border of the main synaptic domain of the sensory afferents and the short arrow marks the anti-LL35N and Lan3-2 positive medial axons. (D) Diagrammatic representation of the longitudinal ganglionic muscles which is dorsal to the synaptic neuropile. Inset represents the neuropile region shown in E and F. (E) Anti-LL35N positive muscle processes in the ganglion. (F) Lan10-1 positive muscle cells and processes in the ganglion. Anterior is up. Bar equals 20 um.


VENTRAL
SENSORY AFFERENT DOMAIN
A
ROOTS
CONNECTIVES
B
C
DORSAL
D
D
MUSCLE
ROOTS
CONNECTIVES
E
F

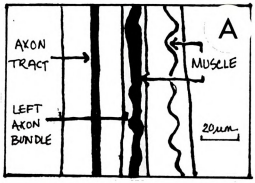
appearance using mAb Laz10-1 which recognizes a muscle-specific extracellular matrix molecule (Thorey and Zipser, 1991). The CNS muscle cells are the first differentiated cell type that so far has been described in the leech embryonic nervous system, preceding the sensory afferents by more than four days of development. However, they do not express LL35 in the 14 day embryo, as determined by staining with anti-LL and anti-LL35N. Thus, it is clear that these muscle cells exist long before they begin to express the lactose-binding protein.

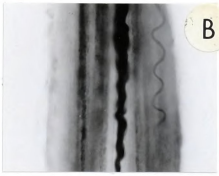
Co-expression of lactose-binding protein on sensory afferents and processes of CNS muscle in the adult nerve cord is shown in the connective which is a much thinner structure than the ganglion (Figure 24A and B). The fortuitous tilt in the connective allowed the superficial muscles processes to be photographed in the same focal plane as the axonal tract (Figure 24B). A group of anti-LL35N labeled axons appeared to be located in the center of each large bundle of axons (only the axon tract in the left bundle is shown). Muscle processes arranged around and between the axon bundles were consistently stained by anti-LL35N. Parallel analysis of rabbit preimmune serum did not stain any structures within the connective (Figure 24C).

Cross-sectional analysis of LL35 distribution in the adult leech

To survey the distribution of LL35 in peripheral organs in the leech, cryosections of an adult leech were stained with affinity-purified anti-LL35N. Figure 25 illustrates the major structures

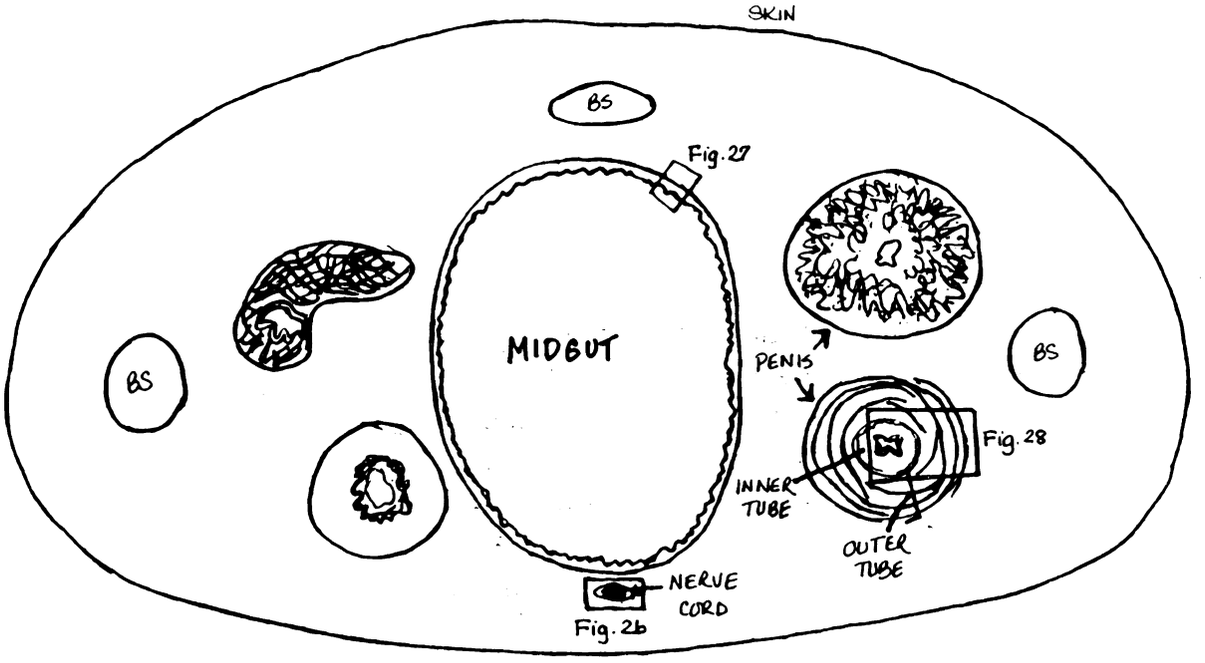
Figure 24. LL35 expression on axon tracts and muscle processes in adult interganglionic connectives. (A) Diagrammatic representation of the anti-LL35N positive structures shown in (B). (B) An anti-LL35N positive axon tract is shown in left lateral axonal bundle of the connective. Anti-LL35N positive muscle processes are arrange around the axonal bundles. Anti-LL35N muscle processes between right and left lateral bundles and a muscle process lying dorsal to the right axon bundle are shown. (C) A connective treated with rabbit preimmune serum. Bar equals 20 um.


A
AXON TRACT
MUSCLE
LEFT AXON BUNDLE
20μm


B


C

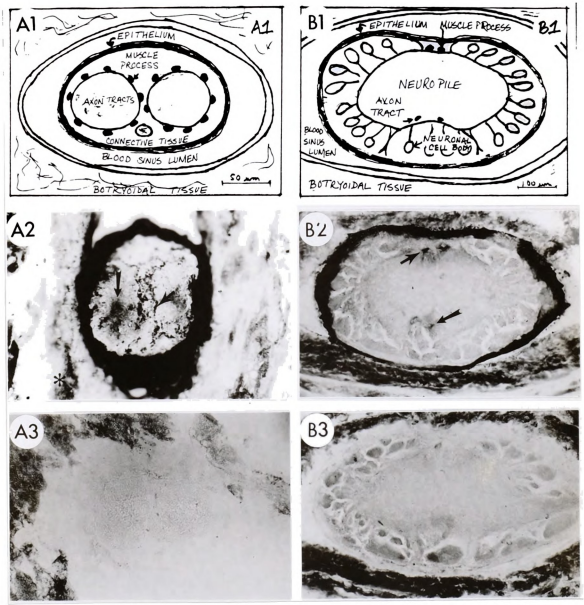
Figure 25. Schematic drawing of a cross-section of an adult leech. This section is taken from the anterior half of the leech which contains its sexual organs. The large lumen of the midgut is located in the center of the section. It is surrounded by the male and female sexual organs, which are looped structures, and blood vascular sinuses (BS). All of which is suspended in botryoidal tissue, a system of connected sinuses (not shown). Boxes indicate regions of cross-sections shown in Figures 26 through 28. The nerve cord (Fig. 26) is located within a blood sinus lying ventral to the gut (Fig. 27). The outer and inner tubes of the male organ (penis; Fig. 28) are indicated. Dorsal is up.


SKIN
BS
Fig. 27
MIDGUT
BS
BS
PENIS
Fig. 28
INNER TUBE
OUTER TUBE
NERVE CORD
Fig. 26

seen in cross-sections of the midbody region of the leech. The midgut occupies the center of the section. It is surrounded by sexual organs and blood sinuses that are suspended in botryoidal tissue (not shown). The botryoidal tissue consists of fine capillaries of the coelomic blood sinus system surrounded by globular, pigmented cells, forming a system of connected sinuses (Bradbury, 1959). The nerve cord, consisting of ganglia linked by the interganglionic connectives, is enclosed within a blood sinus that lies ventral to the alimentary canal. The three insets represent the nerve cord, gut, and penis cross-sections pictured in Figures 26, 27, and 28, respectively.

In cross-sections of the nervous system, LL35 was specifically localized to epithelia, muscle and an axonal subset, confirming the distribution of LL35 expression determined from examination of whole-mounted CNS ganglia. This pattern was observed in cross sections of the nerve cord, cut at the level of the interganglionic connectives (Figure 26A1-A3) and CNS ganglia (Figure 26B1-B3). As indicated diagrammatically, cross-sections of the interganglionic connectives (Figure 26A1) display two large lateral bundles and a small medial bundle (Faivre's nerve) of axons and glial processes. These bundles of axon tracts are surrounded by muscle cell processes and are suspended in a dense network of connective tissue. The illustration of ganglionic cross-sections (Figure 26B1) shows the relationship between the neuronal cell bodies and the synaptic neuropile in the ganglion. The synaptic neuropile is enveloped by neuronal cell bodies that are themselves enclosed within glial packets. These central neurons project

Figure 26. LL35 distribution in cryosections of adult leech nerve cords. Diagrammatic illustrations of a cross-section of the connective or ganglion are shown in A and B, respectively. Forty micron cryosections of connectives or ganglia were immunocytochemically stained with anti-LL35N (A2 and B2), no primary antibody (A3), rabbit preimmune serum (B3). Long arrow points to stained axon tracts; short arrow marks a muscle process; and star indicates naturally pigmented botryoidal tissue that is not immunostained. Anterior is up. Bar equals 50 um in A and 100 um in B.


A1
A1
EPITHELIUM
MUSCLE PROCESS
AXON TRACTS
CONNECTIVE TISSUE
BLOOD SINUS LUMEN
BOTRYOIDAL TISSUE
50 μm
B1
B1
EPITHELIUM
MUSCLE PROCESS
NEURO PILE
AXON TRACT
BLOOD SINUS LUMEN
NEURONAL CELL BODY
BOTRYOIDAL TISSUE
100 μm
A2
B2
A3
B3

processes into the neuropile and join with other central and peripheral axons to form synapses or axon tracts within the neuropile. The pair of CNS muscle cells lie dorsal to the neuropile. The entire nerve cord, both interganglionic connectives and ganglia, is covered by a continuous layer of squamous epithelial cells. The nerve cord is enclosed within the ventral blood sinus and is surrounded by green and brown pigmented botryoidal tissue. The most prominent expression of LL35 was found on the continuous layer of squamous epithelial cells surrounding the interganglionic connectives and ganglia (Figure 26A2 and B2). The apparent staining peripheral to the epithelial cells reflects the natural pigmentation of the botryoidal tissue as demonstrated in corresponding sections staining with no primary antibody (Figure 26A3) or preimmune serum (Figure 26B3).

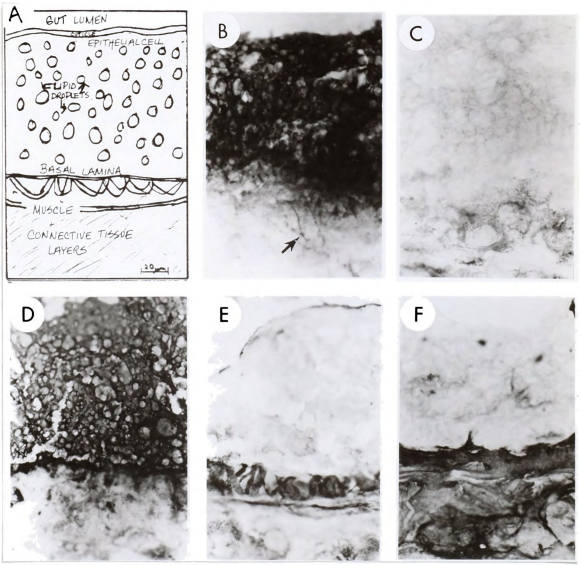
As seen in Figures 26A2 and B2, axons expressing LL35 occupied the ventral half of the large lateral bundles of axons and the ventral aspect of the ganglionic neuropile as is typical for sensory afferent neurons (Bajt et al., 1990; Hockfield and McKay, 1983; Peinado et al., 1987; Peinado et al., 1990). LL35 also was found on processes of muscle cells surrounding the axonal tracts of the interganglionic connective and lying dorsal to the neuropile. In contrast, LL35 was not expressed by other cell types in the nerve cord: the cell bodies of central neurons (26B1 and B2), the four types of macroglial cells (Coggeshall and Fawcett, 1964) and the fibroblasts in the connective tissue layer surrounding the axonal tracts (Bajt et al., 1990; Thorey and Zipser, in preparation).

In the gut, only epithelial cells and axons expressed LL35. Shown diagrammatically, the epithelium of the gut is a folded, unicellular layer of columnar epithelial cells (Hammersen and Pokahr, 1972) (Figure 27A). Adjacent epithelial cells are connected by desmosomes at the luminal end and the boundary between cells is irregularly discontinuous (Hammersen and Pokahr, 1972). Epithelial cells are attached to a basal lamina below which are layers containing connective tissue and muscle. In well-fed leeches such as these, the epithelial cells expand in size and contain numerous lipid droplets, which serve as an energy source during periods of starvation (Jennings and Van Der Lande, 1967).

Anti-LL35N staining of gut epithelial cells was profound and appeared to be greatest in the basal portion of the cell (Figure 27B). The lipid droplets did not enclose LL35, but appeared to be surrounded by LL35. The only staining visible in the underlying muscle and connective tissue layers were neuronal processes in the neuronal plexus (Abraham and Minker, 1958, Hogg and Zipser, 1983) below the epithelium. Rabbit preimmune serum did not stain any of the structures in corresponding sections of the midgut (Figure 27C). As had been previously reported (Hogg et al., 1983), mAb Lan3-2 also stains the gut epithelium (Fig 27D). The thickness of the epithelial cell layer was independently verified by staining parallel sections with Lan3-2.

LL35 was not expressed by gut muscle cells found below the epithelial layer. Staining the gut with muscle-specific mAb Laz10-1 (Thorey and Zipser, 1991), revealed bands of muscles just below the basal laminae (Fig 27E). These muscles and the deeper circular

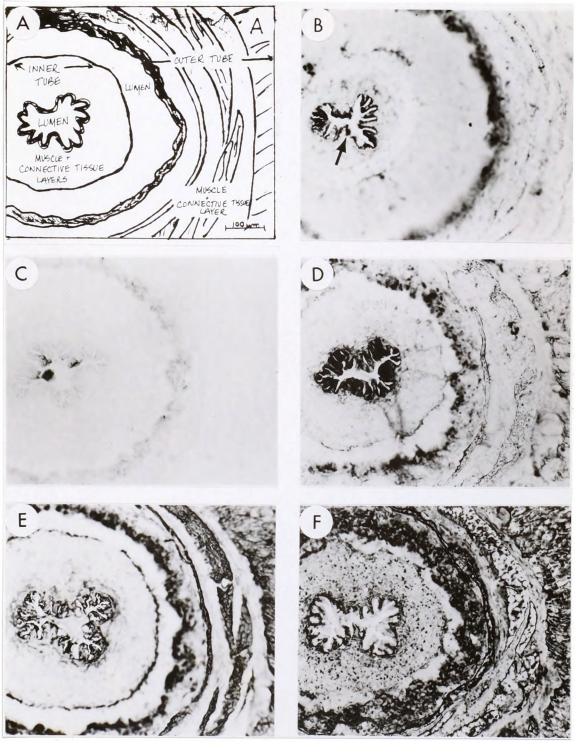
Figure 27. LL35 distribution in the epithelial layer of the midgut. (A) Diagrammatic illustration of the unicellular layer of columnar epithelial cells in the midgut. Forty micron cryosections of leech midgut were immunocytochemically stained with: (B) anti-LL35N; (C) rabbit preimmune serum; (D) sensory afferent mAb, Lan3-2; (E) muscle mAb, Laz10-1; (F) connective tissue mAb, Laz9-84. Arrow points to anti-LL35N stained neuronal processes. Gut lumen is up. Bar equals 20 um.


A
GUT LUMEN
EPITHELIAL CELL
LIPID DROPLETS
BASAL LAMINA
MUSCLE
CONNECTIVE TISSUE LAYERS
10
B
C
D
E
F

and longitudinal muscles in the gut did not express LL35. This is in contrast to the expression of LL35 by CNS specific muscle cells. LL35 also is not expressed by cells of the connective tissue layer, which secrete the 130 kD connective tissue protein recognized by mAb Laz9-84 (Figure 27F) (Bajt et al., 1990).

The restricted expression of LL35 to epithelial cells and neurons in peripheral organs was confirmed in cross-sections of the leech penis. As indicated diagrammatically, the leech penis consists of an inner and outer tube (Figure 28A). The outer tube has a thick wall consisting of longitudinal muscle cells. The wall of the inner tube also is muscular but is approximately four times thinner. When the muscle cells of the outer tube contract and shorten, the inner tube is forced through the gonopore, which leads to penile eversion. The inner surfaces of the inner and outer tube, as well as the outer surface of the inner tube, are lined with epithelial cells. LL35 is most prominently expressed by the epithelial cells lining the lumen of the inner tube (Figure 28B) and less prominently by the epithelial layer on the outer surface of the inner tube. In addition, areas with a diameter of 0.5 to 1 micron throughout the outer tube were strongly stained by anti-LL35N. The diameter and frequency these stained areas suggest that they represent single sensory afferents or small bundles of sensory afferents. Penile sensory afferents have been described (Passani et al., 1991). The presence of LL35 on sensory afferents was confirmed by staining whole-mounts of penis sheath (not shown). Parallel staining of cryosections with rabbit preimmune serum demonstrated that only the inner surface of the outer tube was nonspecifically stained (Figure 28C).

Figure 28. LL35 distribution in the leech penis. (A) Diagrammatic illustration of the leech penis showing the relative positions of the inner and outer tubes. Forty micron cryosections of leech penises were immunocytochemically stained with: (B) anti-LL35N; (C) rabbit preimmune serum; (D) sensory afferent mAb, Lan3-2; or (E) muscle mAb, Laz10-1; (F) connective tissue mAb, Laz9-8. Bar equals 100 um.


A
A
INNER TUBE
OUTER TUBE
LUMEN
LUMEN
MUSCLE & CONNECTIVE TISSUE LAYERS
MUSCLE CONNECTIVE TISSUE LAYER
100 µm
B
C
D
E
F

The distribution of epithelial cells on the inner lumen was independently confirmed by staining with mAb Lan3-2 (Figure 28D). As in the gut, LL35 was not expressed in the different layers of penile muscle, visualized with Laz10-1 (Figure 28E), nor was LL35 contained in the different layers of connective tissue, visualized with mAb Laz9-84 (Figure 28F). Thus, in peripheral organs expression of the lactose-binding protein, LL35, is restricted to epithelial cells and neurons.

DISCUSSION

Using affinity-purified polyclonal antibodies to LL35, I have shown that LL35 is immunocytochemical localized to neurons and epithelial cells (Table 4). Neurons and epithelial cells differentiate from the same blastomeres during development (Weisblat and Shankland, 1985), so it is not surprising that these two related tissues each express LL35. LL35 also is expressed by longitudinal ganglionic muscles in the adult nerve cord. This is intriguing, because all other types of muscles cells are LL35 negative.

LL35 has a highly restrictive distribution in the nervous system. It is only found in a small subset of neurons. This small neuronal subset belongs to the large population of sensory afferent neurons whose cell bodies are associated with epithelial tissues. Different subsets of the leech sensory afferents neurons can be distinguished based on the expression of different surface molecules (Peinado et al., 1987). These subset-specific markers have been identified as carbohydrate in character (Bajt et al., 1990). This is the first example of a subset of leech sensory afferents being identified by a carbohydrate-binding protein.

Likewise, in the vertebrate nervous system, carbohydrate-binding proteins are specific markers for neuronal subsets. Members of the L14 and L30 galactose-binding lectins are present in the vertebrate nervous system (Regan et al., 1986). The L30 neuronal lectin has a more restricted distribution than the L14 group. The L30 and L14 neuronal lectins are expressed by subsets of sensory

Table 4. TISSUE DISTRIBUTION OF LL35 IN THE LEECH

Organ	Cell Type	Anti-LL35N Staining	
		Adult	Embryo
CNS	axon subset	+	++
	epithelia	++	+
	muscle	+	-
	connective tissue	-	-
	glia	-	-
Gut	nerve	+	
	epithelia	++	
	muscle	-	
	connective tissue	-	
Skin	epithelia	+	
Penis	nerve	+	
	epithelia	++	
	muscle	-	
	connective tissue	-	

axons that terminate in the superficial layers of the dorsal horn of the spinal cord. L14 is also present in all spinal motoneurons and has been suggested to be a marker for spinal motoneuron differentiation (Hynes et al., 1990). On the other hand, L30-like lectins may be a specific marker for or have a specific function in a subset of sensory neurons.

Vertebrate Ca-independent galactose-binding proteins are developmentally regulated. The same lectin that is expressed at high levels during the development of one tissue and subsequently at low levels in the adult may have the reverse temporal expression in another tissue (Beyer and Barondes, 1982). Likewise, LL35 is developmentally regulated. During early embryogenesis, LL35 is expressed in high amounts by the neuronal subset and is absent from the CNS muscle at a time when epithelial cells have only begun to express LL35 weakly. In contrast, in the adult leech, LL35 is only weakly expressed by the neuronal subset and it is more clearly visible in the ganglionic muscle, while the expression of LL35 in epithelial cells is intense.

The tissue distribution of LL35 bears a striking resemblance to the distribution of vertebrate class of Ca-independent galactose-binding proteins. Based on the intensity of staining by anti-LL35N, leech epithelial tissues (skin, lining of the gut, endothelium) were extremely rich in LL35. Ca-independent galactose-binding lectins are abundant in the skin of all vertebrates, and in frog skin these proteins account for as much as 5% of the soluble protein (Marschal et al., 1992). Their secretion in response to stressful stimuli (Bevins and Zasloff, 1990) suggests a defensive function. They may

form a chemical defense barrier to prevent the colonization or invasion of bacteria or fungi in the mucus layers on the skin. Other functions have been suggested, such as modulation of cell adhesion to basement membranes or cell proliferation.

The vertebrate intestine is also rich in galactose-binding lectins comprising up to 1% of the soluble protein (Leffler et al., 1989). A chemical defense barrier of galactose-binding proteins in the intestine seems less likely. Playing a regulatory role in glycosylation by affecting the interaction of enzymes with beta-galactoside substrates has been suggested (Ruggiero-Lopez et al., 1992). One intestinal galactose-binding protein has been demonstrated to be immunologically similar to εBP and to bind IgEs. This lectin may serve as a transepithelial carrier of IgE and IgE complexes and may play a role in mediating food allergic disorders (Brassart et al., 1992). In the leech, the apparent association of LL35 with vesicles implies that it may be involved in some endocytotic or exocytotic process, or in vesicular traffic within the epithelium.

Involvement in transepithelial trafficking is an attractive possibility for LL35 in the endothelium covering the leech nerve cord. The leech nerve cord is not vascularized but lies within a blood sinus beneath the gut. Nutrients must diffuse or be transported to the nerve, glial, and muscle cells within the nerve cord. LL35 may facilitate the movement of nutrients from the hemocoel to the cells within the nerve cord.

Interestingly, the lactose-binding lectins found in the vertebrate skin and intestine are either monomers or dimers of the

L14 class Ca-independent galactose-binding lectins (Leffler et al., 1989; Marschal et al., 1992; Muramoto and Kamiya, 1992). Lactose-binding proteins of the L30 group have not been reported. In leech skin and intestine, monomers of L14-like lectins may also be present. However, the leech skin and intestine also contain a L30-like lactose-binding lectin, LL35.

Sequencing data and complementary DNA analysis indicate that all of the vertebrate L30 lectins are homologous (Cherayil et al., 1990; Cherayil et al., 1989; Jia and Wang, 1988; Oda et al., 1991; Raz et al., 1988; Raz et al., 1989; Robertson et al., 1990; Sakakura et al., 1990; Woo et al., 1990; and reviewed in Wang et al., 1991). This implies that these lectins, located in the skin and in the immune, nervous, and pulmonary systems, have similar functions in cell differentiation and proliferation, morphogenesis, transformation, and homeostasis. However, a function for these lectins still remains to be established. Recently, a L30 lectin from the nematode was identified and sequenced (Hirabayashi et al., 1992). This L30 nematode lectin differs from the vertebrate L30 lectins in that it is a covalent tandem repeat of two L14 lectins. It is suggested that the nematode L30 lectin may be involved in cuticle formation. A beta-galactose-binding lectin that exists as a dimer of two 34 kD proteins was isolated from the gonads of the mollusk *Aplysia*. This lectin, APL, was shown to enhance neurite outgrowth and viability of cultured *Aplysia* neurons (Wilson et al., 1992). Lectins from exogenous sources previously have been demonstrated to modulate the growth of isolated neurons (Chiquet and Acklin, 1986; DeGeorge and Carbonetto, 1986; Grumbacher-Reinert, 1989). However, an

interesting implication from the observations of the effects of *Aplysia* lectin on the *Aplysia* neurons is that the differentiation or maintenance of neurons in *Aplysia* may be hormonally regulated. The *Aplysia* lectin could be involved in hormonal regulation of neurons during periods of reproductive behavior. Seasonal variations in lectin production has been observed in mollusks (Muramoto et al., 1991).

It remains to be determined whether LL35 has enzymatic activity. Glycosyltransferases are functionally similar to lectins in that they bind to specific carbohydrate chains, but they also mediate the transfer of saccharides from a nucleotide-sugar to an oligosaccharide chain. Cell surface glycosyltransferases have been proposed to behave as cellular recognition molecules (Roth et al., 1971). In the absence of the nucleotide sugar they bind to specific sequences and terminate that binding in the presence of the appropriate nucleotide sugar. Galactosyltransferases have been shown to participate in the migration of neural crest cells and initiation of neurites on substrates of laminin (Begovac and Shur, 1990; Runyan et al., 1986) and during neurulation *in vivo* (Hathaway and Shur, 1992). Galactosyltransferases also modulate the function of other neuronal adhesion molecules by affecting their interaction with the cytoskeleton (Balsamo and Lilien, 1990; Balsamo et al., 1991; Gaya-Gonzalez et al., 1991).

Currently, there are only three neuronal model systems in which a functional role has been directly demonstrated for carbohydrate recognition in the nervous system: chick neural crest migration (Hathaway and Shur, 1992); pioneer axons in the cockroach

(Wang and Denburg, 1992); and leech sensory afferents (Zipser and Cole, 1991; Zipser et al., 1989). However, there is no direct evidence demonstrating an interaction between endogenous glycoconjugates and complementary lectins *in vivo*. The presence of LL35 on a small subset of sensory afferents and on CNS muscle, but not on peripheral muscles, suggests that LL35 participates in nervous system-specific functions. In cultured leech embryos, the developing nervous system is readily permeable to macromolecules (Zipser and Cole, 1991; Zipser et al., 1989). Thus, through direct application of isolated LL35 or Fabs of anti-LL to the embryonic leech, the function LL35 can be investigated directly in a virtually intact nervous system in its normal microenvironment. These types of studies will provide insight into the role of carbohydrate recognition in the nervous system.

SUMMARY AND CONCLUSIONS

Carbohydrate recognition in the nervous system is postulated to mediate many aspects of neurogenesis including neuronal migration, axonal growth, and synapse formation (reviewed in Jessell et al., 1990). Carbohydrate recognition is involved in patterning of the leech nervous system (Zipser et al., 1989; Zipser and Cole, 1991). The overall objective of this thesis was to identify and characterize candidate carbohydrate recognition molecules that potentially are involved in mediating neuronal pattern formation in the leech. Two of these molecules, a 130 kD glycoprotein expressed on glial cells and a lactose-binding protein expressed on a subset of neuronal and epithelial cells, are described in this thesis.

In Chapter 1, the onset of expression of the glial glycoprotein is described during the development of the leech nervous system. The glial glycoprotein was used as a marker to demonstrate that glial processes are located at key sites of morphogenic movement and neuronal differentiation in the leech embryonic nervous system. A midline glial fascicle, bearing the glial glycoprotein, resides at the primary axis of symmetry in the embryo, alongside which teloblasts move as they generate columns (or bandlets) of stem cells. The n-bandlets straddle the midline glia and are known to produce most of the central neuroblasts. The midline glia fascicule eventually defasciculates as neuroblasts begin to aggregate into neuromeres. These defasciculated processes expand into the neuromeres, appearing to mold the future central neuropile. The

aggregating neuroblasts will initiate primary axons towards the midline glia. As the neuromeres mature, midline glial processes thin out to demarcate the orientation of the future connectives, the major longitudinal axon tracts that project along the midline. Next, segmental but still primordial glia appear in the neuromeres. Initially, these embryonic glial cells also project processes longitudinally, then transversely, demarcating the other two major axonal pathways - the commissures traversing the neuromeres and peripheral roots. Finally, processes of the adult glial cells proliferate as massive axon growth invades the central and peripheral nervous systems. Thus, glial processes with different developmental histories accompany different aspects of leech neurogenesis and appear to prefigure the nervous system. In other systems, glia have been shown to promote the differentiation and the guidance of neurons. It remains to be seen whether the glial specific 130 kD glycoprotein is a receptor mediating these typical glial functions during the development of the leech nervous system.

Carbohydrate recognition involves a glycoconjugate binding to a carbohydrate-binding protein (lectin), however, there is no information on leech lectins. In Chapter 2, the isolation and characterization of two lactose-binding proteins with molecular weights of 35 kD and 63 kD, termed Leech Lectin 35 (LL35) and Leech Lectin 63 (LL63) from membrane fractions of the leech are described. LL35 and LL63 are related immunologically, however, they can be separated based on their different sugar affinities and tissue distributions. Because the nervous system contains LL35 and not LL63, most attention was focused on the characterization of

LL35. LL35's saccharide binding activity is calcium-independent, active over a wide pH range, and sensitive only to galactose derivatives, with strong preferences for beta-galactosides. Detergent and reducing agents were necessary to insure complete extraction and retention of LL35 binding activity. These characteristics are similar to a class of calcium-independent galactose-binding lectins first described in vertebrates, but which recently have been described also in the invertebrate, *Caenorhabditis elegans*, a nematode (Hirabayashi et al., 1992).

In Chapter 3, the immunocytochemical location of LL35 in the adult and embryonic leech is described. LL35 is developmentally regulated. In the embryo, LL35 is most prominent on a small subset of sensory afferent neurons in the embryo. In the adult, LL35 is most prominent on epithelial cells. Also in the adult, LL35 is expressed on muscle processes present in the central nervous system. Muscle cells in various peripheral organs are devoid of LL35. Because LL35 is selectively expressed on a neuronal subset, it may mediate axonal projections during development. LL35 presence solely on muscle cells in nerve cords, the leech's central nervous system, but not on peripheral muscles supports the suggestion that LL35 is involved with nervous system-specific functions.

The leech nervous system is an excellent model system to study the role of carbohydrate recognition. The leech has a simple nervous system that is well-characterized. Groups of neurons are chemically coded with different carbohydrate epitopes (Bajt et al., 1990) and, as demonstrated in this thesis, carbohydrate-binding proteins (lectins). These candidate carbohydrate recognition

molecules are developmentally regulated and easily manipulated in cultured embryos (Zipser et al., 1989; Zipser and Cole, 1991). Because up to 100 ug of LL35 can be isolated from one leech, the function of this calcium-independent galactose-binding lectin can be readily studied in this classical neurobiological system. Thus, through culturing embryonic leeches in the presence of isolated LL35 or Fabs of antiserum generated against LL35, the function LL35 can be investigated directly in a virtually intact nervous system in its normal microenvironment. These types of studies in the near future will provide insight into the role of carbohydrate recognition in the nervous system.

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