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Differential Glycosylation of Tractin and LeechCAM, Two Novel Ig Superfamily Members, Regulates Neurite Extension and Fascicle Formation

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Abstract. By immunoaffinity purification with the mAb Lan3-2, we have identified two novel Ig superfamily members, Tractin and LeechCAM. LeechCAM is an NCAM/FasII/ApCAM homologue, whereas Tractin is a cleaved protein with several unique features that include a PG/YG repeat domain that may be part of or interact with the extracellular matrix. Tractin and LeechCAM are widely expressed neural proteins that are differentially glycosylated in sets and subsets of peripheral sensory neurons that form specific fascicles in the central nervous system. In vivo antibody perturbation of the Lan3-2 glycoepitope demonstrates that it can selectively regulate extension of neurites and filopodia. Thus, these experiments provide evidence that differential glycosylation can confer functional diversity and specificity to widely expressed neural proteins.

Neural cell adhesion molecules (CAMs)[1] belonging to the Ig superfamily are highly complex glycosylated molecules with often very restricted expression patterns and multiple functional specificities (Rathjen and Jessell, 1991; Walsh and Doherty, 1993). They have been shown to help mediate the correct patterning of neural connections by promoting selective outgrowth of specific neuronal populations and by organizing similar neurons into coherent fasciculated projections that may serve as guides for subsequently differentiating neurons (Goodman and Shatz, 1993; Johansen and Johansen, 1997). Their importance for selective axon pathway formation and maintenance is underscored by the consistent finding that their up- or down-regulation, perturbation, or ectopic expression in many cases leads to disrupted and disorganized axonal pathways and increased navigational errors (Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996). In addition, increasing evidence suggests that neural CAMs also participate in activity-dependent plasticity during development, as well as in synaptic plasticity in adults (Rutishauser and Landmesser, 1996; Fields and Itoh, 1996). A defining feature of the molecular structure of the neural CAMs of the Ig superfamily is the variability of their extracellular regions, which in most cases contain multiple tandemly arranged domains. This suggests that they may have several different binding sites that allow them to interact with an array of different proteins. Based on their overall domain organization and primary structure, these proteins can be categorized into molecules composed of (*a*) Ig-like domains only, (*b*) Ig-like domains in conjunction with fibronectin type III (FNIII)–like domains, and (*c*) Ig-like domains in combination with structural motifs other than that of FNIII (Brümmendorf and Rathjen, 1993; Vaughn and Bjorkman, 1996). Some of the CAMs have transmembrane and intracellular domains, whereas others are attached to the membrane by glycosylphosphatidylinositol anchors. In addition, the diversity in the structure of neural CAMs is amplified with the existence of many splice variants and various posttranslational modifications within each subgroup (Rathjen and Jessell, 1991).

One posttranslational modification that potentially could serve an important regulatory function for these molecules is differential glycosylation. It has long been recognized that the structural diversity of cell surface carbohydrates makes them ideal candidates for mediating cell-specific recognition processes (Roseman, 1971; Lis and Sharon, 1993; Dwek, 1995). That this is the case has been most clearly demonstrated by the process of lymphocyte homing, which is mediated by selectins that are capable of recognizing and binding ligands expressing specific oligosaccharide structures (Springer, 1994; Lasky, 1995). In the

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1. *Abbreviations used in this paper*: CAM, cellular adhesion molecule; CNS, central nervous system; FNIII, fibronectin type III.

nervous system distinct carbohydrate epitopes such as that recognized by anti-HRP antibody in insects and the HNK-1/L2 epitope in vertebrates have been demonstrated to be widely expressed on glycoproteins (Jessell et al., 1990). In addition, several carbohydrate epitopes of more restricted expression and distribution have been identified. For example, in the vertebrate olfactory system, stage- and position-specific carbohydrate antigens were found to be topographic markers for selective projection patterns of olfactory axons (Key and Akeson, 1991; Schwarting et al., 1992). A striking example of how the developmental regulation of glycosylation can affect neural pathway formation is provided by the modulation of the polysialic acid content of NCAM in the plexus region of the chick limb bud where its up-regulation allows the axons to defasciculate into their proper pathways (Tang et al., 1992, 1994). Furthermore, a carbohydrate moiety of a membrane-associated glycoprotein was shown to play a role in the segregation of afferent and efferent cortical axons in the white matter (Henke-Fahle et al., 1996). Thus, specific carbohydrate structures on neural proteins are promising candidates for assisting in patterning neural connections during development.

A particularly advantageous system in which to study the potential function of specific oligosaccharide modifications of neural proteins regulating pathway formation is in the projections of sensillar neurons in leech. Using mAbs and dye injections, the axons of sensillar neurons have been shown to project in tightly fasciculated bundles through the periphery into the central nervous system (CNS) where they bifurcate and segregate into four well-defined and stereotypically located fascicles in each of the central connectives (Johansen et al., 1992; Jellies et al., 1994; Briggs et al., 1995). At least five different mAbs (Lan3-2, Lan2-3, Lan4-2, Laz2-369, Laz7-79) that recognize different glycoepitopes specific to the entire population as well as distinct subsets of these neurons have been identified (McKay et al., 1983; Peinado et al., 1987; Bajt et al., 1990; Johansen et al., 1992; Zipser et al., 1994). Of these mAbs, the Lan3-2 antibody, which labels all sensillar neurons, has been the most extensively studied since it has been demonstrated that Fab fragments of Lan3-2 antibody can perturb normal fascicle formation in vitro (Zipser et al., 1989; Song and Zipser, 1995*a*), directly implicating a functional role for this epitope in pathway formation. The other four mAbs label subsets of the Lan3-2–positive sensillar neurons that selectively express different glycoepitopes that appear to be correlated with their choices of particular pathways. For example, early in embryogenesis the Lan4-2 antibody recognizes only a few peripheral neurons, the projections of which are confined to extending along only one of the four Lan3-2–positive fascicles (Johansen et al., 1992). Furthermore, perturbation studies with the mAbs Laz2-369 and Laz7-79 show that only the subset of neurites expressing the corresponding glycoepitopes is functionally affected by the incubation (Song and Zipser, 1995*b*). Thus, there is compelling evidence that a hierarchy of distinct carbohydrate structures expressed by specific neurons is involved in regulating neuronal pathway formation in this system. However, an impediment to the further analysis of these aspects has been a lack of knowledge of the molecules carrying the glycosylated epitopes.

In this study we have used immunoaffinity purification techniques with the Lan3-2 antibody to identify the molecules carrying the Lan3-2 and Lan4-2 glycoepitopes. We show that both epitopes are expressed on two novel Ig superfamily members, which we have named Tractin and LeechCAM. Tractin has a highly unusual structure: it contains six Ig-like domains, four FNIII-like domains, an acidic domain, twelve repeats of a novel proline- and glycine-rich motif, a transmembrane domain, and an intracellular domain with an ankyrin binding motif. Tractin possesses a potential cleavage site that in conjunction with other evidence suggests that the NH_2-terminal half of the molecule is secreted. LeechCAM has five Ig-like domains, two FNIII-like domains, and a transmembrane domain and may be a leech homologue of NCAM (Cunningham et al., 1987), FasII (Harrelson and Goodman, 1988), and Ap-CAM (Mayford et al., 1992). Tractin and LeechCAM are expressed by both peripheral and central neurons but are differentially glycosylated with the Lan3-2 and Lan4-2 epitopes only in the peripheral sensory neurons and their axonal projections. In vivo functional studies injecting purified Lan3-2 antibody into the germinal plate show that normal neurite extension and fascicle formation of the sensillar sensory neurons are impaired by the antibody, while the development and projections of central neurons expressing Tractin and LeechCAM but not carrying the Lan3-2 glycoepitope are indistinguishable from controls. These findings suggest that differential glycosylation of widely expressed neural CAMs can functionally regulate neuronal outgrowth and fascicle formation of distinct neuronal subpopulations.

Materials and Methods

Experimental Preparations

For the present experiments we used the two hirudinid leech species *Hirudo medicinalis* and *Haemopis marmorata.* The leeches were either captured in the wild or purchased from commercial sources. Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition (mM): 110 NaCl, 4 KCl, 2 $CaCl_2$, 10 glucose, 10 Hepes, pH 7.4. In some cases, 8% ethanol was added and the saline solution was cooled to 4°C to inhibit muscle contractions. Breeding, maintenance, and staging of *Hirudo medicinalis* embryos at 22°–25°C were as previously described (Fernández and Stent, 1982; Jellies et al., 1987), except that embryos were maintained in embryo water that was made as sterile-filtered solutions of 0.0005% commercial sea salt (Instant Ocean). Embryonic day 10 (E10) was characterized by the first sign of a tail sucker, while E30 is the termination of embryogenesis. There are ~10–20 embryos in each cocoon and these sibling embryos develop synchronously.

Protein Purification and Microsequencing

Purification of the Lan3-2 glycoproteins was achieved by constructing an immunoaffinity column of protein G–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) to which purified mAb Lan3-2 had been covalently coupled by dimethyl pimelimidate dihydrochloride (Pierce Chemical Co., Rockford, IL) according to the method of Schneider et al. (1982) at a ratio of 2.5 mg mAb per ml of matrix. The mAb Lan3-2 was purified from hybridoma culture supernatant grown in DME (GIBCO BRL; Gaithersburg, MD) containing the serum-free supplement Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN) by binding to a protein G–Sepharose matrix. Purified Lan3-2 antibody eluted from the protein G–Sepharose matrix was >99% pure as judged by SDS-PAGE and silver staining of the gel. A nonspecific purified mouse IgG (Sigma Chemical Co., St. Louis, MO) protein G–Sepharose column was constructed similarly. All subsequent steps were performed at 4°C unless stated otherwise.

200 *Haemopis* leech nerve cords were homogenized in 2 ml of extraction buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% NP-40, 0.2% Triton X-100, pH 7.4) containing protease inhibitors. The homogenate was incubated on ice for 1 h and cleared by centrifugation at 13,000 *g* for 20 min and at 100,000 *g* for 45 min. The cleared homogenate was then incubated with 1 ml of the nonspecific mouse IgG-bound protein G–Sepharose for 4–8 h and centrifugated for 30 s at 2,000 *g*. The resulting supernatant was incubated with 1 ml of mAb Lan3-2–bound protein G–Sepharose overnight. This slurry was then applied to a Bio-Rad column (Bio Rad Laboratories, Hercules, CA), which was sequentially washed with 5–10 ml of each of the following buffers containing protease inhibitors: (*a*) extraction buffer; (*b*) 20 mM Tris-HCl, 200 mM NaCl, 0.2% NP-40, 0.2% Triton X-100, pH 8.2; (*c*) 50 mM Tris-HCl, 1 M NaCl, 0.5% NP-40, pH 8.2; (*d*) 50 mM Tris-HCl, 150 mM NaCl, pH 8.2; and (*e*) 20 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholic acid, pH 8.2. After these washes the column was disconnected from the chromatography system and rinsed with a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% NP-40, pH 8.2, and the bound Lan3-2 antigen was eluted with 1 ml of 0.5 M methyl α-D-mannopyranoside in the same buffer after incubation for 45 min. The elution process was repeated once and the eluted solutions were combined before dialysis against a buffer containing 10 mM NH_4CO_3, 0.02% SDS, pH 7.8. The resulting eluate was concentrated with a Centricon-50 spin column (Amicon Corp., Beverly, MA) at 5,000 *g*.

For protein microsequencing the Lan3-2 immunoaffinity-purified proteins were separated by 7.5% SDS-PAGE, followed by electroblotting onto Immobilon P^{SQ} membrane (Millipore Corp., Bedford, MA), and the transferred proteins were stained by 0.1% amido black in 10% acetic acid. The amido black–labeled electroblot was put in a sealed plastic bag and sent to the Wistar Institute Protein Microsequencing Facility (3601 Spruce Street, Philadelphia, PA 19104). At the Wistar Institute the broad 130-kD band was cut out and in situ digested with trypsin, the cleaved peptides were separated by microbore HPLC, and the fractions were screened by mass spectrometry. Seven fractions were selected for gas phase microsequencing of which one fraction yielded two peptide sequences for a total of eight.

Antibodies and Antibody Production to Synthetic Peptides

Three previously reported mAbs, Lan3-2 (IgG_1) and Lan4-2 (IgG_1) (Zipser and McKay, 1981; McKay et al., 1983), as well as an antibody to acetylated tubulin (IgG_{2B}) (Sigma Chemical Co.), were used in these studies. In addition, new antibodies were made to synthetic peptides based on the microsequence obtained from the purified Lan3-2 antigen. Three peptides were synthesized: pep2, **C**YNLDYEGNFHFANVMEEDHR-NH_2; pep3, MEEVEIPCEA**C**-NH_2 (both by QCB Inc., Hopkinton, MA), and pep6, *S*HHYNLEWEKPETGGSPIR**C**-NH_2 (by Chiron Mimotopes, Emeryville, CA). A cysteine (*bold*) was added to the end of each peptide for coupling purposes and two residues (*italics*) were modified by acetylation. The peptides were covalently coupled to keyhole limpet hemocyanin (Pierce Chemical Co.) carrier protein with sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce Chemical Co.) as per manufacturer's instructions. Two rabbits were injected with 200–400 μg of each of the coupled peptides in Freund's complete adjuvant (GIBCO BRL), and then boosted at 21-d intervals using Freund's incomplete adjuvant as described in Harlow and Lane (1988). After the second boost, serum samples were collected 7 and 10 d after injection. The sera were analyzed for specificity by comparing the staining obtained with the antisera and the preimmune sera on nitrocellulose filters spotted with the synthetic peptides coupled to BSA. Specific antisera were obtained for all three peptides and were titrated from undiluted to a 1:5,000 dilution in Blotto (0.5% Carnation nonfat dry milk in TBS). In addition to the rabbit antisera, the mAb 4G5 was generated against pep2 by injecting Balb C mice with 50 μg of the keyhole limpet hemocyanin–coupled peptide at 21-d intervals. After the third boost, spleen cells of the mice were fused with Sp2 myeloma cells and a monospecific hybridoma line was established using standard procedures (Harlow and Lane, 1988). 4G5 ascites were obtained by injecting four mice intraperitoneally with antibody-producing hybridoma cells. The 4G5 mAb is of the IgG_1 subtype. All procedures for mAb production were performed by the Iowa State University Hybridoma Facility.

Molecular Cloning and Sequence Analysis

Rabbit antisera to pep2 and pep6 sequence at a dilution of 1:4,000 were used to screen a random primed *Hirudo* CNS-enriched cDNA λ-ZAP II expression library essentially according to the procedures of Sambrook et al. (1989). A total of 10^6 plaques was screened at a density of 30,000 plaque-forming units per 150-mm plate. Positive clones were in vivo excised to generate pBluescript phagemids according to the method provided by the manufacturer (Stratagene, La Jolla, CA). One partial Tractin cDNA was identified by the pep2 antiserum and two partial LeechCAM cDNA clones were identified by the pep6 antiserum in these screens. To obtain the full sequence of the cDNAs for Tractin and LeechCAM, the same cDNA library was rescreened using ^{32}P-labeled fragments of the originally identified clones. The fragments were radiolabeled using random priming according to the manufacturer's procedure (Prime-a-Gene kit; Promega, Madison, WI), and the library was screened using standard procedures (Sambrook et al., 1989).

DNA sequencing was performed using a DNA Sequencer 377A (Applied Biosystems, Foster City, CA) at the Iowa State University Nucleic Acid Sequencing Facility using commercially available universal and reverse sequencing primers (Stratagene) or specific primers synthesized at the Iowa State University Nucleic Acid Synthesis Facility based on known Tractin and/or LeechCAM sequences. The nucleotide and predicted amino acid sequences were analyzed using the Genetics Computer Group (GCG package, version 8; Madison, WI) suite of programs (Devereux et al., 1984). The Tractin and LeechCAM sequences were compared with known and predicted proteins in the SwissProt and GenBank databases using the FASTA and TFASTA programs within the GCG package. In addition, a BLAST search was performed using the National Center for Biotechnology Information BLAST e-mail server (Altschul et al., 1990) comparing the Tractin and LeechCAM sequences with SwissProt, PIR, and GenPept databases.

Northern Blot Analysis

Total RNA was prepared from whole leech according to the method of Chomczynski and Sacchi (1987) with minor modifications. 5–6 g of adult *Hirudo* leeches frozen with liquid nitrogen was ground in a coffee grinder with dry ice to a fine powder. The leech powder was mixed thoroughly with 60 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate, 0.1 M β-mercaptoethanol, pH 7.0), to which the following solutions were sequentially added with thorough mixing: (*a*) 6 ml of 2 M sodium acetate, pH 4; (*b*) 60 ml of phenol (Tris-HCl saturated); and (*c*) 12 ml of chloroform-isoamyl alcohol mixture (49:1). The suspension was vigorously shaken and centrifuged at 10,000 *g* for 20 min at 4°C. The aqueous phase was extracted and precipitated with 1 vol (~60 ml) of isopropanol at −20°C for at least 1 h before centrifugation at 10,000 *g* for 20 min at 4°C. The resulting RNA pellet was dissolved in 6 ml of denaturing solution, and the above acid, guanidinium thiocyanate-phenol-chloroform extraction and isopropanol precipitation were repeated. The RNA pellet was washed with 70% ethanol, vacuum dried, and dissolved in diethyl pyrocarbonate–treated H_2O. PolyA+ mRNA was isolated using an oligo-dT column matrix (New England Biolabs, Beverly, MA) based on standard protocols (Sambrook et al., 1989). 20 μg of polyA+ mRNA was fractionated on 1.2% agarose formaldehyde gels, transferred to nitrocellulose, and hybridized to [^{32}P]dCTP-labeled Tractin or leechCAM probes according to standard methods (Ausubel et al., 1987). High stringency hybridization and washing conditions were used (Ausubel et al., 1987), and the filters were exposed to X-OMAT XAR film (Eastman Kodak Co., Rochester, NY) at −80°C with an intensifying screen.

Biochemical Analysis

SDS-PAGE, Western Blotting, and Two-dimensional Gel Electrophoresis. SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with 0.5× buffer containing 5% methanol. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2-μm nitrocellulose and using either anti–mouse or anti–rabbit HRP-conjugated secondary antibody (Bio Rad Laboratories) (1:3,000) for visualization of primary antibody diluted 1:2,000 in Blotto for immunoblot analysis. The signal was developed with DAB (0.1 mg/ml) and H_2O_2 (0.03%) and enhanced with 0.008% $NiCl_2$. In some experiments, gels were fixed and silver stained using the Bio-Rad Silver Stain Kit as per the manufacturer's instructions. The Western blots were digitized using the NIH-Image software, a high resolution cooled CCD camera (Paultek, Grass Valley, CA), and a Pixel-Buffer framegrabber (Perceptics, Knoxville, TN) or an Arcus II scanner (AGFA, Ridgefield, NJ).

Two-dimensional gel electrophoresis was done according to von Boxberg (1988) using the Bio-Rad Mini PROTEAN II 2-D cell. In brief, for first dimension IEF, each sample contained 3 μl of Lan3-2 antigen from the original purification (equivalent to antigen from six leech nerve cords) in 10 mM NH_4CO_3, 0.02% SDS, pH 7.8, which was mixed with 17 μl of IEF sample buffer (8.5 M urea, 2% NP-40, 5% β-mercaptoethanol; 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte 5–8 [Bio Rad Laboratories]). The IEF gels were run at a constant current of 200 μA for 15 min followed by a setting of 500 V constant voltage for 3.5 h. The two-dimensional electrophoresis was performed with 7.5% SDS-PAGE. The electroblot transfer and immunodetection of the proteins were as described above.

Immunoprecipitation. Immunoprecipitations with Lan3-2 antibody were performed at 4°C. Dissected *Haemopis* leech nerve cords were homogenized in extraction buffer (see above) and the homogenate (20 μl) was incubated with the nonspecific mouse IgG conjugated to protein G–Sepharose matrix for 2 h. The resulting supernatant was then incubated with purified Lan3-2 antibody conjugated to protein G–Sepharose matrix (10 μl) for 1 h. After a brief spin for 20 s at 2,000 rpm, the supernatant was discarded, and the immunoaffinity matrix was resuspended and washed sequentially with 400 μl for 5 min of: (*a*) extraction buffer; (*b*) 50 mM Tris-HCl, 1 M NaCl, 0.2% NP-40, pH 8.2; (*c*) 20 mM Tris-HCl, 2 M NaSCN, 0.1% NP-40, pH 8.2; (*d*) 20 mM Tris-HCl, 4 M urea, 0.1% NP-40, pH 7.5; (*e*) 0.2 M glycine-HCl, 0.1% NP-40, pH 2.5; and (*f*) extraction buffer. The final pellet was resuspended in 20 μl of SDS-PAGE sample buffer and boiled for 5 min before centrifugation and analysis of the supernatant by SDS-PAGE and immunoblotting.

Immunohistochemistry

Dissected *Hirudo* embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The embryos were incubated overnight at room temperature with diluted antibody (Lan3-2, 1:75; Lan4-2, straight; acetylated tubulin mAb, 1:1,000; 4G5 ascites, 1:1,000; rabbit or mouse pep6 antiserum, 1:1,000) in PBS containing 1% Triton X-100, 10% normal goat serum, 0.001% sodium azide; washed in PBS with 0.4% Triton X-100; and incubated with HRP-conjugated goat anti–mouse or goat anti–rabbit antibody (1:200 dilution; Bio Rad Laboratories). After washing in PBS, the HRP-conjugated antibody complex was visualized by reaction in DAB (0.03%) and H_2O_2 (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole mounts in Depex mountant (BDH, Poole, UK). Double-labeled preparations were obtained by a subsequent incubation in the other primary antibody and by using fluorescently conjugated subtype-specific secondary antibodies. A rabbit anti–mouse IgG Texas red–conjugated secondary antibody (Cappel Laboratories, Malvern, PA) was used for Lan3-2, and a rabbit anti–mouse IgG_{2B} FITC-conjugated secondary antibody (Cappel Laboratories) was used for the acetylated tubulin antibody. Fluorescently labeled preparations were mounted in glycerol with 5% *n*-propyl gallate. The labeled preparations were photographed on an Axioskop (Carl Zeiss, Inc., Thornwood, NY) using Ektachrome 64T or Ektar 100 daylight film (Eastman Kodak Co.). The color positives were digitized using Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA) and a Coolscan slide scanner (Nikon Inc., Garden City, NY). In Photoshop the images were converted to black and white and image processed before being imported into Freehand (Macromedia) for composition and labeling. In some cases, digital images were obtained using a high resolution cooled CCD camera, a PixelBuffer framegrabber, and the NIH-Image software.

Lan3-2 Perturbation Experiments

1–2 μl of purified Lan3-2 or purified mouse IgG_1 (Sigma Chemical Co.) control antibody from a 0.2 mg/ml stock solution was injected beneath the germinal plate of E8 *Hirudo* embryos. In addition to antibody, the stock solution contained 10% Ringer solution and 0.2% fast green (Sigma Chemical Co.), allowing for visual confirmation of pressure-injected antibody under the stereo microscope. For the injections the embryos were immobilized in crevices in Sylgard-coated tissue-culture dishes while anesthetized with 8% ethanol in 10% Ringer solutions. After the injection the embryos were transferred to embryo water without ethanol and allowed to develop for 24 h at 25°C. At this time the embryos were dissected, fixed, and processed for antibody labeling. In most experiments the embryos were labeled or relabeled overnight by incubation in Lan3-2 antibody supernatant, and the labeling was visualized with either HRP- or Texas red–conjugated anti–mouse IgG secondary antibody. However, in some cases of Lan3-2–injected embryos, only the Texas red–conjugated secondary antibody was applied. In addition, after examination of Lan3-2–perturbed fluorescently labeled embryos, some were demounted, reprocessed, and labeled with acetylated tubulin mAb, and the labeling was visualized with FITC-conjugated anti–mouse IgG_{2B} secondary antibody. 31 embryos from three different cocoons were examined: 15 were injected with purified Lan3-2 antibody, 12 with IgG_1 mouse control antibody, and 4 served as uninjected controls (1 uninjected control embryo failed to develop and was not considered in the analysis). In each experiment, sibling embryos at synchronized developmental stages from the same cocoon were compared at the different experimental conditions. For measuring the area occupied in the neuropil by the sensory afferents, we used a method similar to that of Zipser and Cole (1991). In brief, digital images of the ganglionic afferents were obtained using the NIH-Image software under a ×63 objective. The area in pixels covered by the neurites and their filopodia was traced using the outline tool and converted to square microns. To allow for comparisons between different preparations, the same four ganglia (g2–g5) were imaged in each specimen.

Results

Purification of Lan3-2 130-kD Antigen by Immunoaffinity Chromatography

Both the Lan3-2 and Lan4-2 antibodies were originally made toward adult *Haemopis* CNS (Zipser and McKay, 1981), but they label growth cones and developing axon fascicles of sensory neurons (Fig. 1) in all leech species examined representing two different orders (Johansen et al., 1992). The antigens are surface glycoproteins and, on immunoblots of adult CNS extract from *Haemopis*, Lan3-2 recognizes several protein bands of different molecular weights including a broad 130-kD band (McKay et al., 1983; Johansen et al., 1992). In contrast, Lan4-2 recognizes only a 130-kD protein band, and cross-immunoprecipitation experiments with Lan3-2 and Lan4-2 have demonstrated that the 130-kD antigens of these antibodies are closely molecularly interrelated (Johansen et al., 1992). The significance of the other bands recognized by Lan3-2 is presently unclear, since immunoblots of embryonic CNS suggest that in E8–E10 embryos, when the Lan3-2–positive fascicles are being formed, only the 130-kD version of the antigens is expressed (McGlade-McCulloh et al., 1990). Thus, these experiments suggest that the 130-kD protein may be the key molecular form of the Lan3-2 antigens involved in the formation of the axon fascicles, being the only form expressed when the fascicles are first pioneered. For these reasons we set out to identify and molecularly clone the 130-kD protein(s) recognized by the Lan3-2 and Lan4-2 antibodies.

Since the Lan3-2 antibody recognizes a glycoepitope (McKay et al., 1983), it was not possible to screen directly a bacteriophage-based expression vector library using Lan3-2. Therefore, it was necessary to first purify the antigen, which makes up only ~0.004% of extractable leech nerve cord protein (Johansen et al., 1992). As a first step we purified Lan3-2 antibody over a protein G–Sepharose column from supernatant of Lan3-2 antibody-producing hybridoma cells grown in serum-free media. The purified antibody was covalently coupled to protein G–Sepharose to construct an immunoaffinity column to which extracts of dissected *Haemopis* leech nerve cords solubilized in Triton X-100/NP-40 buffer were applied. The solubilized protein was precleared by incubation with nonspecific mouse IgG coupled to protein G–Sepharose, and the final Lan3-2

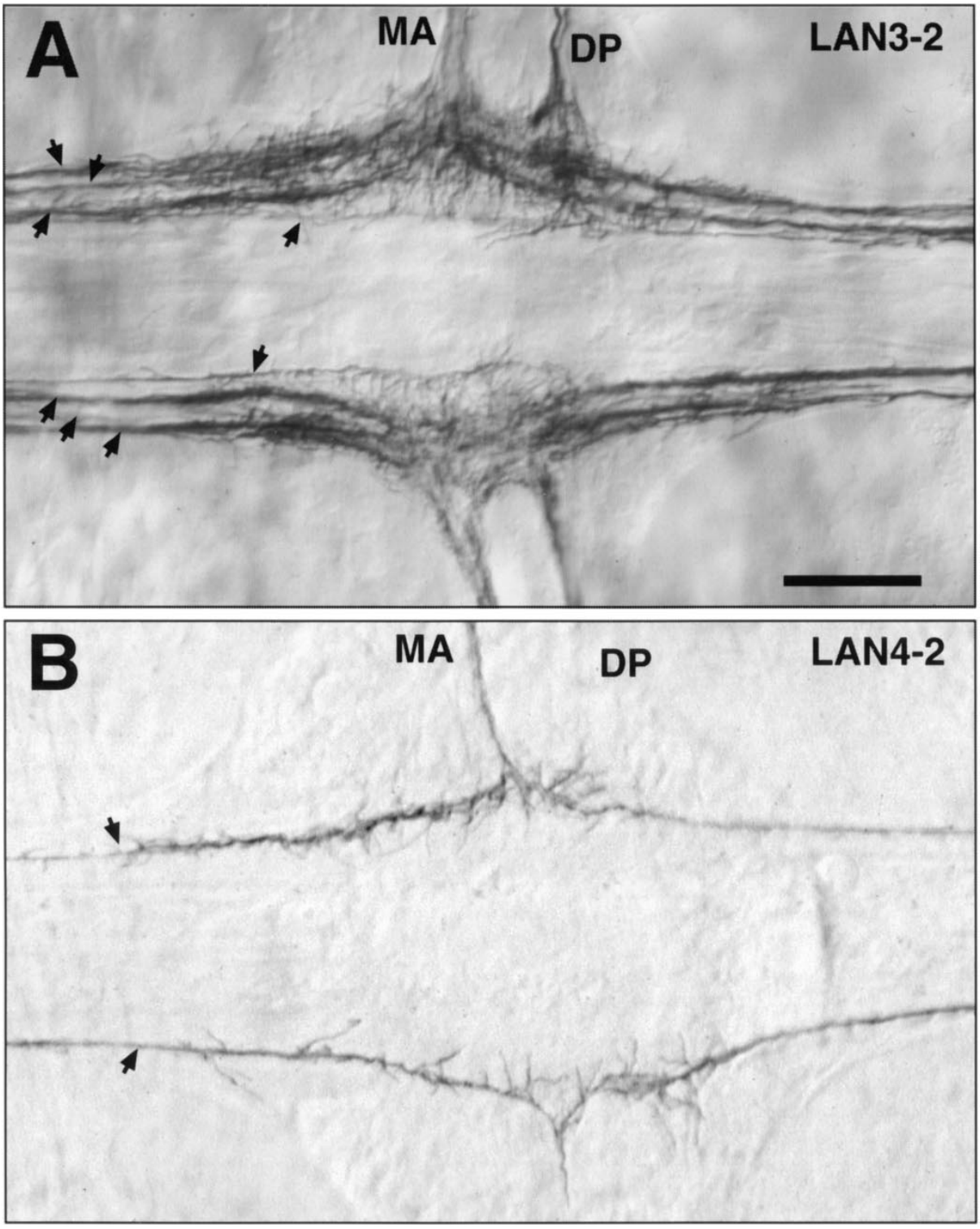


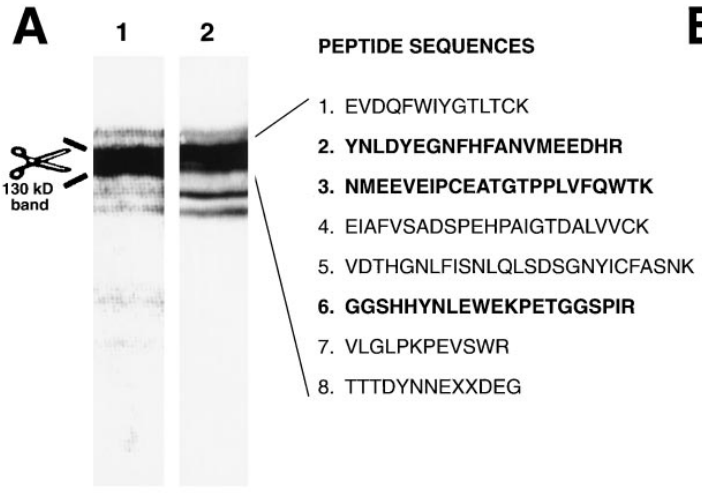
Figure 1. Axonal tracts labeled by the mAbs Lan3-2 and Lan4-2 in *Hirudo* CNS. (*A*) Lan3-2 labels a glycoepitope expressed by peripheral sensory neurons, the axons of which enter the CNS through the median anterior (*MA*) and dorsal posterior (*DP*) nerves and segregate into four distinct axonal tracts (*arrows*). The leech nervous system is bilaterally symmetrical, and anterior is to the left in this and all the following figures. (*B*) Lan4-2 labels a different glycoepitope expressed by a small subset of the Lan3-2–positive neurons, the axons of which segregate into a single axon tract (*arrows*). Bar, 25 μm.

antibody column was washed stringently with 1 M NaCl containing detergent. However, specifically eluting the bound antigen from the column proved to be difficult as the affinity of the antibody for the antigen was so high that neither low nor high pH conditions eluted the Lan3-2 antigen. A variety of other eluates used (4 M $MgCl_2$, 4 M urea, and 3 M NaSCN) also proved to be unsuccessful. Finally, by taking advantage of the fact that methyl α-D-mannopyranoside can block Lan3-2 antibody labeling on immunoblots (McKay et al., 1983), we found that 0.5 M methyl α-D-mannopyranoside could be used to compete off the Lan3-2 binding. This method of elution was not very efficient but proved to be highly specific. After dialysis and concentration, SDS-PAGE, and blotting onto polyvinylidene difluoride membrane, ~10 μg of immunopurified antigen was obtained from 200 nerve cords by this method. Fig. 2 *A* shows the purified antigen on a Western blot stained with amido black from which microsequence was obtained together with a corresponding immunoblot labeled with Lan3-2 antibody. Only the broad 130-kD band was cut out and analyzed for the reasons stated above. The purified protein was trypsin digested in situ, and the resulting peptides were separated by microbore HPLC and screened by mass spectrometry. Seven peaks were selected for gas phase microsequencing, from which the eight sequences shown in Fig. 2 *A* were deduced (one of the peaks contained two peptide sequences). Searches of the databases showed that all eight peptides represented novel sequences; however, pep2, 3, 5, and 7 exhibit sequence homology with the Ig-like domains of various neural CAMs of the L1 subfamily (Hortsch, 1996). In addition, pep6 shows homology to FNIII-like domains of the NCAM/FasII/ApCAM subfamily (Cunningham et al., 1987; Harrelson and Goodman, 1988; Mayford et al., 1992), whereas pep1, 4, and 8 have no homology to any known protein sequence in the databases.

The observation that some of the peptide sequences showed homology to different subfamilies of CAMs raised the possibility that we had obtained peptide sequences from more than one protein that comigrate in the broad 130-kD band recognized by Lan3-2. To account for this possibility, we adopted a cloning strategy where we made mono- and polyclonal antibodies in mice and rabbits to synthetic peptides based on the sequence from pep2, 3, and 6 (Fig. 2 *A*). As shown in Fig. 2 *B*, antisera to all three peptides recognized a broad 130-kD band on immunoblots of *Haemopis* nerve cord extracts, indicating that the peptides were indeed derived from the targeted proteins. Furthermore, the peptide antibodies cross-reacted with similarly sized proteins from nerve cord extracts from *Hirudo* (data not shown). Consequently, we used these rabbit antisera to the peptides to screen a *Hirudo* randomly primed cDNA library.

Molecular Cloning and Characterization of Tractin and LeechCAM

From ~10^6 clones screened with pep2 antiserum, a single partial cDNA clone was identified. The predicted amino acid sequence of this clone contained the exact peptide sequence of pep2 (Figs. 3 *A* and 4), confirming its identity. Subsequently, the cDNA library was rescreened with radiolabeled nucleotide probes generated from the 5′ and 3′ ends of the original cDNA clone. In this way, five additional independent and overlapping cDNA clones were isolated, which encompassed the entire coding sequence (Fig. 4). The predicted sequence is for a protein containing 1,880 amino acids with several novel features (Figs. 3 *A* and 4) that we have named Tractin since it is expressed on axon tracts. Sequence analysis suggests that the first 25 amino acids are a cleaved signal peptide (Von Heijne, 1983). Another hydrophobic region near the COOH-terminal end is indicative of a putative transmembrane domain and is followed by a short cytoplasmic tail. The extracellular part of Tractin contains four structural domains (Fig. 4) beginning with a region of six Ig-like domains. Ig-like domains consist of two β sheets that are stabilized by a characteristic disulfide bond and can be categorized into different types (V, C1, C2, or I) depending on key amino acid features (Harpaz and Chothia, 1994; Vaughn and Bjorkman, 1996). The Ig-like domains of Tractin are predicted to belong to



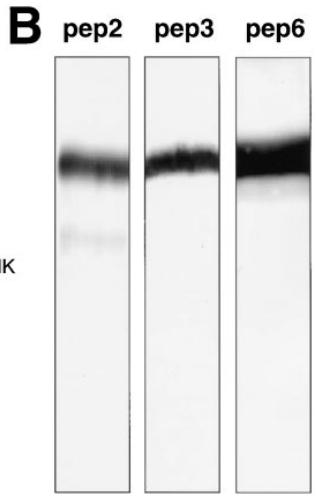


Figure 2. Peptide sequences and new antibodies from purified Lan3-2 130-kD antigen in *Haemopis.* (*A*) Western blot of Lan3-2 immunoaffinity-purified antigen from *Haemopis* CNS labeled with amido black (lane *1*) compared with an immunoblot of the same material labeled by Lan3-2 antibody (lane *2*). The broad 130-kD band was cut out as indicated on the figure and microsequenced. Eight peptide sequences were deduced as shown to the right. (*B*) Western blots of *Haemopis* CNS proteins labeled by rabbit antisera that were made to synthetic peptides based on the sequences from pep2, pep3, and pep6 shown in bold in *A.* All three peptide antisera were specific to the broad 130-kD band. 200 kD is at the top of the blots in *A* and *B*, and 25 kD is at the bottom.

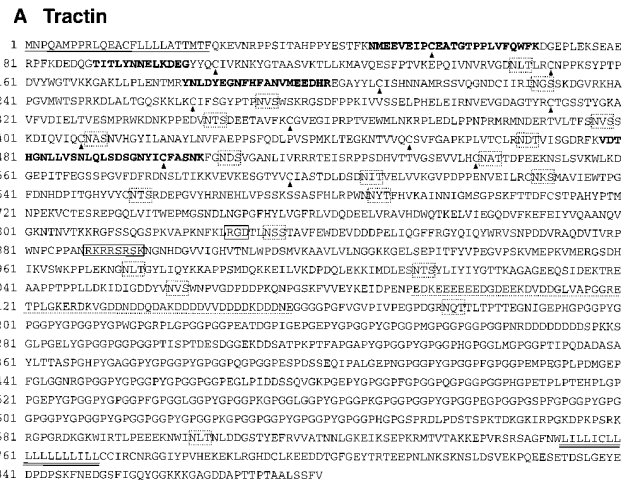


Figure 3. The predicted protein sequence of Tractin and LeechCAM in *Hirudo.* The cDNAs for Tractin (*A*) and LeechCAM (*B*) encode two novel members of the Ig superfamily. The cysteines of the Ig-like domains are indicated by arrowheads; potential N-linked glycosylation sites are indicated by stippled boxes; the hydrophobic signal sequences are underlined; and the putative transmembrane domains are double underlined. In addition, the sequences matching the deduced peptide sequences from the microsequencing of immunopurified *Haemopis* Lan3-2 antigen are shown in bold. Where the sequences are not an exact match, this difference could be due to a misassignment during microsequencing and/or species differences. The predicted Tractin protein sequence (*A*) consists of 1,880 residues or 1,855 without the signal sequence with an estimated molecular mass of 197 kD. It contains an RGD integrin binding motif (*boxed*), a putative RKRRSRSK peptide cleavage site (*boxed*), and a 60-residue highly acidic domain (*stippled underline*). The predicted LeechCAM sequence (*B*) is for a protein of 858 amino acids or 841 without signal sequence with an estimated molecular mass of 95 kD. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers U92813 (Tractin) and U92814 (LeechCAM).

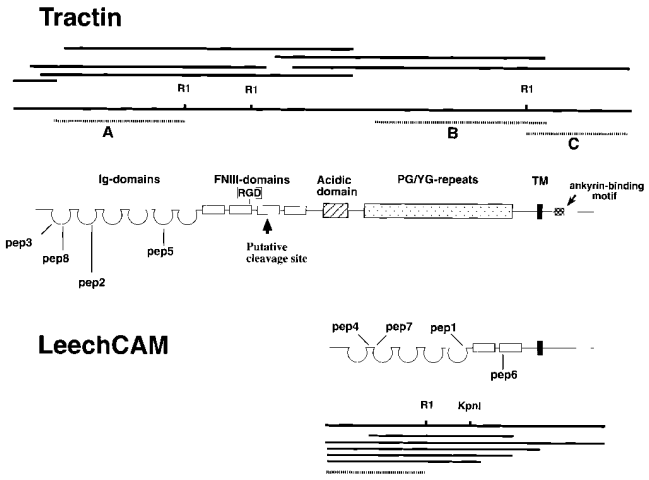


Figure 4. Diagrams of the Tractin and LeechCAM proteins. The protein sequence of Tractin is organized into six Ig-like domains, four FNIII-like domains, an acidic domain, a PG/YG repeat–containing domain, a transmembrane domain, and an intracellular domain with an ankyrin binding motif. The locations of the RGD integrin binding motif in the second FNIII-like domain and the putative peptide cleavage site in the third FNIII-like domain are indicated. Above the diagram of Tractin and below the LeechCAM protein sequence are partial restriction maps of the cDNAs relative to the independent, overlapping cDNA library clones from which the complete sequences were compiled (*black lines*). The protein sequence of LeechCAM consists of five Ig-like domains, two FNIII-like domains, a transmembrane domain, and a short intracellular tail. As indicated on the figure, the Tractin and LeechCAM sequence each matched four of the eight originally microsequenced peptides from the Lan3-2 immunopurification. In addition, the sequences used for Northern blot analysis are indicated as stippled lines below the restriction maps. Three different sequences (*A*, *B*, and *C*) were used to probe Tractin Northern blots.

the I-set. The six Ig-like domains are followed by four FNIII-like domains (Hynes, 1990). The second FNIII-like domain contains an RGD tripeptide sequence that is a characteristic binding sequence for integrins (Hynes, 1992). In the region after the fourth FNIII-like domain, there is an acidic block of 64 amino acids of which more than half of the residues are either aspartate or glutamate. A dotplot analysis of the tractin sequence against itself shows that about a fourth of the protein is made up of a repeat sequence (Fig. 5 *A*). A sequence alignment (Fig. 5 *B*) shows that this region contains 12 repeats of a 30–amino acid motif rich in prolines and glycines in which 10 positions are invariant. The residues of the repeats are most highly conserved in the first half, whereas the length and sequence of the repeats are more variable in the second half. The consensus sequence for the repeats has a proline at each third residue and is made up of 10 repetitions of the triplet PG/YG. The 12 repeat segments are interspersed by short linkers of variable length. There are no known homologies in the protein databases to either the acidic or the PG/YG repeat region. The cytoplasmic tail has a stretch of residues (Fig. 5 *C*) that conforms to the consensus sequence for ankyrin binding domains (Davis and Bennett, 1994; Dubreuil et al., 1996), suggesting that Tractin may interact with the cytoskeleton. In addition, the extreme COOH-terminal end of Tractin contains the sequence SSFV that matches the XS/TXV motif that is predicted to associate with PDZ domains of the PSD-95/discs-large/ZO-1 family of membrane skeletal proteins (Doyle et al., 1996). Interestingly, this XS/TXV PDZ domain binding motif is also found in several neural cell adhesion molecules such as NrCAM and *Drosophila* FasII, and in a neuroglian isoform (Kornau et al., 1995; Hortsch et al., 1990). In the extracellular part Tractin has 19 possible N-linked glycosylation sites.

The predicted molecular mass of Tractin is 200 kD, or 197 kD after cleavage of the signal peptide. This is much larger than the size of the 130-kD Lan3-2 antigen. However, the protein contains a putative cleavage site of RKRRSRSK in the third FNIII-like domain (Fig. 3 *A*); a similar sequence motif is also found in FNIII-like domains in CAMs of the L1 subgroup where in several cases it has been demon-

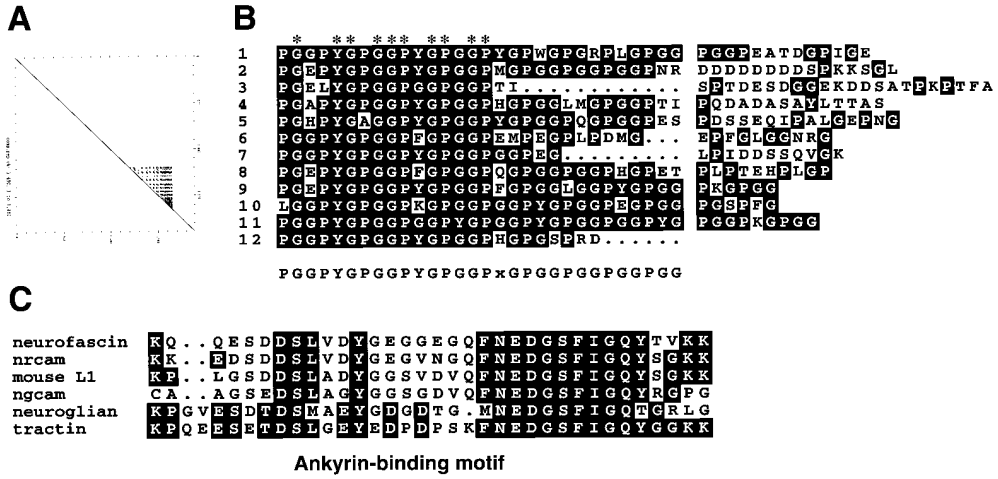


Figure 5. The PY/GG repeat region of Tractin and sequence comparison of the ankyrin binding motif. (*A*) Dot plot of the Tractin amino acid sequence. The sequence was analyzed for similar sequences within itself by the computer program COMPARE with a stringency of 10 and window length of 8. Points at which the two sequences are homologous are indicated by dots. The plot reveals an extended region encompassing ~25% of the entire amino acid sequence with an internal repeat structure. (*B*) A sequence alignment of the region identified in *A* demonstrates that it is made up of 12 repeats of a 30–amino acid motif rich in prolines and glycines in which 10 positions are invariant (*asterisks*). The consensus sequence for the repeats shown at the bottom is made up of 10 repetitions of the amino acid triplet PY/GG. The 12 repeat segments are connected by short linker sequences. Proline, glycine, and tyrosine residues are highlighted in white letters on a black background. (*C*) Sequence alignment of the ankyrin binding motif of the intracellular domain with comparable sequences from members of the L1 subfamily of CAMs: neurofascin (S26180), nrcam (P35331), L1 (P11627), ngcam (Q03696), and *Drosophila* neuroglian (P20241). Conserved residues of these sequences with Tractin are highlighted in white letters on a black background.

strated to serve as an actual cleavage site (Burgoon et al., 1991, 1995; Kayyem et al., 1992; Volkmer et al., 1992). Cleavage of Tractin at this site would give rise to a secreted protein of 97 kD that, with glycosylation taken into account, would correlate well with the observed size of the Lan3-2 antigen on immunoblots. Northern analysis of leech mRNA suggests that Tractin may be alternatively spliced into a major and a minor transcript of 9.3 and 8.5 kb, respectively (Fig. 6). Both of these transcripts are recognized by Tractin sequence probes from the Ig domain region, the PG/YG repeat region, as well as the cytoplasmic tail region (data not shown), and are more than large enough to encode for the entire sequence of Tractin. This finding, together with the fact that neither Lan3-2, pep2, nor pep3 antibodies recognize a full-length 197-kD version of the protein on immunoblots, suggests that Tractin may be cotranslationally cleaved into a secreted as well as a 100-kD transmembrane protein.

Comparison of the eight microsequenced peptides with the full-length Tractin protein shows that only four of these peptides (pep2, 3, 5, and 8) are matched by Tractin sequence (Figs. 3 *A* and 4). This suggests that at least one other protein was copurified with Tractin. We therefore rescreened the *Hirudo* cDNA expression library with pep6 antiserum and obtained two independent partial cDNA clones. Radiolabeled nucleotide probes generated from the 5′ and 3′ ends of these cDNA clones were then used to obtain three additional overlapping cDNAs that encompassed the entire coding sequence. The predicted sequence is for a protein of 858 amino acids with five Ig-like domains, two FNIII-like domains, a putative transmembrane domain, and a cytoplasmic tail (Figs. 3 *B* and 4). The first 17 residues define a hydrophobic stretch and are likely to be a cleaved signal peptide (Von Heijne, 1983). That the correct protein was cloned was verified by the presence of the pep6 sequence within the second FNIII-like domain

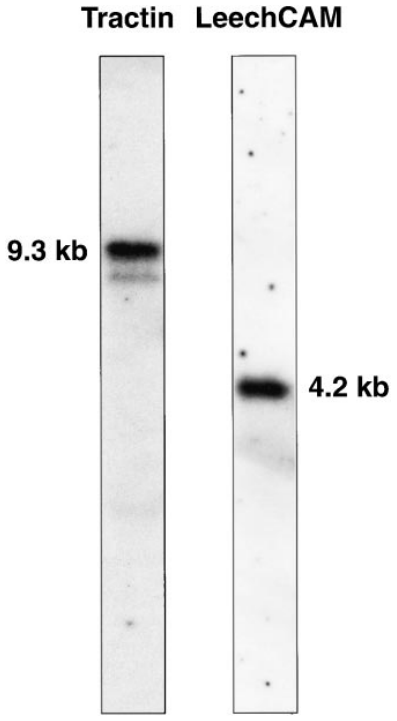


Figure 6. Northern blot analysis of Tractin and LeechCAM mRNA. 20 μg of total Hirudo polyA$^+$ mRNA was fractionated on a 1.2% agarose formaldehyde denaturing gel, transferred to nitrocellulose, and probed under high stringency conditions using random primer–labeled Tractin and LeechCAM sequences. For Tractin, sequences from a 5′ end EcoRI fragment coding for the first five Ig-like domains were used, and for LeechCAM, sequences from a 5′ EcoRI fragment coding for the first three Ig-like domains were used (see Fig. 4). For Tractin, a major band at 9.3 kb and a minor band at 8.5 kb were detected, whereas a single band of 4.2 kb was observed for LeechCAM.

(Figs. 3 *B* and 4). That the sequences are not an exact match could be due to a misassignment during microsequencing and/or to species differences. The microsequence was obtained from *Haemopis* protein whereas the cDNA is from *Hirudo* (the degree of protein sequence identity between two other proteins, a neurofilament and a voltage-gated K-channel, that have been cloned in both *Hirudo* and *Haemopis* is ~95%; unpublished results). The domain organization of the protein is similar to that of NCAM, FasII, and ApCAM, to which it has sequence homology in the range of 26–30% based on identical amino acids, suggesting it is the leech homologue of these proteins. Consequently, we have named the protein LeechCAM. The calculated molecular mass of LeechCAM is 97 kD, or 95 kD after cleavage of the signal peptide. It has 10 potential N-linked glycosylation sites in the extracellular region and is recognized by pep6 antibody as a 130-kD protein on immunoblots (Fig. 2 *B*), suggesting that 35 kD or ~27% of its molecular mass is due to glycosylation. On Northern blots of leech mRNA, it is identified as a single transcript of 4.3 kb (Fig. 6). In addition to the pep6 sequence, the sequence of pep1, 4, and 7 is found within LeechCAM, thus accounting for all eight of the originally sequenced peptides.

Differential Glycosylation and Immunocytochemical Localization of Tractin and LeechCAM

Immunoaffinity chromatography purification with Lan3-2 antibody yielded two proteins, which raised the question of whether both Tractin and LeechCAM carry the Lan3-2 epitope or whether one or the other is an interacting protein without the Lan3-2 epitope that may have been copurified. To address this question we immunoprecipitated leech CNS proteins with purified Lan3-2 antibody covalently coupled to a protein G–Sepharose matrix. We then took advantage of the very high affinity binding of the Lan3-2 antibody to its antigen by sequentially washing the precipitate with 1 M NaCl, 4 M $MgCl_2$, 3 M NaSCN, and 4 M urea. These very stringent washing conditions would be expected to break most noncovalent protein–protein associations. The Lan3-2 immunoprecipitate was then boiled, separated by SDS-PAGE, and immunoblotted. Fig. 7 *A* shows that a 130-kD band was recognized by Lan3-2, Tractin, and LeechCAM, as well as by Lan4-2 antibody. This indicated that Tractin and LeechCAM were most likely copurified because both proteins are glycosylated with the Lan3-2 epitope. In addition, Tractin and LeechCAM antibody to the protein core only recognized the 130-kD proteins and not the other molecular mass bands recognized by Lan3-2 antibody, suggesting that these are different proteins being glycosylated with the Lan3-2 epitope in the adult nervous system (Fig. 7 *A*).

Unfortunately, neither the mAb 4G5 nor any of the new antisera raised against core protein sequences of either Tractin or LeechCAM were able to immunoprecipitate either of these proteins specifically. Thus, to confirm the presence of both the Lan3-2 and Lan4-2 epitopes on Tractin and LeechCAM, we separated leech CNS proteins from the original purification (Fig. 2 *A*) by two-dimensional gel electrophoresis with IEF in the first dimension and SDS-PAGE in the second. The separated proteins were transferred to nitrocellulose and probed separately with Lan3-2, Lan4-2, mAb 4G5 toward pep2 of Tractin, and mouse polyclonal antiserum against pep6 of LeechCAM. By this procedure we were able to clearly separate the LeechCAM and Tractin proteins. LeechCAM antiserum recognizes a diffuse spot on the gel (Fig. 7 *B*, *arrowhead*), which is not labeled by Tractin antibody. Conversely, Tractin recognizes the 130-kD protein in a broad

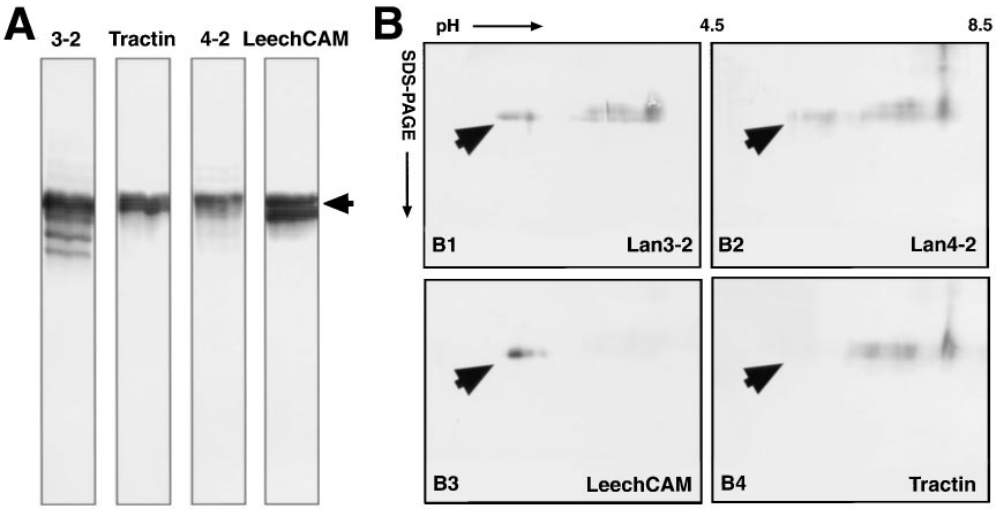


Figure 7. Tractin and LeechCAM are separate proteins that are both glycosylated with the Lan3-2 and Lan4-2 epitopes. (*A*) Immunoblots of Lan3-2–immunoprecipitated *Haemopis* nerve cord proteins. The immunoprecipitate was sequentially washed with 1 M NaCl, 4 M $MgCl_2$, 3 M NaSCN, and 4 M urea before separation by 10% SDS-PAGE. A broad 130-kD band (*arrow*) was recognized by both Lan3-2 and Lan4-2 and by Tractin (pep2) and LeechCAM (pep6) antisera (200 kD is at the top and 25 kD is at the bottom of the blot). (*B*) Immunoblots of immunoaffinity-purified Lan3-2 antigen from *Haemopis* nerve cords separated by two-dimensional-gel electrophoresis. The purified nerve cord proteins were subjected to IEF in the first dimension (range: pH 4.5–8.5) and 7.5% SDS-PAGE in the second (200 kD is at the top and 25 kD is at the bottom of the blot). LeechCAM antiserum (*B3*) recognizes an oblong diffuse 130-kD spot (the arrow indicates the location of this position in all four panels) that is not labeled by the Tractin mAb 4G5 (*B4*). In contrast, the Tractin mAb 4G5 labels 130-kD protein in a broad range of more basic pHs (*B4*) that are not labeled by the LeechCAM antiserum (*B3*). Both Lan3-2 and Lan4-2 label proteins in a pattern matching the combined pattern of Tractin and LeechCAM antibody (*B1* and *B2*).

range of pH, which is not labeled by LeechCAM antibody. However, the combined pattern of LeechCAM and Tractin antibody labeling matches that of both Lan3-2 and Lan4-2. This suggests that Tractin and LeechCAM are separate proteins that both can be glycosylated with the Lan3-2 and Lan4-2 epitopes. However, it should be noted that we only clearly resolved the proteins in the 130-kD region; the other and less efficiently purified proteins recognized by Lan3-2 (Fig. 2 *A*, lane *1*) were in most cases below the threshold of detection in this analysis. The predicted IEF points for LeechCAM and the secreted Tractin core proteins are pH 4.7 and 5.5, respectively. This is consistent with their relative position on the immunoblots although their observed values are shifted toward more basic pHs. This observation and their distribution over a wide range of pH, instead of being focused into tight spots, probably reflect heterogeneous glycosylation of the core proteins as has been previously described for other glycosylated CAMs (Rathjen et al., 1987*a*,*b*; Wolff et al., 1988). Furthermore, since all eight microsequenced peptides were found in the sequence of Tractin and LeechCAM, they may represent the only two 130-kD proteins carrying these glycoepitopes, although the existence of one or more additional proteins cannot be rigorously excluded.

We examined the developmental expression of Tractin and LeechCAM by labeling E10 *Hirudo* embryos with Tractin (mAb 4G5)– and LeechCAM (mouse antiserum)–specific antibodies (Fig. 8) (similar results were obtained with the rabbit antisera; data not shown). We found that Tractin was detected by the mAb 4G5 on the soma and axons of all neurons of both the central and peripheral nervous system (Fig. 8 *A*). We suspect this also to be the case for LeechCAM; however, the immunocytochemical labeling with the mouse polyclonal antiserum was not as robust as for the Tractin mAb, allowing us only to identify positively labeling of central neurons, sensilla, and a few of the other peripheral neurons such as the nephridial nerve cell (Fig. 8 *B*). At this developmental stage, Lan3-2 labels all peripheral sensillar neurons and their projections (Fig. 8 *C*), whereas Lan4-2 labels only a small subset of these that fasciculate into a single tract (Fig. 8 *D*). These results indicate that Tractin and LeechCAM are widely expressed proteins in the leech nervous system; however, they are differentially glycosylated to carry the Lan3-2 glycoepitope in the sensillar neurons only and, in a distinct subset of these sensillar neurons, they are additionally modified to also carry the Lan4-2 glycoepitope.

In Vivo Perturbation of Neurite Extension and Fascicle Formation of Sensillar Neurons by Lan3-2 Antibody

To test whether the differential glycosylation of Tractin and LeechCAM functions in neuritogenesis and/or fascicle formation of the sensillar neurons in vivo, we injected purified Lan3-2 antibody (0.2–0.5 μg) beneath the germinal plate cavity of sibling E8 embryos (Fig. 9). Assuming a volume of ~20 μl for an E9 embryo germinal plate, this would result in an effective antibody concentration of 10–25 μg/ml, which is comparable to the highest concentrations used in the in vitro perturbation studies by Zipser et al. (1989) and Song and Zipser (1995*a*). Embryos, either not injected or injected with a nonspecific purified mouse IgG_1 fraction

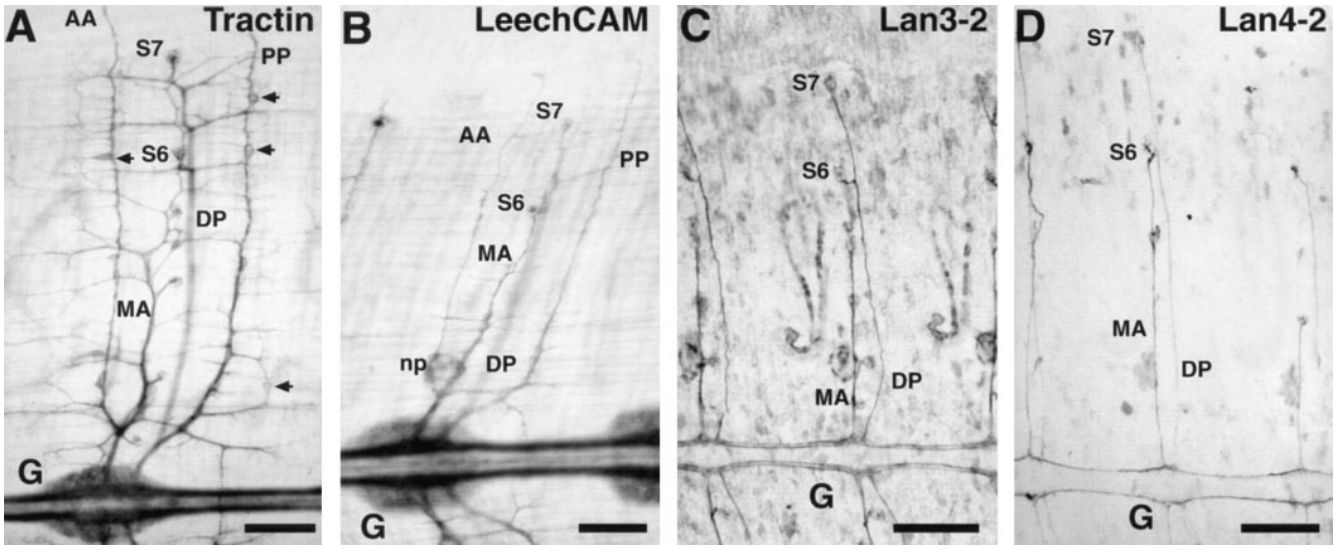


Figure 8. Tractin and LeechCAM are widely expressed proteins that are differentially glycosylated with the Lan3-2 and Lan4-2 epitopes in distinct subpopulations of peripheral sensory neurons. (*A–D*) Hemisegments of *Hirudo* embryos at approximately the same developmental stage (E10–11) labeled by Tractin (mAb 4G5 to pep2), LeechCAM (mouse antiserum to pep6), Lan3-2, and Lan4-2 antibody. Tractin antibody (*A*) labels the soma and projections of all neurons in both the central (*G*) and peripheral nervous system. S6 and S7 indicate the location of the dorsal sensilla, whereas some of the somata of nonsensillar peripheral neurons are indicated by arrows. LeechCAM antibody (*B*) labels central (*G*) and sensillar neurons (*S6* and *S7*) and their projections in the four peripheral nerves (*AA*, *MA*, *DP*, and *PP*), in addition to unidentified cells in tissue forming the nephridiopore (*N*). Lan3-2 (*C*) recognizes a glycoepitope expressed on the soma and axons of all sensillar neurons. Their axons project into the CNS (*G*) through the MA and DP nerves where they segregate into four distinct fascicles. Lan4-2 (*D*) recognizes a different glycoepitope expressed by a subset of the Lan3-2–positive sensillar neurons, the axons of which segregate into a single tract in the CNS (*G*). Bars: (*A* and *B*) 75 μm; (*C* and *D*) 100 μm.

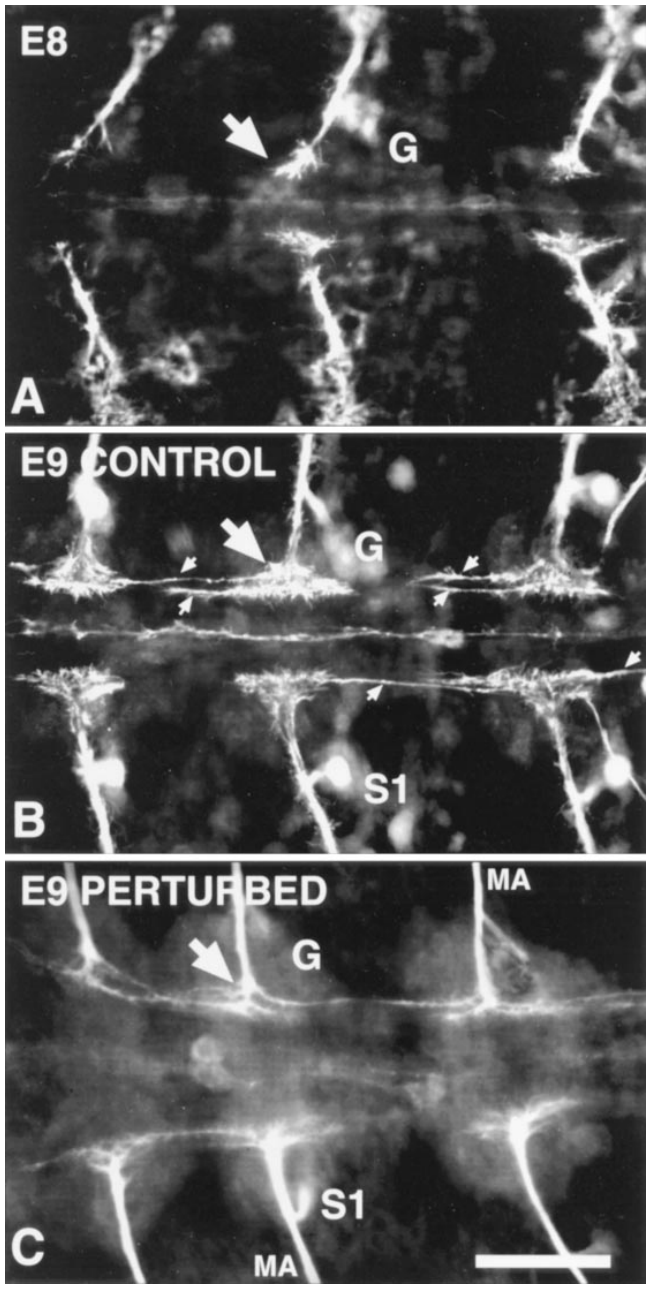


Figure 9. In vivo perturbation by Lan3-2 antibody of neurite extension and fascicle formation of sensillar neurons from three segments. (*A*) Anterior ganglia labeled by Lan3-2 antibody, showing the degree of development at the time Lan3-2 antibody or control mouse IgG_1 antibody were injected into sibling embryos at E8. The location of the primordial middle ganglion is indicated by the G, and the arrow points to the choice point for segregation into fascicles of the growth cones of S3 sensillar neurons that have just entered the ganglionic neuropil. At this stage only the S3 sensillum has differentiated. (*B*) Uninjected control embryo from the same cocoon as the embryo in *A* was dissected, fixed 24 h later at E9, and labeled with Lan3-2 antibody. The control embryo shows the extensive elaboration of growth cones and filopodia at the choice point for segregation of axons into different fascicles (*arrow*). Several fascicles are forming (*small arrows*) in both the anterior and posterior direction. The most ventral sensillum (*S1*) has differentiated at this stage and extends axons to the CNS. (*C*) Sibling embryo of that shown in *B* injected with 0.5 μg of purified Lan3-2 antibody at E8. As in *B*, the embryo was dissected and fixed 24 h later at E9, and the Lan3-2 labeling was visualized with Texas red–conjugated second antibody. The axons of the sensilla in the MA nerve are still fasciculated; however, at the choice point (*arrow*) in the ganglia (*G*), the extension of growth cones and filopodia is greatly reduced and fascicle formation is truncated. Bar, 40 μm.

in equal amounts to that of the Lan3-2 antibody, served as controls. After injection the intact embryos were incubated for 24 h in embryo water before dissection and fixation. In some experiments with Lan3-2–injected embryos, the fixed embryos were relabeled with Lan3-2 antibody and Texas red–conjugated second antibody, while in others only fluorescently conjugated secondary antibody was applied. The results from both methods were indistinguishable, demonstrating that the injected Lan3-2 antibody is stable and has full access to its epitope. At the time of injection at E8, the growth cones of the sensillar neurons in anterior segments have just reached the ganglionic neuropil before their bifurcation into fascicles in the CNS (Fig. 9 *A*). During normal development in the ensuing 24 h, more and more sensillar neurons extend neurites into the CNS (Jellies et al., 1994). These neurites have numerous filopodia that branch extensively in the neuropil as the projections segregate into distinct fascicles (Fig. 9 *B*). In contrast, in the presence of Lan3-2 antibody the extension of neurites and filopodia at the bifurcation point is severely impaired, as shown in Fig. 9 *C*, where the sensillar projections clearly are stunted, although indications of fascicles are discernible. Of the 15 Lan3-2–injected embryos, this phenotype was observed in four embryos, whereas six showed intermediate levels of perturbation, and five embryos showed no discernible difference from controls. Since none of the 12 embryos injected with IgG_1 control antibody were distinguishable from uninjected controls, the perturbation phenotype seen in a majority of the embryos is likely to be a consequence of the presence of Lan3-2 antibody (application of a χ^2-test shows that the phenotype distribution of Lan3-2–injected embryos is significantly different from that of the control distribution [$P < 0.01$]). As an estimate of the degree of inhibition of neurite extension and branching, we measured the average area occupied by the sensory afferents in the neuropil of four ganglia in each of the four embryos most clearly perturbed by Lan3-2. The average area in these embryos was 577 ± 188 μm^2 (n = 32, ± SD) compared with 1,402 ± 240 μm^2 (n = 16, ± SD) in ganglia from two unperturbed embryos, corresponding to an ~60% reduction in the average area occupied by neurites and filopodia. Although the extension of neurites in the ganglionic neuropil is clearly inhibited by Lan3-2 antibody, sensillar neurons do appear to differentiate in the periphery under these conditions and remain fasciculated in the peripheral nerves. Consequently, the effect of Lan3-2 antibody perturbation of sensillar neurons may be mainly exerted within the CNS. That rudimentary fascicles do form during Lan3-2 antibody incubation may be due to the fact that we have not obtained complete inhibition of the function of the Lan3-2 epitope or that some axons are able to rely on other cues that can partly compensate for the blockage of the Lan3-2 epitope. To verify that the antibody perturbation was a specific effect on sen-

sillar neurons expressing the Lan3-2 glycoepitope on their surface and not a general effect on neurons in the CNS, we relabeled Lan3-2–perturbed embryos with an antibody to acetylated tubulin that labels central neurons but not sensillar neurons (Jellies et al., 1996) and visualized them with FITC-conjugated second antibody. As shown in Fig. 10, the development of central neurons, which also express Tractin and LeechCAM but without the Lan3-2 glycoepitope, appears completely normal in embryos injected with Lan3-2 antibody. The projections of the central neurons in both the neuropil and in the peripheral nerves (Fig. 10 *B*) are orderly and indistinguishable from uninjected controls (Fig. 10 *A*). Thus, these experiments suggest that antibody perturbation of the Lan3-2 glycoepitope on sensillar neurons can selectively regulate extension of neurites and filopodia of these neurons at the choice point for bifurcation and fascicle selection in the CNS neuropil, which in many cases leads to abnormal and truncated fascicle formation.

Discussion

In this study we have identified and molecularly cloned the coding sequences for two proteins, LeechCAM and Tractin, which are differentially glycosylated with the Lan3-2 and Lan4-2 glycoepitopes in peripheral sensory neurons in leech. Whereas LeechCAM appears to be a leech homologue of NCAM, FasII, and ApCAM, the organization of Tractin has several unique features. The possession of six Ig-like domains plus four FNIII-like domains places Tractin in the Ig superfamily. However, no other Ig superfamily member contains sequences similar to the acidic region or the PG/YG repeats of Tractin, suggesting that Tractin may define a novel subgroup of these molecules. The organization of Tractin into six Ig-like and four FNIII-like domains in the extracellular part is similar to that of the TAG-1/Axonin-1/F3/F11 subgroup of CAMs (Brümmendorf and Rathjen, 1993); however, on the sequence level this region of Tractin is most homologous (33%) to *Drosophila* neuroglian (Bieber et al., 1989) and other members of the L1 subgroup. Other features shared with the L1 group of CAMs (Hortsch, 1996) are the presence of an ankyrin binding motif in the cytoplasmic tail and of a putative protease cleavage site in the third FNIII-like domain. That Tractin is cleaved at this site is supported by the finding that antibodies to NH_2-terminal sequence of Tractin do not recognize a full-length version of the protein on immunoblots but only a 130-kD glycosylated fragment. Furthermore, previous studies using phase separation with Triton X-114 of the Lan3-2 antigens have shown that a major proportion of the 130-kD proteins partition as loosely associated peripheral membrane proteins (Bajt et al., 1990). The nature of such a peripheral attachment is not known; however, Tractin has an RGD integrin binding motif in the second FNIII-like domain just upstream of the putative cleavage site that could potentially anchor it to the cell surface via association with integrins. A similar mechanism has been proposed for the secreted TAG-1/axonin-1 isoforms (Felsenfeld et al., 1994) that are without glycosylphosphatidylinositol linkers but that also have an RGD motif in their second FNIII-like domains (Ruegg et al., 1989*a*,*b*; Furley et al., 1990; Karagogeos et al., 1991).

Cleavage at the third FNIII-like domain of Tractin would in addition yield a 100-kD transmembrane protein, the extracellular domain of which would be made up largely of the region of the 12 PG/YG repeats. The sequence of this region, although considerably more structured, is reminiscent of that of collagen, which also has sequence rich in glycines and prolines and contains the iterated motif GX_1X_2 where X_1 and X_2 often are prolines (Miller and Gay, 1987). Collagen is a major constituent of extracellular matrix and basal lamina and can form fibrils made up of triple α helices. The PG/YG repeats alternatively can be considered to be composed of the triplet GPG/Y, which would conform with the collagen motif's requirement of a glycine at every third residue that facilitates α helix formation. Thus, while the designation of the repeat as PG/YG is most consistent with the overall sequence alignment, it is conceivable that the sequence may structurally have some of the same functional properties as collagen. The linker regions between the repeats may allow the sequence to fold back upon itself and form an intertwined α domain structure (Branden and Tooze, 1991) or, alternatively, the repeats may interact with other molecules forming the basal lamina. Furthermore, if the prolines were hydroxylated as in collagen, the PG/YG repeat region potentially could be glycosylated. Thus, this novel domain may constitute membrane-

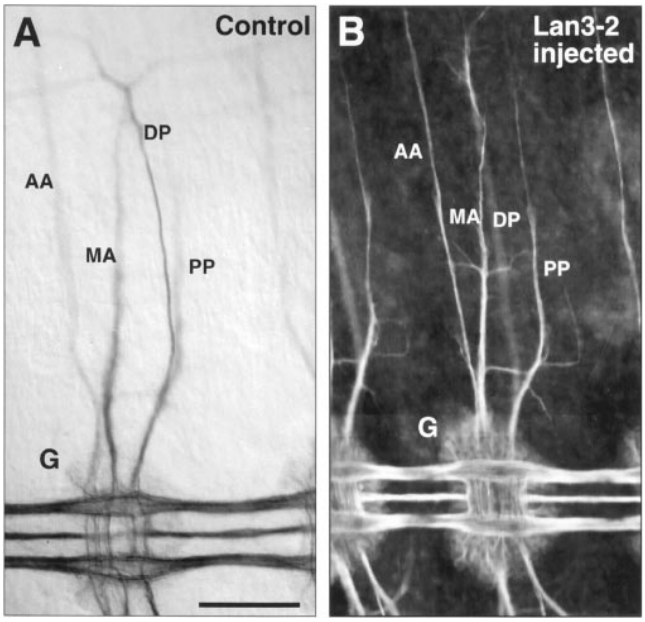


Figure 10. Central neurons and their projections develop normally in Lan3-2–injected embryos. (*A*) Hemisegment of an E9 control embryo labeled with an antibody to acetylated tubulin that shows the ganglionic nerve tracts and peripheral projections of central neurons. The figure is a Nomarski image of antibody labeling visualized with HRP-conjugated secondary antibody. (*B*) Hemisegment of an E9 embryo labeled with acetylated tubulin antibody that was injected with 0.5 μg of Lan3-2 antibody as in Fig. 9 *C*. The organization and extent of central neuron projections is indistinguishable from that of uninjected controls (*A*). The figure is a fluorescence image of antibody labeling visualized with FITC-conjugated second antibody. *G*, location of the ganglion; *AA*, *MA*, *DP*, and *PP*; position of the four peripheral nerves. Bar, 100 μm.

attached sequence that has properties similar to that of extracellular matrix molecules and that may be linked to the cytoskeleton through interactions with ankyrin. Future structural and functional analysis should provide insight into whether this domain participates in organizing extracellular matrix around the neurons and axons, and whether it serves as a substrate for axon outgrowth and guidance.

We show by using antibodies specific to the core protein sequences of Tractin and LeechCAM that these proteins are widely expressed by both central and peripheral neurons in leech embryos. Furthermore, immunocytochemical labeling with the Lan3-2 antibody demonstrated that Tractin and LeechCAM are differentially glycosylated with the Lan3-2 epitope only in sensillar and extrasensillar peripheral sensory neurons (see also Jellies et al., 1995), and that in a small subset of these neurons both proteins are additionally glycosylated with the Lan4-2 epitope. This implies that specific glycosyltransferases are selectively expressed in subpopulations of these neurons and that different polypeptides can serve as substrates for the modifications. Interestingly, in addition to the Lan4-2 epitope, the Lan2-3, Laz2-369, and Laz7-79 glycoepitopes also have been shown to be present on 130-kD proteins and expressed by separate subsets of peripheral sensory neurons (Peinado et al., 1987; Bajt et al., 1990; Zipser et al., 1994). The identity of the neurons expressing the different epitopes is not known, but it has been proposed that neurons sharing a common sensory modality, such as various types of chemoreceptors, mechanoreceptors, and photoreceptors that are mixed within the sensilla, may express specific carbohydrate markers that correlate with their axonal outgrowth and segregation into specific pathways (Zipser et al., 1994). Direct evidence that the different oligosaccharide epitopes can regulate neuronal outgrowth and fascicle formation of distinct neuron subpopulations has been provided by antibody perturbation studies. In this report we show that purified Lan3-2 antibody injected into the germinal plate of the living intact embryo inhibits the extension of neurites and filopodia at the choice points where the sensillar axons segregate into separate fascicles. These results are similar to and confirm those obtained in vitro with incubation of dissected cultured germinal plates with Fab fragments of Lan3-2 antibody, which also leads to an impairment of neurite extension and a reduction in the number of fascicles (Zipser et al., 1989; Song and Zipser, 1995*a*). Evidence that this perturbation is specific to the blockage of the carbohydrate epitope is provided by experiments showing that cleaving of carbohydrate moieties with the glycosidase N-glycanase or incubation with mannose coupled to albumin has the same effect as that of antibody perturbation (Zipser and Cole, 1991). In contrast with the inhibition of neurite extension by Lan3-2 antibody, in vitro incubation with Fab fragments of the Laz2-369 and Laz7-79 antibodies has an outgrowth-promoting effect on the neurites and filopodia that is specific to the subpopulation of neurons expressing each of these epitopes (Song and Zipser, 1995*b*). The proteins carrying the Laz2-369 and Laz7-79 epitopes are furthermore immunoprecipitated by Lan3-2 antibody (Bajt et al., 1990), suggesting that they may represent additional modifications to either or both of the Tractin and LeechCAM proteins. Thus, Tractin and LeechCAM represent protein cores that are differentially glycosylated in distinct subset of neurons where the specific oligosaccharide structures may functionally assist in regulating the outgrowth and patterning of their axonal projections.

Based on the Lan3-2 perturbation experiments in vitro, it has been proposed that the Lan3-2 glycoepitope is involved in regulating the defasciculation of sensillar neurons at the choice points where they segregate into different fascicles in the CNS (Zipser et al., 1989). In other systems, modulation of the polysialic acid content of NCAM in chick has been implicated as a key regulator of a general form of axonal defasciculation (Rutishauser and Landmesser, 1996). However, in leech embryos we found no evidence based on immunocytochemical labeling that the expression of the Lan3-2 epitope is dynamically regulated along the axons, indicating a different functional mechanism. Genetic experiments in *Drosophila* with up- and down-regulation of the CAM FasII, to which LeechCAM is a likely homologue, show that in loss of function mutants the axons that normally fasciculate together fail to do so, whereas in gain of function phenotypes normally separate pathways are joined together and become fused (Grenningloh et al., 1991; Lin et al., 1994). However, as with the Lan3-2 epitope on Tractin and LeechCAM, the level of expression of FasII appears uniform along the axons and fascicles, suggesting that modulation of FasII function by interactions with other molecules is required at the choice points (Fambrough and Goodman, 1996). A gene with such a function that has been recently identified is *beaten path*, a locus that genetically interacts with *FasII* (Fambrough and Goodman, 1996). *beaten path* codes for a secreted protein that by mutational analysis can be shown to regulate the selective defasciculation at specific choice points of motoneurons. In a similar manner, local interactions involving the Lan3-2 epitope may be regulating defasciculation of sensillar neurons and facilitating the segregation of their axons into specific tracts. Alternatively, the Lan3-2 glycoepitope may be involved in promoting axonal outgrowth and neurite extension, the inhibition of which could also account for the observed perturbation phenotype. There are many examples of CAMs stimulating axonal growth (Doherty and Walsh, 1994), and it has been demonstrated that chromophore-assisted laser inactivation of FasII in grasshopper prevents the initiation of Ti1 peripheral neuron axonal outgrowth but has no effect on axon fasciculation (Diamond et al., 1993). Furthermore, oligo-mannosidic carbohydrates expressed on L1 have been shown to determine its interaction with NCAM and to modulate neurite outgrowth (Horstkorte et al., 1993).

In summary, increasing evidence indicates that interactions between surface oligosaccharides and carbohydrate binding proteins mediate many important cellular processes in nonneuronal cells (Springer, 1994; Lasky, 1995); however, progress in studying such interactions within the nervous system has been slow partly due to the lack of probes (Jessell et al., 1990). In leech sensillar neurons, antibodies have identified at least five different glycoepitopes that are expressed by distinct populations of these neurons. Functional studies indicate that these epitopes may regulate different aspects of axonal outgrowth as well as the patterning of projections that are specific to the neuron subpopulations expressing the respective carbohy-

drate structures. The identification and cloning of two of the proteins being differentially glycosylated in this way promises to facilitate the further analysis of how the different physical and biochemical properties of specific carbohydrates may lead to functional diversity and specificity of widely expressed neural proteins.

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