Differential glycosylation of Tractin and LeechCAM, two novel Ig-superfamily members, regulates neurite extension and fascicle formation

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Differential glycosylation of Tractin and LeechCAM, two novel Ig-superfamily members, regulates neurite extension and fascicle formation

by

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This is to certify that the Doctoral dissertation of

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For the Graduate College
To Hongxia and Jingyi (Janne), for their unfailing love.
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INTRODUCTION

The proper functioning of a nervous system requires the appropriate wiring up of the connections in development. The specificity of the connections is first partially established independent of the neuronal activity and fine-tuned and remodeled depending upon the activities. During development, the growing axons usually navigate long distances to find their correct target areas, in response to a variety of guidance signals in their environment. The growth cones, the enlarged tips of growing axons, have the responsibility to explore the surrounding guidance cues, and transduce and integrate the signals into the cell which then translates the signals into meaningful steering decisions. During the last decade or so, especially the last few years, our understanding of the cellular and molecular mechanisms of axon guidance has greatly increased.

A few excellent reviews appeared recently which summarized the new development in the field. Goodman and Shatz (1993) gave a comprehensive overview of the cellular mechanism by which the neuronal specificity is built up. More recent reviews emphasizing the molecular aspects are found in Keynes and Cook (1995), Goodman (1996), Tessier-Lavigne and Goodman (1996), and Johansen and Johansen (1997). This chapter is not meant to be all-inclusive. Here I shall briefly summarize the cellular mechanisms of axon guidance, and then examine the newly found molecules implicated. Following that is an introduction of the leech nervous system as a model to study the role of carbohydrate mediated cell recognition in axon guidance.

Four different cellular mechanisms in axon guidance

It is now well established that the guidance cues can be either permissive/attractive, or inhibitory/repulsive; they can either be short range, or long range. The four combinations of all these would be short range attractive, short range repulsive, long range attractive
(chemoattractive) and long range repulsive (chemorespulsive) (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). Short range guidance is mediated by the interactions between the growth cones and their contacting cells and extracellular matrix; long range guidance, on the other hand, depends on the sensing of diffusible factors secreted from the targets. One must bear in mind, however, that the division between these four guidance cues can be arbitrary, and one molecule can be either attractive or repulsive depending on the axons and their receptors, and furthermore, the different cues can often be utilized by the same axon pathways. For example, the concept of selective fasciculation mediated by cell adhesion molecules was thought as the best example of short range attractive guidance, but now there is accumulating evidence suggesting that fasciculation may actually be the default pathway when axons encounter some repulsive cues (see the sections on Semaphorin I and Eph Receptors below).

The hypothesis that the target can secret molecules that attract the innervating axon dates back to Spanish neurologist Santiago Ramon y Cajal (1893). The evidence to demonstrate this, however, had to wait for nearly a century to come, when Lumsden and Davis (1983) used their newly developed collagen gel co-culture assay to show that trigeminal ganglion axons are attracted by a diffusible factor released by their peripheral target, the maxillary epithelium. In the spinal cord, it was demonstrated similarly that the floor plate releases a diffusible activity that attract the ventrally growing commissural axons (Tessier-Lavigne et al., 1988). Netrins and Netrin receptor DCC are the molecules shown to mediate the long range attraction in nematode, fly and vertebrates (Serafini et al., 1994; Keino-Masu et al., 1996).

In contrast to the idea of attractive guidance, the notion that axons can be guided through inhibitory or repulsive cues was relatively new. The first solid evidences to show the short range inhibition were provided through the growth cone collapse assay developed by Raper and colleagues (Kapfmammer and Raper, 1987) and the strip assay by Bonhoeffer's group (Walter et al., 1987). Pini (1993) was the first to prove that chemorepulsion through a
di usu ble factor released from the septum was responsible for the guidance of lateral olfactory tract axons away from the midline. Now the examples of inhibitory/repulsive guidance include the avoidance of central axons by peripheral axons and vice versa (Kapfhammer and Raper, 1987), the avoidance of posterior tectum by temporal retinal axons (Walter et al., 1987; Drescher et al., 1995), the avoidance of floor plate cells by trochlear motor axons (Colamarino and Tessier-Lavigne, 1995), as well as the avoidance of ventral spinal cord by NGF responsive temperature and pain receptors (Fitzgerald et al., 1993; Messersmith et al., 1995)

**Netrins act as both attractive and repulsive cues**

Netrins are a family of conserved long range guidances cues. The first members of netrin proteins were originally purified from chick embryonic brains on the basis of their ability to mimic the out-growth promoting activity of the floor plate cells on the commissural axons (Serafini et al., 1994; Kennedy et al., 1994). Evidence that netrin-1 is the likely floor plate chemoattractant came from examination of its expression pattern and in vitro assays. First, netrin-1 is expressed solely at the floor plate whereas netrin-2 is expressed more widely and at lower levels in the ventral two-thirds of the spinal cord (Serafini et al., 1994). Second, the medium conditioned with COS cells expressing netrin-1 can stimulate the axon outgrowth just like the floor plate, mimicking the chemotrophic effect. Most importantly, these transfectant cells can mimic the floor plate in collagen gel assay to induce the turning of the commissural axons toward the cell aggregates (Kennedy et al., 1994).

Netrin-1 and netrin-2 are 72% similar to each other and they are 50% homologous to nematode C. elegans UNC-6, a secreted molecule required along with two other genes, *unc-5* and *unc-40*, for circumferential projections of commissural axons in both the dorsal and the ventral direction (Hegecock et al., 1990; Ishii et al., 1992). The mutation of *unc-6* disrupts both the dorsal and ventral migrations. UNC-6 is expressed in ventral midline neurons and other ventral cells such as epidermal myoblasts and cephalic sheaths (Wadsworth et al., 1996).
In *unc-5* mutants, only the dorsal migration is impaired; while *unc-40* mutation affects mainly the ventral (and to a lesser extent the dorsal) growth (Hegcock et al., 1990). UNC-5 is a transmembrane protein with 2 Ig domains and 2 thrombospondin-1 like domains (Leung-Hagesteijn et al., 1992). The genetic data all support a model in which UNC-6 acts as a bi-functional molecule, attracting some axons while repelling some others. UNC-5 is a likely receptor (or a component of receptor complex) for the repellent cue, since first, *unc-5* ectopic expression by some neurons that usually don’t express it and that normally project ventrally or longitudinally cause the dorsal growth of their axons. Furthermore, this ectopic effect is totally dependent upon the *unc-6* (Hemlin et al., 1993). UNC-40 is likely a receptor involved in guiding the ventral growth (Chan et al., 1996; see below). The finding that the *unc-6* dorsal and ventral phenotypes can be correlated with specific mutations in different UNC-6 domains further suggests that the attractive and repulsive activities of UNC-6 might be related to distinct domains (Wadsworth et al., 1996).

The structural similarity to UNC-6 suggests a similar repulsive function of the newly found chemoattractant netrin-1. And this was indeed confirmed by Colamarino and Tessier-Lavigne (1995), who found that floor plate can repel the axons of trichlear motor neurons which normally grow dorsally away from the floor plate in vivo. COS cells secreting recombinant netrin-1 mimic this effect, suggesting that the bi-functionality of UNC-6/netrin is conserved between invertebrates and vertebrates.

The function of netrins has also been assayed in Drosophila, whose genome harbors two netrins tandemly, *Netrin-A* and *Netrin-B*. The fly netrins also function at the midline in a manner similar to that of nematode and vertebrates (Mitchell et al., 1996; Harris et al., 1996). These netrins are expressed at the midline (and also subsets of neurons, muscles and epidermal tissues); and in embryos lacking the two Drosophila netrin genes, CNS commisures are partially missing or thinner. This CNS phenotype is rescued by expressing either one of the netrins in the midline. Pan-neural ectopic expression of netrins causes defects similar to loss-
of-function mutations, suggesting that netrin expression patterns are crucial for the appropriate function. At present, it is not known if Drosophila netrins also can function as repulsive cue as nematode and vertebrate netrins do.

The function of vertebrate netrin-1 was further confirmed in vivo with a loss-of-function mutation identified through a gene-trap screen (Serafini et al., 1996). An analysis of the mice homozygous for a hypomorphic mutation shows severe defects in spinal commissural axons, suggesting a role for netrin-1 in guiding these axons consistent with previous data. Several forebrain commissures are also found to be abnormal, suggesting additional function for netrin-1. However, trochlear motor axons, which can be repelled by recombinant netrin-1, are largely normal. This raises the possibility of additional repulsive cues for these axons. The evidence for the existence of a distinct repelling activity comes from a collagen gel co-culture assay showing that floor plate cells from the mutant embryos retain the ability to repel the trochlear motor axons.

Both the structural and functional conservation of netrins from nematode to chordates suggest a similar signaling mechanism. This is now well documented by the studies which characterized the netrin receptors in nematode, fly and rat (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). In nematode C. elegans, mutations in the unc-40 gene affect the ventral migration of axons and epidermal cells in a similar way as unc-6 mutations (Hedgecock et al., 1990; see above); whereas the unc-6:unc-40 double mutation does not enhance either unc-6 or unc-40 phenotype. The genetic data were interpreted to suggest that UNC-40 might act as a receptor for UNC-6. Now the cloning of unc-40 shows that UNC-40 is a nematode homolog of the vertebrate cell adhesion molecules DCC (Deleted in Colorectal Cancer) and neogenin, which form a subgroup of the immunoglobulin superfamily which is characterized by having four Ig domains and six FNIII (fibronectin type III) repeats (Chan et al., 1996). UNC-40 is expressed by motile cells and pioneer neurons and is shown to work autonomously, consistent with its possible function as a receptor. This notion is backed by the
studies of rat DCC (Keino-Masu et al., 1996). DCC was isolated originally as a candidate tumor suppressor gene that is lost at high frequency in colorectal cancers (Fearon et al., 1990). DCC was shown to be expressed in the developing mouse, chick, and Xenopus nervous system (reviewed in Keino-Masu et al., 1996). The actual function of DCC in the nervous system was not known until recently when it was shown that netrin-1 binds DCC-expressing cells in vitro (Keino-Masu et al., 1996). Furthermore, DCC is expressed by the commissural neurons and axons which are known to be guided by netrins. The concept of DCC as a netrin receptor is further tested with function-blocking antibody which perturbs the axon outgrowth effects of netrin-1. These results are supported by genetic analysis of the Drosophila DCC homolog frazzled (Kolodziej et al., 1996). The Drosophila frazzled mutant was identified through an enhancer-trap screen for mutations that affect nervous system development. The frazzled mutants show gaps in longitudinal axon tracts, they also have defects in commissures, a phenotype very much like the netrin double mutants. Frazzled protein is expressed on commissural and longitudinal axons. Molecular analysis with transgenic flies shows that frazzled acts cell-autonomously. All these data, taken together, strongly support the hypothesis that in C. elegans, flies, and rodents, DCC/UNC-40/frazzled function as a netrin/UNC-6 receptor. DCC/UNC-40/frazzled do have a cytoplasmic domain however, its features do not suggest a mechanism by which the signals are transduced. It remains to be determined which molecules act downstream of the DCC receptor. It will also be of interest to see if vertebrates use a UNC-5 homolog in mediating the repulsive activity of netrin-1.

The semaphorin/collapsin family in repulsion

The first members of this family were cloned through two very different approaches. Semaphorin-I (formerly Fasciclin IV) was identified by its dynamic expression pattern in grasshopper CNS (Kolodkin et al., 1992). Collapsin-1 was purified as a dorsal root ganglia growth-cone collapsing activity from chick brains. Collapsin-1 was found to be homologous to
semaphorin. Based on the sequence similarities, PCR strategies were taken to clone more semaphorin family members in Drosophila, chicken, mouse, and human (Kolodkin et al., 1993; Luo et al., 1995; Puschel et al., 1995).

Semaphorin/collapsin is a family of transmembrane and secreted proteins of about 100 kDa. The hallmark of semaphorin/collapsin is the semaphorin domain of about 500 amino acids with 14-16 conserved cysteines, and blocks of other conserved residues. The transmembrane semaphorins have an additional ~80 amino acids stretch, a transmembrane domain, and a short cytoplasmic domain; while the secreted semaphorins have a single immunoglobulin domain, and a 70-120 amino acid C-terminal region. Recently, still another semaphorin member was found to have a thrombospondin-like domain (see Tessier-Lavigne and Goodman, 1996). Different semaphorins are expressed in distinct but overlapping regions in the developing nervous system (and other systems also, suggesting additional functions).

The function of semaphorins in repulsive/inhibitory axon guidance is now firmly established for several semaphorin members, namely grasshopper Sema I, Drosophila Sema II, and rat Sema III/chick collapsin-1, each by a different approach. Grasshopper Sema I, a transmembrane Sema member, is expressed on a strip of epithelial tissue in the limb bud. This strip is perpendicular to the initial direction of outgrowth of a pair of afferent pioneer TII neurons. Normally, when the pioneer neuron pair encounter the Sema I strip, they make a sharp turn initially both dorsally and ventrally, then extend ventrally as a fasciculated bundle along the distal portion of the strip. They then make another turn and extend proximally across the strip. Sema I antibody perturbs this fasciculation in limb bud culture. This phenomenon was re-interpreted to suggest that Sema I acts as an inhibitory cue that stall and then steer these axons by inhibiting axons from entering the strip first, by preventing branching of the axonal bundle (thus promoting the fasciculation). Since the axons finally enter the strip, the strip can not be an absolute inhibitor (Kolodkin et al., 1993).
Drosophila Sema II function was tested with a gain-of-function mutation, since loss-of-function mutation of Sema II doesn't show a readily visible phenotype (Methes et al., 1995). The transient expression of Drosophila Sema II by a subset of CNS neurons and by a single thoracic muscle in the periphery suggests this secreted molecule might have some function. In transgenic flies that ectopically express Sema II in muscles that usually do not express it, two identified growth cones RP3 and DC1 were found to be inhibited from forming synaptic arborizations on their target muscles. Sema II does not repel the axons from entering its expression region. The results show that semaphorin II in vivo can function as a selective target-derived signal that inhibits synaptic arborizations.

Unlike grasshopper Sema I and Drosophila Sema II, which seem to function as inhibitory cues, the vertebrate Sema III has a strong repelling function. This was tested elegantly by an in vitro assay (Messersmith et al., 1995). In the dorsal root ganglia, distinct classes of sensory neurons can be differentiated by their physiological properties, axonal diameters, pattern of projections into the spinal cord, and finally, their responsiveness to different neurotrophic growth factors. For example, temperature and pain receptor sensory neurons have small diameter afferents, and terminate in the dorsal-most laminae (I and II), and they are NGF responsive. The large group of Ia muscle spindle afferents, terminate on the motor neurons in the ventral portion of the spinal cord, and they are responsive to neurotrophin 3 (NT-3). Fitzgerald et al (1993) showed directly that a diffusible factor is secreted from the ventral spinal cord that inhibits the neurite outgrowth of dorsal root ganglia. Since collapsin-1, a chick homolog of mouse/rat/human Sema III, was previously shown to induce the collapse of DRG growth cones (Luo et al., 1993), and Sema III is expressed by the ventral spinal cord (but not the floor plate), Messersmith et al (1995) set out to check if Sema III was responsible for the inhibitory activity of the ventral spinal cord demonstrated by Fitzgerald et al. (1993). In culture assays, Sema III expressing COS cells can mimic the ventral spinal cord's ability to repel the axons of the NGF responsive thermo- and noci-receptor neurons, but not NT-3.
responsive Ia neurons. This suggest an in vivo function of Sema III in patterning the sensory axon projections in the spinal cord, presumably by restricting the ventral growth of the NGF responsive axons that terminate in the dorsal-most part, without effect on the axons that terminate in the ventral spinal cord. More recently, the exact function of Sema III in the spinal cord was examined in Sema III knock-out mice (Behar et al., 1996). In the Sema III mutants, some sensory axons project into the regions of the spinal cord where Sema III is normally expressed, thus directly demonstrating an in vivo function for Sema III. Sema III mutant also shows defects in other regions of the nervous system and in bones and heart.

Eph receptor tyrosine kinases and ligands

The Eph family of the receptor tyrosine kinases (RTK), named after its first identified member, is the largest known family of the RTKs with at least 13 distinct members. Members of the Eph receptor family are expressed dynamically and spatially restrictively during embryogenesis. A role of Eph receptors in axonal pathfinding was originally suggested by the finding that murine Nuk receptor (also called Hek5/Erk/Sek3/Cek5 in different literature) is expressed on initial axonal outgrowths (Henkemeyer et al., 1994). The ligands for the Eph receptors have been identified recently. The seven published Eph receptor ligands comprise a family, with members sharing 23-56% homology to each other. One striking feature of the Eph ligands is that all are membrane associated, either through a transmembrane domain or through glycosyl-phosphatidylinositol (GPI) linkers. The association with the membrane is required for receptor-ligand interaction. This suggests that the Eph receptors/ligands are ideal candidates for mediating locally restricted signaling. Another eminent feature for the Eph signaling is that it does not elicit conventional growth responses as other RTK usually do. Recent studies have accumulated evidence suggesting that Eph receptors and ligands might be involved in axonal bundling or guidance, perhaps by providing repulsive signals, and by providing positional
cues for establishing retinotectal projection patterns (Winslow et al., 1995; Drescher et al., 1995; Cheng et al., 1995; reviewed in Tessier-Lavigne, 1995).

The first hint came from the studies of AL-1, a ligand for Eph receptor Rek7 by Winslow et al. (1995), who found in a cortical neuron-astrocyte co-culture assay that AL-1 expressed on astrocytes promotes cortical neuron axonal fasciculation by interacting with Rek7 expressed on the neurons, as both soluble AL-1 and extracellular domain of Rek7 can cause the defasciculation. AL-1 probably acts as a repulsive signal that promotes fasciculation by default. The second line of work was done in the retinotectal system, where the function of Eph receptors/ligands are best understood. RAGS (repulsive axon guidance signal) was purified through a search of molecules responsible for the repulsive activity of the posterior tectal membrane to the temporal retinal axons (Drescher et al., 1995; Walter et al., 1987). RAGS is the chick homolog of AL-1. RAGS is GPI linked and expressed in a posterior to anterior gradient, and most importantly, cell membranes from COS cells expressing the recombinant RAGS can mimic the posterior tectal membrane to repel retinal axons. Unlike the native posterior tectal membrane, RAGS expressed on COS cells are equally potent in repelling nasal and temporal axons. Another line of evidence that Eph ligand can act as a guidance molecule came from the finding that Mek4, another Eph RTK, is expressed in retinal ganglion cells and their axon terminals in a topographic gradient complimentary to the topographic expression of its ligand, ELF-1 (Cheng et al., 1995). Mek-4 is expressed in an increasing nasal-temporal gradient; whereas ELF-1 is expressed in a decreasing posterior-anterior gradient in the tectum. The existence of opposing gradients of receptor and ligand makes them excellent candidates for being the positional labels for topographic map development. The actual function of ELF-1 in retinal axon guidance and mapping was examined in vitro and in vivo respectively in a study by Nakamoto et al. (1996). In vitro, ELF-1 does act as a repellent for retinal axons. Moreover, ectopic expression of ELF-1 in vivo mediated by retroviral vectors in the tectum disturbs the retinal axon map. Furthermore, the ELF-1 has specific effects on temporal, but not nasal,
retinal axons. Comparing the ELF-1 and RAGS expression and activity, it was proposed that ELF-1 has a role in determining the nasal versus temporal specificity, while RAGS might have a role in refining topographic specificity near the posterior end of the tectum (Nakamoto et al., 1996).

**Receptor protein tyrosine phosphatases**

Just as RTKs are implicated in axon guidance, so are receptor protein tyrosine phosphatases (RPTPs). Two features suggest a role of RPTPs in axon guidance. First, the presence of Ig- and FNIII domains in the extracellular domain similar to other CAMs such as NCAM, Fas II, neuroglian, L1, suggest that RPTPs might act as cell adhesion molecules. Second, four of five known RPTPs in flies are predominantly expressed on developing axons. Vertebrate RPTP CRYPα has been found in the growth cones (Stoker et al., 1995). That RPTPs are indeed axon guidance molecules is supported by genetic analysis of loss-of-function mutations of several RPTPs in Drosophila (Desai et al., 1996; Krueger et al., 1996). All the analyses were performed on the motor axons that innervate the musculature. The motor axons exiting the CNS are sorted into five branches: the intersegmental nerve (ISN), 4 segmental nerves a, b, c and d (SNa, SNb, SNc and SNd). Normally, SNb exits the common motor pathway, enters the ventral target region and then makes contacts with specific ventral muscles. In DLAR (a Drosophila homolog of LAR RPTP) mutants, SNb axons bypass their normal target muscles and continue to extend distally, following the ISN (although as a distinct fascicle) along its dorsal trajectory (Krueger et al., 1996). In RPTP69D loss-of-function mutants, SNb pathway displays a variety of aberrant phenotypes, for example, some of the SNb axons bypass their normal targets and continue to grow dorsally within ISN (whereas in DLAR mutants, the SNb axons do appear to separate from the ISN but do follow ISN). Some of the SNb axons that bypass their normal ventral targets can make a U-turn and leaves the ISN to go back to their normal targets. In the so-called “detour” phenotype, some SNb axons can
make correct contacts with their normal targets but through incorrect routes. DPTP99A mutation, although itself does not display severe phenotype, enhances the phenotypes of DPTP69D when in the double mutants (Desai et al., 1996). These two in vivo studies directly implicate RPTPs in motor axon pathfinding. Furthermore, DLAR and PTP69D might have distinct functions: PTP69D may be mainly involved in the defasciculation from the ISN, while DLAR might have more direct role in recognition of the correct target signals.

Cell adhesion molecules

Classical cell adhesion molecules that have been implicated in axonal growth and guidance are categorized into three major classes: integrins, cadherins and immunoglobulin superfamily (IgSF) members. Cell adhesion molecules have been the subject of several excellent reviews, they include Edelman and Crossin (1991), Hortsch and Goodman (1991), Doherty and Williams (1994), Chiba and Keshishian (1996), Fields and Itoh (1996) and Vaughn and Bjorkmann (1996). More specific reviews dealing with one family of molecules include: for IgSFs, Brumendorf and Rathjen (1993), Harpaz and Chothia (1994); for cadherins, Redies and Takeichi (1996); and for integrins, Hynes (1992), Brugg (1996). Here I highlight some of the recent advances showing the complexities and importance of cell adhesion molecules in axonal growth and guidance.

Integrins are heterodimeric receptors for extracellular matrix; cadherins are a family of Ca\(^{++}\) dependent cell adhesion molecules with a number of cadherin repeats in the extracellular domain. In contrast to cadherin, integrins and IgSF members bind in a Ca\(^{++}\)-independent way. By definition, IgSFs all have one or more immunoglobulin-like domains which show homology to the variable or constant domains of antibodies. At the primary sequence level, they are traditionally identified by the presence of two cysteines separated by 55-75 amino acids (which form a disulfide bond in the folded structure). Structurally, the Ig-like domains are composed of seven to nine β-strands arranged into two anti-parallel sheets (Vaughn and
Bjokman, 1996). While some IgSF members have only Ig domains, many IgSFs are found to consist of tandem Ig-like domains connected in series with other structural motifs, such as the fibronectin type III repeats that surprisingly have a similar β-strand structure as the Ig-domains, although at the amino acid sequence level, there is no detectable homology between Ig-like and FNIII domains. Interestingly, some of the signaling molecules implicated in various aspects of axon guidance also posses elements of Ig and/or FNIII, e.g. Eph TKRs and receptor tyrosine phosphatases (see previous sections).

The dynamic expression patterns of the cell adhesion molecules in developing nervous systems and in vitro assay studies suggest a functional role for them in neurite outgrowth promotion and selective fasciculation. There are ample examples of in vitro studies showing that purified cell adhesion molecules are good substrates for permitting axonal outgrowth, antibodies have also often been used in in vitro assays to show that the various CAMs are involved in mediating the fasciculation process (reviewed in Rathjen and Jessell, 1991). Recently, these in vitro investigations are being complemented and confirmed by in vivo studies taking several approaches. In Drosophila where advanced genetics is available, transgenic flies carrying loss-of-function and gain-of-function mutations were analyzed (Lin and Goodman, 1994; Lin et al., 1994; Chiba and Keshishian, 1995); and in mouse, the knock-out strategy has begun to be used to study the function of CAMs in neural development (Cremer et al., 1994; Tomasasiewicz et al., 1993). In chick and Xenopus, where the embryos are more accessible for experimental manipulations, in vivo antibody perturbation results have shed some light on the roles different CAMs play in the guidance of commissural (Stockeli and Landmesser, 1995) or retinal axons (Stone and Sakaguchi, 1996). In addition to the antibody blocking method, a strategy utilizing the retrovirus-mediated ectopic expression of mutant CAMs was taken by Holt and collegues (Riehl et al., 1996 ) to show the function of N-cadherin in the initiation of axon growth. Last but not least, in human, where none of the above approaches is applicable, the investigations of CAMs have benefited from the genetic and
molecular analysis of mutations of CAMs that give rise to neurological diseases (reviewed in Wong et al., 1995; Hortsch, 1996). Below, I shall briefly review these in vivo functional studies illustrating different strategies.

Drosophila Fas II, an IgSF member related to vertebrate NCAM, is expressed by a subset of three longitudinal axons (Grenningloh et al., 1991). Loss-of-function of Fas II leads to abnormal defasciculation of the axons which normally express Fsa II; transgenic flies carrying a Fas II expressed by these axons can rescue the abnormal defasciculation; in addition, over-expression of Fas II results in the fasciculation of some axons that usually would defasciculate (Lin et al., 1994). Thus, an in vivo function of Fas II in these axons would be to mediate the selective fasciculation. Furthermore, increased expression of Fas II by motor axons and ectopic expression of Fas II on other cells they encounter lead to four classes of defects of the motor axons (Lin and Goodman, 1994). Thus, the level and pattern of Fas II expression in vivo must be tightly regulated to modulate the ability of growth cones to respond to guidance cues.

Like Fas II, mouse NCAM function has been researched through loss-of-function mutations. Two mouse NCAM loss-of-function mutations have been generated: in one, a major NCAM isoform is deleted (Tomasiewicz et al., 1993); in the other, Cremer et al. (1995) knocked out all the NCAM isoforms. While both mutations unexpectedly resulted in viable and fertile mice, they do show some subtle defects in the nervous system. For example, localized and distinct defects in the CNS include the morphology of the ventricular zone, olfactory bulb, cerebellum, retina, and hippocampus (Tomasiewicz et al., 1993); whole NCAM inactivation also leads to defects in spatial learning (Cremer et al., 1994). More intensive analysis is needed to reveal subtle defects that might not be seen at first sight.

For chick and Xenopus researchers, mutants are rare and a better approach was to inject specific antibodies against CAMs into a localized area and assay the effects. This is best illustrated by results from two groups. In the chick, Stoeckli and Landmesser (1995) studied
the axonin-1, Ng-CAM, and Nr-CAM, three members of the immunoglobulin superfamily, in the guidance of commissural axons. Axonin-1 and Ng-CAM are expressed by the commissural axons and Nr-CAM by the floor plate cells. Perturbation of axonin-1 (by injections of either specific antibody or a soluble form of axonin-1) not only causes defasciculation of the commissural axons, but also pathfinding errors: ~50% of commissural axons fail to cross the midline. The pathfinding errors are separable from that of the defasciculation since injections of Ng-CAM antibody mainly cause defasciculation but do not prevent the commissural axons from crossing the midline. Injections of Nr-CAM antibody result in the same commissural pathfinding errors as perturbing axonin-1. These results directly implicate axonin-1 and Ng-CAM in the fasciculation, and axonin-1 and Nr-CAM in pathfinding of commissural axons; furthermore, it suggests an interaction of Nr-CAM and axonin-1 with the latter being the receptor (Stoeckli and Landmesser, 1995). The function of N-cadherin and β1-integrins in Xenopus retinotectal projection has been examined similarly by injecting blocking antibodies (Stone and Sakaguchi, 1996). While the injection of either β1-integrin antibody or N-cadherin antibody does cause slight pathfinding errors, injecting both antibodies together has a much stronger effect. Pathfinding errors both in the optic tract and within the optic tectum are scored. This shows that both β1-integrins and N-cadherin are required for the projection of the retinotectal pathway.

Finally, human L1 mutations have been shown to be responsible for several clinically related neurological diseases, including X-linked hydrocephalus, MASA syndrome (Mental retardation, Aphasia, Shuffling gait and Adducted thumbs), and SPG1 (Spastic paraplegia type I) (Wong et al., 1995; Hortsch, 1996). The mutations found include reading frame shifts, truncations, and missense mutations. Although a correlation between the mutations and the severity of different phenotypes are complex to analyze, the phenotypes are consistent with a role of L1 in neuronal migration and axonal guidance in development.
Carbohydrates in axon guidance and leech as a model system to study the role of carbohydrate-mediated recognition

Although some CAMs such as fasciclinis (reviewed in Hortsch and Goodman, 1991), LAMP (Pimenta et al., 1995), and TAG-1 (Dodd et al., 1988) have very restricted expression, most molecules studied thus far are relatively widely expressed. So the specificity is unlikely conferred by the adhesion molecules per se. The general perception in the field is that ubiquitously expressed IgSF members can modulate growth of ALL types of neurons. 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). Alternative splicing however, is unlikely to generate all the specificity needed to match the specificity of growth cone guidance and pathway selection. One fascinating possibility however, is that carbohydrate structures, specifically expressed on subsets of these molecules may be responsible for a higher order of specificity.

It has long been recognized that the structural diversity of cell surface carbohydrates make them ideal candidates for mediating cell specific recognition processes (Roseman 1970; 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 oligosaccharides (Springer, 1994; Lasky, 1995). In the 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 (PSA) content of N-CAM in the plexus region of the chick limb bud where its upregulation 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 monoclonal antibodies (mAbs) and dye-injections, the axons of sensillar neurons have been shown to project in tightly fasciculated bundles through the periphery into the 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). At least five different mAbs (Lan3-2, Lan2-3, Lan4-2, Laz2-369, Laz7-79) which 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, 1995a), 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, 1995b). Thus, there is compelling evidence that a hierarchy of distinct carbohydrate structures expressed by specific neurons are 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, respectively. 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 and we provide evidence that the N-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 homolog of NCAM (Cunningham et al., 1987), FasII (Harrelson and Goodman, 1988), and ApCAM (Mayford et al., 1992). Surprisingly, Tractin and LeechCAM are expressed by both peripheral and central neurons but are differentially glycosylated with the 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 hirudiniid 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 (in mM): 110 NaCl, 4 KCl, 2 CaCl₂, 10 glucose, 10 HEPES, pH 7.4. In some cases 8% ethanol was added and the saline solution 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 water that was made as sterile-filtered solutions of 0.0005% commercial sea salt (Instant Ocean), wt/wt. Embryonic day 10 (E10) was characterized by the first sign of a tail sucker, while E30 is the termination of embryogenesis. There are about 10-20 embryos in each cocoon and these sibling embryos develop synchronously within a few percent of development.

Protein purification and microsequencing

Purification of the Lan3-2 glycoproteins was achieved by constructing an immunoaffinity column of protein G-Sepharose (Pharmacia) to which purified mAb Lan3-2 had been covalently coupled by dimethyl pimelimidate dihydrochloride (Pierce) according to the method of Schneider et al. (1982) at a ratio of 2.5 mg mAb/ml of matrix. The mAb Lan3-2 was purified from hybridoma culture supernatant grown in serum-free medium (Nutridoma) by binding to a protein G-Sepharose matrix. A non-specific purified mouse IgG (Sigma) 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 MgCl₂, 1 mM CaCl₂, 0.2% NP-
40, 0.2% Triton X-100, pH 7.4) containing protease inhibitors. The homogenate was incubated on ice for 1 hour 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 non-specific mouse IgG bound protein G Sepharose for 4-8 hr, centrifuged for 30 sec at 2000 g, and the resulting supernatant incubated with 1 ml of mAb Lan3-2 bound protein G Sepharose overnight. This slurry was then applied to a Bio-Rad column which was sequentially washed with 5-10 ml of each of the following buffers containing protease inhibitors: 1) extraction buffer; 2) 20 mM Tris-HCl, 200 mM NaCl, 0.2% NP-40, 0.2% Triton X-100, pH 8.2; 3) 50 mM Tris-HCl, 1 M NaCl, 0.5% NP-40, pH 8.2; 4) 50 mM Tris-HCl, 150 mM NaCl, pH 8.2; and 5) 20 mM Tris-HCl, 150 mM NaCl, 0.5% Na-DOC (deoxycholic acid, sodium salt, Sigma), pH 8.2. After these washes the column was disconnected from the chromatography system, 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 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 combined before dialysis against a buffer containing 10 mM NH₄CO₃, 0.02% SDS, pH 7.8. The resulting eluate was concentrated with a Centricon-50 spin column at 5000g.

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), and the transferred proteins stained by Amido Black (0.1% 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 he broad 130 kDa band was cut out, in situ digested with trypsin, the cleaved peptides separated by microbore HPLC, and the fractions 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 and Lan4-2 (Zipser and McKay, 1981; McKay et al., 1983) as well as an antibody to acetylated tubulin (Sigma) 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, CYNLDYEGNFHFANVMEEDHR-NH₂; pep3, MEEVEIPCEAC-NH₂ (both by QCB Inc., Hopkinton, MA) and pep6, SHHYNEWEKPETGGSPIRC-NH₂ (by Chiron Mimotopes, Emeryville, CA). A cysteine (in bold) was added to either the N-terminal or the C-terminal end of each peptide for coupling purposes, each peptide ended with an -NH₂ amide group, and two residues (in italics) were modified by acetylation. The peptides were covalently coupled to keyhole limpet haemocyanine (KLH) (Pierce) carrier protein with Sulfo-SMCC (Sulfosuccinimidyl 4-(N-maleimidoraethyl) cyclohexane-1-carboxylate, Pierce) as per the instructions of the manufacturer. Two rabbits were injected with from 200 µg to 400 µg of each of the coupled peptides, and then boosted at 21 day intervals as described in Harlow and Lane (1988). After the second boost serum samples were collected seven and ten days after injection. The sera was 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 1:5000 dilution in Blotto (0.5% Carnation non-fat dry milk in TBS). In addition to the rabbit antisera a mAb, 4G5, was obtained to pep2 by injecting balb C mice with 50 µg of the KLH-coupled peptide at 21 day intervals. After the third boost spleen cells of the mice were fused with myeloma cells and a monospecific hybridoma line established using standard procedures (Harlow and Lane, 1988). 4G5 ascites was obtained by injecting 4 mice interperitoneally with antibody producing hybridoma cells. The 4G5 mAb is of the IgG₁
Molecular cloning and sequence analysis

Rabbit antisera to pep2 and pep6 sequence at a dilution of 1:4000 were used to screen a random primed Hirudo CNS-enriched cDNA lambda-ZAP II expression library essentially according to the procedures of Sambrook et al. (1989). A total of 10^6 plaques were screened at a density of 30,000 plaque-forming units/150 mm plate. Positive clones were in vivo excised to generate pBluscript phagemids according to the method provided by the manufacturer (Stratagene). 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 32P-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) and the library screened using standard procedures (Sambrook et al., 1989).

DNA sequencing was performed using Applied BioSystem DNA Sequencer 377A at ISU Nucleic Acid Sequencing Facility using commercially available sequencing primers (universal and reverse primers from Stratagene) or specific primers synthesized at the ISU Nucleic Acid Synthesis Facility based on known Tractin and/or LeechCAM sequences. The nucleotide and predicted amino acid sequences were analyzed using the GCG (Genetics Computer Group 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 NCBI 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 denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate, 0.1M β-mercaptoethanol, pH 7.0) to which the following solutions were sequentially added with thorough mixing: 1) 6 ml of 2 M sodium acetate, pH 4, 2) 60 ml of phenol (Tris-HCl saturated), and 3) 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 volume (about 60 ml) of isopropanol at -20°C for at least 1 h before being centrifuged at 10,000 g for 20 minutes 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 DEPC (diethyl pyrocarbonate, Sigma)-treated H₂O. PolyA+ mRNA was isolated using an oligo-dT column matrix (New England BioLabs) 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 a [³²P]dCTP-labeled Tractin or LeechCAM fragments according to standard methods (Ausubel et al, 1987). High stringency hybridization and washing conditions were employed (Ausubel et al, 1987), and the filters were exposed to X-OMAT XAR film (Kodak) at -80°C with an intensifying screen.

Biochemical analysis

SDS-PAGE, Western blotting, and 2-D gel electrophoresis. SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was
performed as in Towbin et al. (1979) with 0.5X 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) (1:3000) for visualization of primary antibody diluted 1:2000 in Blotto for immunoblot analysis. The signal was developed with 3,3’ diaminobenzidine (0.1 mg/ml) and H2O2 (0.03%) and enhanced with 0.008% NiCl2. The Western blots were digitized using the NIH-image software, a cooled high resolution CCD-camera (Paultek), and a PixelBuffer framegrabber (Perceptics) or an Arcus II scanner (AGFA).

2-Dimensional gel electrophoresis was done according to Boxberg (1988) using the Bio-Rad Mini PROTEAN II 2-D cell. In brief, for first dimension isoelectrofocusing each sample contained 3 μl of Lan3-2 antigen from the original purification (equivalent to antigen from 6 leech nerve cords) in 10 mM NH4CO3, 0.02% SDS, pH 7.8 which was mixed with 17 μl of isoelectric focusing 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). The isoelectric focusing 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 second 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 cord were homogenized in extraction buffer (see above) and 20 μl of homogenate incubated for 2 hr with the nonspecific mouse IgG conjugated to protein G Sepharose matrix. The resulting supernatant was then incubated for 1 hr with purified Lan3-2 antibody conjugated to protein G Sepharose matrix. After a brief spin for 30 sec at 2000 rpm the supernatant was discarded and the immunoaffinity matrix resuspended and washed sequentially with 200-400 μl of: 1) extraction buffer; 2) 50 mM Tris-HCl, 1 M NaCl, 0.2% NP-40, pH 8.2; 3) 20 mM Tris-HCl, 2M NaSCN, 0.1% NP-40, pH 8.2; 4) 20 mM Tris-HCl, 4M urea, 0.1% NP-40, pH 7.5; 5) 0.2M glycine-HCl, 0.1% NP-40, pH 2.5; and 6)
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 0.1M phosphate buffer, pH 7.4. The embryos were incubated overnight at room temperature with diluted antibody (Lan3-2, 1:75; Lan4-2, straight; ACT, 1:1000; 4G5 ascites 1:1000; rabbit pep6 antiserum, 1:1000) 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 (Bio-Rad, 1:200 dilution). After washing in PBS the HRP-conjugated antibody complex was visualized by reaction in 3,3′ diaminobenzidine (0.03%) and H₂O₂ (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole-mounts in Depex mountant. Double labeled preparations was 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) was used for Lan3-2 and a rabbit anti-mouse IgG₂B FITC-conjugated secondary antibody (Cappel) for the ACT-antibody. Fluorescently labeled preparations were mounted in glycerol with 5% n-propyl gallate. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T or Ektar 100 daylight film. The color positives were digitized using Adobe Photoshop and a Nikon Coolscan slide scanner. 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 Paultek cooled CCD camera, a Pixel Buffer framegrabber (Perceptics) and the NIH-Image software.
Lan3-2 perturbation experiments

1-2 µl of purified Lan3-2 or purified mouse IgG₁ control antibody from a 0.2 mg/ml stock solution were injected beneath the germinal plate of E8 Hirudo embryos. In addition to antibody the stock solution contained 10% Ringer and 0.2% fast green (Sigma) allowing for visual confirmation of injected antibody under the stereo microscope. The injections were performed by pressure controlled by a Microspitzer pressure apparatus. 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 10% Ringer 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 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 where demounted, reprocessed, and labeled with Acetylated tubulin mAb and the labeling visualized with FITC-conjugated anti-mouse IgG₂B secondary antibody. A total of 41 embryos from three different cocoons were examined. In each experiment sibling embryos at synchronized developmental stages were compared at the different experimental conditions.
RESULTS

Purification of Lan3-2 130 kDa antigen by immunoaffinity chromatography

Both the Lan3-2 and Lan4-2 antibodies were originally made toward adult Haemopis CNS (Zipser and McKay, 1981) but label growth cones and developing axon fascicles of sensory neurons (Figure 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 protein bands with molecular weights of 130, 105, and 90 kDa respectively, whereas Lan4-2 recognizes only a 130 kDa band (McKay et al., 1983; Johansen et al., 1992). Cross immunoprecipitation experiments with Lan3-2 and Lan4-2 have demonstrated that the 130 kDa version of the antigens of these antibodies are closely molecularly interrelated (Johansen et al., 1992). The significance of the two lower 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 kDa version of the antigens is expressed (McGlade-McCuUoh et al., 1990). Thus, these experiments suggest that the 130 kDa 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 kDa protein or proteins 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 directly screen a bacteriophage-based expression vector library using Lan3-2. Therefore, it was necessary to first purify the antigen, which makes up only about 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 hybridomas grown in serum-free media. The purified antibody was covalently coupled to protein G-Sepharose in order to construct an immunoaffinity column to which extracts of
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 MA (median anterior) and DP (dorsal posterior) nerves and segregate into four distinct axonal tracts (arrows). The leech nervous system is bilaterally symmetrical and anterior is to the left in all figures. Bar, 25 \( \mu \)m. (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). Same scale as in (B).
dissected Haemopis leech nerve cords solubilized in Triton X-100/NP-40 buffer were applied. The solubilized protein was precleared by incubation with non-specific mouse IgG coupled to protein G Sepharose, and the final Lan3-2 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 is so high that neither low nor high pH conditions eluted the Lan3-2 antigen. A variety of other eluates used (4M MgCl₂, 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 and after dialysis and concentration, SDS-PAGE, and blotting onto PVDF-membrane approximately 10 µg of immunopurified antigen was obtained from 200 nerve cords by this method. Figure 2A 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 kDa band was cut out and analyzed for the reasons stated above. The purified protein was trypsin digested in situ and the resulting peptides separated by microbore HPLC and screened by mass spectrometry. Seven peaks were selected for gas-phase microsequencing, from which the eight sequences shown in Figure 2A 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), 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.
Figure 2. Peptide Sequences and New Antibodies from Purified Lan3-2 130 kDa Antigen in Haemopis. (A) Western blot of Lan3-2 immunoaffinity purified antigen from Haemopis CNS labeled with amido black (lane 1) compared to an immunoblot of the same material labeled by Lan3-2 antibody (lane 2). The broad 130 kDa 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 kDa band. 200 kDa is at the top of the blots in (A) and (B) and 25 kDa at the bottom.
The observation that some of the peptide sequences show homology to different subfamilies of CAMs raised the possibility that we had obtained peptide sequences from more than one protein which co-migrate in the broad 130 kDa 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 (Figure 2A). As shown in Figure 2B antisera to all three peptides recognized a broad 130 kDa 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 the rabbit antisera to the peptides to screen a Hirudo randomly primed cDNA library.

Molecular cloning and characterization of Tractin and LeechCAM

From approximately 1 x 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 (Figure 3A 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 (Figure 4). The predicted sequence is for a protein containing 1880 amino acids with several novel features. We have named this protein Tractin (Figure 3A and 4). Sequence analysis suggest that the first 25 amino acids are a cleaved signal peptide (Von Heijne, 1983). Another hydrophobic region near the C-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 (Figure 4) beginning with a region of 6 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,
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 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 1880 residues or 1855 without the signal sequence with an estimated molecular mass of 200 kDa. It contains a 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 97 kDa. These sequences are available from EMBL/GenBank/DDBJ under accession number U92813 (Tractin) and U92814 (LeechCAM).
A Tractin

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<th>Amino acid sequence</th>
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<td>NPQAMFRPQACFLLATATMTTPQKEVNRPSPTAHPPYESTFNMHEVHEIPQHATGFLPVPFWKIDGEPELKESEAE</td>
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B LeechCAM

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<tr>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td>PKFDEQGTTITLYNELKEDQYQCVNKYCTAASVTLKMMVQFPTVEPKQCVNVRDGNHLCNPKSTYPT</td>
</tr>
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</table>

[Continued with more amino acid sequences]
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 location of the RGD integrin binding motif in the 2nd FNIII-like domain and the putative peptide cleavage site in the 3rd FNIII-like domain are indicated. Above the diagram of the Tractin protein sequence is a partial restriction map of the cDNA relative to the six independent, overlapping cDNA library clones from which the complete sequence was compiled. 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 Tractin and LeechCAM sequence each matched four of the eight originally microsequenced peptides from the Lan3-2 immunopurification.
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 the I-set. The six Ig-like domains are followed by four FNIII-like domains (Hynes, 1990). The second FNIII-like domain contains a RGD tripeptide sequence which is a characteristic binding sequence for integrins (Hynes, 1992). In the region following 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 dot-plot analysis of the Tractin sequence against itself shows that about a fourth of the protein is made up of a repeat sequence (Figure 5A). A sequence alignment (Figure 5B) 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 ten 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 (Figure 5C) which 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 C-terminal of Tractin contains the sequence SSFV, which matches the \(-X-S/T-X-V-COO^\) motif (where X denotes any amino acid) that is predicted to associate with PDZ domains of the PSD-95/disc-large/ZO-1 family of membrane skeletal proteins (Doyle et al., 1996). Very interestingly, this X-S/T-X-V motif is also found in several neural cell adhesion molecules such as vertebrate NcAMs, Drosophila Fas II, Toll (see the list of Kornau et al., 1995) and neuroglian long isoform (Hortsch et al., 1990). In the extracellular part Tractin has 19 possible N-linked glycosylation sites.

The predicted molecular weight of Tractin is 200 kDa, or 197 kDa after cleavage of the signal peptide. This is much larger than the size of the 130 kDa Lan3-2 antigen. However, the
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 about 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 ten repetitions of the amino acid triplet PY/GG. The twelve 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 sequence from members of the L1 sub-family of CAMs: neurofascin (Accession number: PIR S26180), nrcam (SwissProt P35331), L1 (SwissProt P11627), ngcam (SwissProt Q03696), and Drosophila neuroglian (SwissProt P20241). Conserved residues of these sequences with Tractin are highlighted in white letters on a black background.
protein contains a putative cleavage site of RKRRSRSK in the third FNIII-like domain (Figure 3A), a similar sequence motif is also found in FNIII-like domains in CAMs of the L1 subgroup where in several cases it has been demonstrated 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 kDa 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 (Figure 6). However, both of these transcripts are more than large enough to encode for the entire sequence of Tractin. This together with the fact that neither Lan3-2, pep2 nor pep3 antibodies recognize a full length 197 kDa version of the protein on immunoblots (Figure 2) suggest that Tractin may be co-translationally cleaved into a secreted as well as a 100 kDa 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 (Figure 3A and 4). This suggests that at least one other protein was co-purified 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 five additional overlapping cDNAs which 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 (Figure 3B and 4). The first 17 residues define a hydrophobic stretch and is 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 3B 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 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 EcoR I fragment encoding the first 5 Ig domains and part of the 6th Ig domain was used; and for LeechCAM, a 5’ end EcoR I fragment encoding the first 3 Ig domains was used. For Tractin a major band at 9.3 kb and a minor band at 8.2 kb were detected whereas a single band of 4.2 kb was observed for LeechCAM.
Tractin  LeechCAM

9.3 kb

4.2 kb
organization of the protein is similar to that of NCAM, FasII, and ApCAM to which it has sequence homology in the range from 26-30% suggesting it is the leech homolog of these proteins. Consequently, we have named the protein LeechCAM. The calculated molecular weight of LeechCAM is 97 kDa, or 95 kDa after cleavage of the signal peptide. It has ten potential N-linked glycosylation sites in the extracellular region and is recognized by pep6 antibody as a 130 kDa protein on immunoblots (Figure 2B) suggesting that 35 kDa or about 27% of its molecular weight is due to glycosylation. On Northern blots of leech mRNA it is identified as a single transcript of 4.3 kb (Figure 6). In addition to the pep6 sequence, the sequence of pep1, 4, and 7 are 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 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 co-purified. To address this 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 MgCl2, 3 M NaSCN, and 4 M urea. These very stringent washing conditions would be expected to break most non-covalent protein-protein associations. The Lan3-2 immunoprecipitate was then boiled, separated by SDS-PAGE, and immunoblotted. Figure 7A shows that a 130 kDa 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 not co-purified as interacting proteins but that they are separate entities, both glycosylated with the Lan3-2 glycoepitope. In addition, Tractin and LeechCAM antibody to
Figure 7. Tractin and LeechCAM are Separate Proteins 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 MgCl₂, 3 M NaSCN, and 4 M urea before separation by 10% SDS-PAGE with half the Bis (see Materials and Methods). A broad 130 kDa band (arrow) was recognized by both Lan3-2 and Lan4-2 and by Tractin and LeechCAM antisera. (B) Immunoblots of immunoaffinity purified Lan3-2 antigen from Haemopis nerve cords separated by 2D-gel electrophoresis. The purified nerve cord proteins were subjected to isoelectric focussing in the first dimension (range: pH 4.5-8.5) and 7.5 % SDS-PAGE in the second (200 kDa is at the top and 25 kDa is at the bottom of the blot). LeechCAM antiserum (B3) recognizes an oblong diffuse 130 kDa 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 kDa 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). Furthermore, the 105 and 90 kDa bands recognized by Lan3-2 in adult nerve cords were not detectable in this pH range (B1) indicating that they are different proteins from Tractin and LeechCAM.
the protein core only recognized the 130 kDa proteins and not the other molecular weight bands recognized by Lan3-2 antibody, suggesting that these are different proteins being glycosylated with the Lan3-2 epitope in adult nervous system (Figure 7A).

In order 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 (Figure 2A) by 2D-gel electrophoresis with isoelectric focusing 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, 4G5 (a mAb towards 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 (Figure 7B, arrowhead) which is not labeled by Tractin antibody. Conversely, Tractin recognizes 130 kDa protein in a broad 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. Furthermore, since all eight microsequenced peptides were found in the sequence of Tractin and LeechCAM, they may represent the only two 130 kDa proteins carrying these glycoepitopes early in development. 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 towards more basic pHs. This and their distribution over a wide range of pH instead of being focused into tight spots probably reflects heterogeneous glycosylation of the core proteins as have been previously described for other glycosylated CAMs (Rathjen et al., 1987a; 1987b; Wolff et al., 1988).

We examined the developmental expression of Tractin and LeechCAM by labeling E10 Hirudo embryos with Tractin (mAb 4G5) and LeechCAM (mouse antiserum) specific antibodies (Figure 8). We found that Tractin was detected by the mAb 4G5 on the soma and
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), LeechCAM (mouse antiserum), 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 non-sensillar 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). Bar, 75 μm in (A) and (B), 100 μm in (C) and (D).
axons of all neurons of both the central and peripheral nervous system (Figure 8A). 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 positively identify labeling of central neurons, sensilla, and a few of the other peripheral neurons such as the nephridial nerve cell (Figure 8B). At this developmental stage, Lan3-2 labels sensillar neurons and their projections (Figure 8C) whereas Lan4-2 labels only a small subset of these that fasciculate into a single tract (Figure 8D). These results indicate that Tractin and LeechCAM are widely expressed proteins in the leech nervous system but that they are differentially glycosylated to carry the Lan3-2 glycoepitope in the sensillar neurons only and that in a distinct subset of these 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 function in neuritogenesis and/or fascicle formation of the sensillar neurons in vivo we injected purified Lan3-2 antibody (20-50 ng) beneath the germinal plate cavity of sibling E8 embryos (n=11). Embryos, either not injected (n=4) or injected with a non-specific purified mouse IgG fraction in equal amounts to that of the Lan3-2 antibody (n=8), served as controls. After injection the intact embryos were incubated for 24 hours in embryo water before dissection and fixation. In some experiments for visualization of the sensillar axonal projections fixed embryos were relabeled with Lan3-2 antibody and Texas Red-conjugated second antibody while in others only fluorescently-conjugated second antibody were 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 (Figure 9A). During normal development in the ensuing 24 hours more and more sensillar neurons extend neurites into the CNS. These neurites have numerous filopodia that branch extensively in the neuropil as the projections segregate into distinct fascicles (Figure 9B). 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 Figure 9C where the sensillar projections clearly are stunted, although indications of fascicles are discernible. However, 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 mainly be exerted within the CNS. That rudimentary fascicles do form during Lan3-2 antibody incubation may be due to 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. In order to verify that the antibody perturbation was a specific effect on sensillar 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 actetylated tubulin that labels central neurons but not sensillar neurons (Jellies et al., 1996) and visualized them with FITC-conjugated second antibody. As shown in Figure 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 (Figure 10B) are orderly and indistinguishable from uninjected controls (Figure 10A). 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.
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) Embryo injected with purified mouse IgG\_1 control antibody at E8. The embryo was dissected and fixed 24 hours 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 hours later at E9 and the Lan3-2 labeling 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 are greatly reduced and fascicle formation is truncated. Bar, 40 μm
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 second antibody. (B) Hemisegment of a E9 embryo labeled with acetylated tubulin antibody that was injected with 0.5 μg of Lan3-2 antibody as in Figure 9C. The organization and extent of central neuron projections is indistinguishable from that of uninjected controls (A). The figure is a fluorescense image of antibody labeling visualized with FITC-conjugated second antibody. G shows the location of the ganglion and AA, MA, DP, and PP the position of the four peripheral nerves. Bar, 100 μm for both panels.
DISCUSSION

In this study we have identified and molecularly cloned 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 homolog of NCAM, FasII, and ApCAM the organization of Tractin has several unique features. The possession of six Ig-like plus four FNIII-like domains places Tractin in the immunoglobulin 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 N-terminal sequence of Tractin do not recognize a full length version of the protein on immunoblots but only a 130 kDa 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 kDa 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 a RGD integrin-binding motif in the second FNIII-like domain just upstream of the putative cleavage site that potentially could 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 GPI-linkers but which also have a RGD motif in their second FNIII-like domains (Ruegg et al., 1989a; 1989b; Furley et al., 1990; Karageous et al., 1991).
Cleavage at the third FNIII-like domain of Tractin would in addition yield a 100 kDa transmembrane protein the extracellular domain of which would be made up largely of the region of the twelve 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 GX1X2 where X1 and X2 often is a proline (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 can alternatively be considered as constituted 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 attached sequence that has properties similar to that of extracellular matrix molecules and which 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, 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 have been shown also to be present on 130 kDa 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 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, 1995a). Evidence that this perturbation is specific to the blockage of the carbohydrate epitope is provided by experiments which show 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 to 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 are specific to the subpopulation of neurons expressing each of these epitopes (Song and Zipser, 1995b). 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 PSA 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 Fas II, to which LeechCAM is a likely homolog, 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 Fas II appear uniform along the axons and fascicles, suggesting that modulation of Fas II function by interactions with other molecules is required at the choice points (Fambrough et al., 1996). A gene with such a function has been recently identified in beaten path, a locus that genetically interacts with Fas II (Fambrough et al., 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 facilitate 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 chomophore assisted laser inactivation of Fas II in grasshopper prevents the initiation of Til peripheral neuron axonal outgrowth but has no effect on axon fasciculation (Diamond et al., 1993). Furthermore, oligo-mannosidic carbohydrates expressed by L1 have been shown to determine its interaction with NCAM and to modulate neurite outgrowth (Horstkorte et al., 1993). Consequently, the precise mechanism for the impaired neurite extension and stunted fascicle formation observed as a result of perturbation of the Lan3-2 glycoepitope cannot be resolved on the basis of current experiments.

Increasing evidence indicates that interactions between surface oligosaccharides and carbohydrate binding proteins mediate many important cellular processes in non-neuronal 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 which 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 carbohydrate 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 proteins.
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