Biochemical characterization of Tractin and LeechCAM, two Ig-superfamily members involved in regulation of axonal outgrowth of Leech neurons

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Biochemical characterization of TRACTIN and LEECHCAM, two Ig-superfamily members involved in regulation of axonal outgrowth of Leech neurons

By

Chunfa Jie

A dissertation submitted to the graduate faculty in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY

Major: Molecular, Cellular, and Developmental Biology
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# TABLE OF CONTENTS

**GENERAL INTRODUCTION**  
Dissertation Organization 1  
Background 2  

**POST-TRANSLATIONAL PROCESSING AND DIFFERENTIAL GLYCOSYLATION OF TRACTIN. AN Ig-SUPERFAMILY MEMBER INVOLVED IN REGULATION OF AXONAL OUTGROWTH**  
Abstract 15  
Introduction 16  
Materials and Methods 18  
Results 25  
Discussion 31  
Acknowledgements 36  
References 37  

**DIFFERENTIAL GLYCOSYLATION AND PROTEOLYTICAL PROCESSING OF LEECHCAM IN CENTRAL AND PERIPHERAL LEECH NEURONS**  
Abstract 53  
Introduction 54  
Materials and Methods 55  
Results 60  
Discussion 63  
Acknowledgements 66  
References 66  

**GENERAL CONCLUSIONS**  
**LITERATURE CITED**  
**ACKNOWLEDGEMENTS**  

GENERAL INTRODUCTION

Dissertation Organization

This dissertation presents studies on the biochemical characterization of two proteins, Tractin and LeechCAM. It begins with a background section that first discusses the diverse characteristics of related proteins, and introduces neural cell adhesion molecules (CAMs) of the Immunoglobulin (Ig) superfamily, into which Tractin and LeechCAM are categorized. Then, post-translational modification of proteins and functional roles of various protein features are discussed, with an emphasis on Ig molecules. After this, an overview of the leech nervous system is presented to show its advantages as a well-defined model system. Finally, previous findings on Tractin and LeechCAM and the significance of this study are addressed.

After the general introduction, the dissertation is organized into two papers to be submitted for publication. The first paper describes the post-translational processing and differential glycosylation of Tractin, an Ig member involved in axonal outgrowth. The second paper presents our findings on LeechCAM, which suggest differential glycosylation and proteolytical processing of LeechCAM.

At the end of the dissertation are the sections of general conclusions and literature cited. Literature cited in the general introduction and general conclusions are listed in the latter section.
Background

*Neural cell adhesion molecules of the Ig superfamily*

During development, neural cell adhesion molecules (CAMs) of the Ig superfamily have been shown to be involved in the guidance mechanisms of nerve pathway formation (Keynes and Cook, 1995; Goodman, 1996; Tessier-Lavigne and Goodman, 1996; Walsh and Doherty, 1997). The Ig-superfamily is featured by the variability and complexity of their extracellular regions, which contain multiple tandemly arranged domains (Johansen and Johansen, 1997). Members of this superfamily can be further grouped into the following three categories based on their overall primary structure and domain organization: 1) molecules containing Ig-like domains only; 2) proteins composed of Ig-like domains in conjunction with fibronectin type III (FNIII)-like domains; and 3) molecules made up of Ig-like domains in combination with structural motifs other than that of FNIII. The defining features of Ig-like domains are a disulfide bond formed between two cysteine residues about 55—75 amino acids apart in the folded structure, and a so-called “invariant” tryptophan residue located 10-15 residues C-terminal to the first conserved cysteine (Williams and Barklay, 1988; Kabat et al., 1991). FNIII repeats are a conserved motif of about 90 amino acids, which was originally identified in the extracellular matrix (ECM) protein fibronectin (Hynes, 1990). Theoretically, this organization of multiple domains and motifs may allow them to interact with various proteins. More interestingly, the structural diversity of Ig molecules is further amplified via various post-translational modifications. As a result, the functional versatility of Ig proteins may be further enhanced.
Functional roles of various domains and motifs of Ig molecules

The structural versatility of the Ig proteins has made them suitable for carrying out diverse functional roles. Some Ig molecules have been suggested to be involved in neuronal migration, neurite outgrowth, fasciculation, axonal guidance, and synaptic plasticity (Goodman, 1996; Tessier-Lavigne and Goodman, 1996). For example, Fas II and apCAM have been shown to regulate synaptic remodeling and growth in *Drosophila* and *Aplysia* (Schuster et al., 1996a; 1996b; Davis et al., 1997; Mayford et al., 1992; Zhu et al., 1994, 1995). Another recent study suggests that apCAM mediates growth cone steering by substrate-cytoskeletal coupling (Suter et al., 1998). The diverse functional roles of Ig proteins are possibly attributed to their tandemly arranged domains of Ig proteins. Ig and FNIII domains have been shown to mediate *in vitro* homo- and/or heterophilic interactions among Ig superfamily members, and between Ig molecules and other proteins, such as the interactions between or within L1 subgroup and NCAM subgroups (Horstkorte et al., 1993; Appel et al., 1993; Kiselyov et al., 1997). The functional importance of such interactions in neuronal development have been under intensive study. For example, Nr-CAM has been suggested to promote neurite outgrowth from peripheral ganglia by a mechanism involving axonin-1 as a neuronal receptor (Lustig et al., 1999).

Besides the Ig and FNIII domains, other motifs have also been suggested to be involved in the function of the neural cell adhesion molecules. An ankyrin-binding motif located at the cytoplasmic tail is found in the L1 subfamily members and Tractin (Davis and Bennett, 1994; Huang et al., 1997). It has been shown to mediate the interaction between members in the L1 subfamily and the membrane-binding domain of ankyrin (Davis and Bennett, 1994), which is part of the membrane-associated skeleton. More interestingly, the cytoplasmic tail of Neuroglian, which is a *Drosophila* homolog of L1 and also contains the
ankyrin-binding motif, has been shown to be indispensable in inducing assembly of the membrane-associated skeleton at cell contact sites (Dubreuil et al., 1996).

In addition to ankyrin-binding motif, the XS/TXV motif at the extreme COOH-terminal end has been found in several neural Ig molecules such as Tractin, vertebrate NrCAMs, *Drosophila* Fas II, Toll, and neuroglian long isoform (Huang et al., 1997; Kornau et al., 1995; Hortsch et al., 1990). This motif has been suggested to associate with the PDZ domains of the PSD-95/Dlg/ZO-1 family of membrane skeletal proteins (Doyle et al., 1996; Zito et al., 1997). The interaction between this motif of Fas II and the PDZ domains of Dlg has been suggested to be important for the synaptic clustering of Fas II (Zito et al., 1997) and the formation of a ternary complex (Fas II + Dlg + Shaker potassium channel) (Thomas et al., 1997). The latter case may imply a molecular mechanism of integrating information about structure and chemical transmission at the synapse. Given the suggested functional role of the XS/TXV motif, it is worth noting the phosphorylation of the -2 serine residue of this PDZ-interacting motif in the inwardly rectifying potassium channel Kir 2.3 by PKA. This phosphorylation inhibits the binding of the PDZ-interacting motif to its interacting partner PSD-95 (Cohen et al., 1996).

Another interesting motif is the R-G-D tripeptide, which is possessed by TAG-1/axonin-1 and Tractin (Felssenfeld et al., 1994; Huang et al., 1997). This motif, which is found in the ligands for several integrins, has been proposed to associate the secreted TAG-1/axonin-1 isoforms with integrins (Felssenfeld et al., 1994). Integrins can further interact with cytoskeleton proteins and other signaling molecules (Horwitz et al., 1986; Tapley et al., 1989; Shattil and Brugge, 1991). A recent study suggests that the R-G-D motif in L1 promotes neurite outgrowth via interaction with the α,β integrin (Yip et al., 1998). Besides the above domains and motifs, other notable sequences of Ig molecules may be of functional
importance. A short stretch of acidic amino acid residues of NCAM have been suggested to be involved in a symmetrical double-reciprocal binding (Kiselyov et al., 1997).

Posttranslational Processing

Post-translational processing has been shown to be responsible for ectodomain shedding of various integral membrane proteins, including cell adhesion molecules. Selective post-translational proteolysis from the cell surface leads to the release of the secreted forms from the intact proteins (Hooper et al., 1997; Blobel et al., 1997). In general, the cleavage process takes place close to the extracellular face of the membrane, and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. Many secretases have been identified as metallo- and/or serine proteinases, which are either localized at the cell surface, or are themselves integral membrane proteins. The structural versatility of neural CAMs is further amplified by post-translational processing. In the L1 subfamily, a consensus sequence S-R/K-R has turned out to be the site for proteolytic cleavage of the intact precursor proteins (Burgoon et al., 1991 and 1995; Volkmer et al., 1992; Kayyem et al., 1992). Alignment of Tractin protein sequence with this consensus sequence shows Tractin also possesses several putative cleavage sites (Huang et al., 1997).

Post-translational proteolysis may have diverse functional roles, which remain to be elucidated. Some proteolytic cleavage has been shown to be the prerequisite for the normal biosynthesis of functional proteins. For example, Notch, a transmembrane receptor for controlling the neural cell fate decisions in Drosophila melanogaster, possesses an evolutionarily conserved proteolytic cleavage site. The cleavage results in a C-terminal fragment, which appears to be cleaved off N-terminal to the transmembrane domain, and an N-terminal fragment, which contains most of the extracellular region. The two fragments have been shown to form a heterodimeric receptor via a disulfide bond, which is suggested to
be the active, ligand-accessible form of Notch (Blaumueller et al., 1997). Moreover, the processing of Notch has been suggested to be controlled by the KUZ protease, whose function may be part of the normal biosynthesis of functional Notch proteins (Pan et al., 1997). In addition to Notch, the study of the Sevenless protein, a putative protein tyrosine kinase receptor required for the proper differentiation of the R7 photoreceptor in *Drosophila*, has provided robust evidence for the importance of proteolytic cleavage. The Sevenless protein is first synthesized as a 280kD glycoprotein precursor, which is subsequently cleaved into 220kD N-terminal and 58kD C-terminal subunits. This cleavage also takes place at a site N-terminal to the transmembrane domain. The two resulting subunits remain associated by noncovalent interactions. It is very interesting that the 58kD C-terminal fragment undergoes further cleavage, which leads to either 49 or 48kD C-terminal fragments with concomitant degradation of the rest of the fragment. In this way, the mature Sevenless protein is formed by proteolytic cleavage of a precursor (Simon et al., 1989). Besides the role in the production of mature protein forms, post-translational cleavage products of proteins can lead to functional versatility, as suggested by the functional difference of the Ng-CAM cleavage products (Burgoon et al., 1995).

Glycosylation

It has been well established that the structural diversity of cell surface carbohydrates makes them ideal candidates for mediating cell specific recognition processes (Roseman, 1971; Lis and Sharon, 1993; Dwek, 1995; Rudd and Dwek, 1997). A clearly demonstrated example is the process of lymphocyte homing, in which selectins work as interpreters of cell-specific carbohydrate information by recognizing and binding ligands expressing specific oligosaccharide structures (Springer, 1994; Lasky, 1995). Glycosylation in the nervous system has also been the focus of various studies. One study identifies stage- and position-
specific carbohydrate antigens as topographic markers for selective projection patterns of olfactory axons (Key and Akeson, 1991; Schwarting et al., 1992). In another study it is shown that a carbohydrate moiety of a membrane-associated glycoprotein has dual action on afferent and efferent axons in cortical development (Henke-Fahle et al., 1996).

Strikingly, it has been shown that Ig proteins expressed on the surface of axons and growth cones, possess specific glycoepitopes. Accumulating evidence from different studies suggests that the characteristic glycosylation profiles may be the basis of additional structure heterogeneity and functional roles for these Ig proteins (Volkmer et al., 1992; Horstkorte et al., 1993; Huang et al., 1997; Denzinger et al., 1999). To study the potential role of glycosylation of neural Ig proteins in pathway formation, the projections of sensillar neurons in leech have been under intensive study. The axons of sensillar neurons project in tightly fasciculated bundles through the periphery before reaching the ganglia of the central nervous system (CNS). At the ganglia, the tightly fasciculated bundles bifurcate and segregate into four well-defined and stereotypically located substracts in each of the central connectives (Johansen et al., 1992; Jellies et al., 1994; Briggs et al., 1995). The entire set and the distinct subsets of sensillar neuron population express specific glycoepitopes, which can be distinctively recognized by five different monoclonal antibodies (mAbs) (Lan3-2, Lan2-3, Lan4-2, Laz2-369, Laz7-79). Among the five mAbs, Lan3-2 labels all sensillar neurons while the other four mAbs selectively identify subsets of the Lan3-2 positive sensillar neurons. It has been demonstrated that the antigens of Lan3-2 and Lan4-2 are Tractin and LeechCAM, two Ig molecules, and that the Fab fragments of Lan3-2 antibody can perturb normal fascicle formation in vitro (Zipser et al., 1989; Song and Zipser, 1995a; Huang et al., 1997). These data suggest that the Lan3-2 glycoepitopes of Tractin and LeechCAM are functionally involved in nerve pathway formation. The glycoepitopes selectively recognized by the other four mAbs appear to be correlated with the pathway choices by the corresponding subsets of
the Lan3-2 positive sensillar neurons. For example, one of the four Lan3-2 positive fascicles is specifically chosen by the subset of sensillar neurons recognized by the Lan4-2 mAb during embryogenesis (Johansen et al., 1992). More interestingly, it has been shown with perturbation experiments that the glycoepitopes of Laz2-369 and Laz7-79 functionally affect the corresponding subsets of neurites of leech sensillar neurons (Song and Zipser, 1995b). Besides the above study with the leech sensillar neurons, another example on the role of glycosylation is the modulation of polysialation of NCAM. In the plexus region of the chick limb bud, the up-regulation of the polysialation of NCAM allows the axons to defasciculate into their proper pathways (Tang et al., 1992, 1994). In addition to NCAM, the modification of L1 with mannosidic carbohydrates was shown to determine the interaction between L1 and NCAM. The same oligomannosidic glycans at the cell surface was also implicated in neurite outgrowth (Horstkorte et al., 1993).

Post-translational modification of collagenous sequences

Collagens are proteins assembled in a variety of supramolecular structures in extracellular matrices that can fulfill a variety of biological functions, such as providing a high mechanical strength and activating signal transduction events (Vogel et al., 1997; Schlessinger, 1997). In all the collagen molecules, a major component of the protein is a triple-helical structure of three polypeptide chains (α-chains) with the characteristic sequence repeat of G-X₁-X₂, where X₁ is frequently a proline and X₂ is frequently a hydroxyproline. Based on their structural characteristics, collagens can be divided into two main groups: fibrillar and non-fibrillar collagens (Vuorio and Crombrugghe, 1990). Fibril collagens contain long continuous triple helices while non-fibril ones contain triple helices interrupted by nonhelical segments. The former group of collagens includes types I-III, V and XI while the latter group is more heterogeneous and have been further classified according to their
molecular characteristics, supramolecular structures and the types of extracellular networks containing them (Prockop and Kivirikko, 1995). Some collagen types are composed of homogeneous \( \alpha \)-chains while other types contain heterogeneous \( \alpha \)-chains. In addition, proteins such as type I macrophage scavenger receptor, C1q, acetylcholinesterase, conglutinin, and surfactant proteins, also contain collagenous domains (Vuorio and Crombrugghe, 1990; Kodama et al., 1990).

Interestingly, it has long been recognized that collagens undergo a complex series of intra- and inter-chain cross-linking via lysine derivatives (Light and Bailey, 1982; Eyre, 1987; Ricard-Blum and Ville, 1989; Reiser et al., 1992), which can be classified into two major groups. One group of cross-links is initiated by the enzyme lysyl oxidase while the other one is derived from nonenzymatically glycated lysine derivatives. Given the key role of collagens in the extracellular matrix, it is not surprising that their cross-linking may affect many biological processes. For example, development beyond gastrulation appears to require the deposition of cross-linked collagen (Wessel et al., 1987). Furthermore, some heritable diseases, such as Menke’s disease, have been attributed to the abnormalities of collagen cross-linking (Prockop et al., 1984). The group of nonenzymatic cross-links occurs more adventitiously while the biochemistry of its arising remains elusive. These kinds of cross-links have been shown to be involved in some pathobiological processes. For instance, nonenzymatic glycation of fibrillar collagens as well as of basement membrane collagen is increased in diabetes (Reiser, 1991). However, considerable progress has been made in the study of the enzymatically mediated cross-links, the generation of which has been attributed to a unique mechanism based on aldehyde formation from lysine or hydroxylysine side chains. The location, structures and biosynthetic pathways of some enzymatically mediated cross-links have been well characterized. For instance, cross-linking of the type IV collagen, a non-fibrillar collagen of basement membrane, has been suggested to differ from that of the
fibrillar collagens in terms of the cross-linking loci and the exact nature of the mature cross-links (Reiser et al., 1992). Moreover, the specific lysine aldehyde-derived cross-links demonstrate a selected tissue distribution pattern. It appears that this selected distribution depends primarily on the tissue source and its physiological function rather than on the genetic type of collagen (Reiser et al., 1992).

In addition to the cross-linking discussed above, collagens carry other extensive post-translational modifications including cleavage, hydroxylation of proline and lysine residues, and various glycosylations. In general, fibrillar collagens undergo the following major post-translational modification: after the secretion of procollagens, cleavage of the N-propeptides and C-propeptides of procollagens by a procollagen N-proteinase and a procollagen C-proteinase, respectively; the hydroxylation of X2-position proline and lysine residues to 4-hydroxyproline and hydroxylysine, and that of a few X1-position proline residues to 3-hydroxyproline; and the addition of galactose or both galactose and glucose to some of the hydroxylysine residues (Prockop and Kivirikko, 1995). As to non-fibrillar collagens, there exists some notable difference in post-translational modification. For example, several such collagens are shown to contain N-glycosylation. Glycosylation of collagens IX, XII and XIV has been shown to include the addition of glycoaminoglycan side chains (Prockop and Kivirikko, 1995). Among the non-fibrillar collagens, a well characterized example is collagen XVII, also known as the 180-kD bullous pemphigoid antigen. The cDNA of collagen XVII is predicted to encode a type II transmembrane protein, which has been suggested to possess a domain organization of an intracellular tail, a single membrane-spanning domain and an extracellular c-terminal region by ultrastructural and biochemical analyses (Hopkinson et al., 1992; Giudice et al., 1993; Ishiko et al., 1993). The extracellular region contains 15 collagenous domains, which are linked by a series of non-collagenous sequences. It has been shown that collagen XVII from epidermal keratinocytes occurs as a homotrimeric protein in
two forms: a full-length transmembrane protein and a soluble ectodomain. The soluble ectodomain form is suggested to be derived from post-translational cleavage by a proprotein convertase of the furin/PACE family (Schäcke et al., 1998). The same study also showed that the collagenous extracellular domain is triple-helical and N-glycosylated. In addition, the full-length protein and the enzyme digested fragments appeared larger than their predicted molecular mass on SDS-PAGE (Schäcke et al., 1998). This observation has been suggested to be attributed to the fact that the elongated collagens as rod like molecules migrate slower than matching globular standards. In another in vitro study, a furin-like proprotein convertase was shown to cleave off the C-propeptide of pro-α1(V) collagen (Imamura et al., 1998).

Overview of the nerve system of Leech

Leech has a segmentally iterated nervous system, which has been well defined. Anatomically, the whole leech body is composed of 4 head segments, 21 body segments, 7 caudal fused segments and the nonsegmental prostomium, which are innervated by a head ganglion, 21 body ganglia, and 7 fused tail ganglia, respectively. These ganglia are chained together by connectives, which consist of two large lateral bundles of nerve fibers and a thin medial connective called Faivre’s nerve. The ganglia and the connectives together compose the central nervous system. In the periphery, sensillar neurons are the first ones to differentiate, as shown with the staining of the Lan3-2 monoclonal antibody. It has been shown that sensillar neurons are clusters of sensory neurons of mixed types on the central annulus of each segment: chemoreceptors, photoreceptors, and mechanoreceptors (Muller et al., 1981; Philips and Friesen, 1982; Johansen et al., 1992). In each hemisegment, all the seven sensilla send axons toward the ganglia with a distinct pattern: sensilla S1-S5 extend their axons via the anterior nerve root while S6 and S7 send their axons through the posterior
nerve root. The fasciculated growing axons of the sensillar neurons make 90° turns in
direction and segregate into four distinct tracts of the lateral connective fibers after reaching
the ganglia. Among the seven sensilla, the first one to appear is the sensillum of S3, which is
followed by the neurons in S6 and S7. Then, S1, S2, S4 and S5 sensilla ensue (Johansen et al.
1994). Interestingly, the development of both the central and peripheral nerve systems in
leech progresses in a gradient fashion: each posterior segment lags by about 2.5 hours from
the anterior one. This leads to a developing embryo displaying different developmental stages
which span a period of approximately 2-3 days due to the existence of 32 segments (Johansen
et al., 1992). This feature of the rostro-caudal progression in development provides excellent
opportunities to examine the axonal outgrowth.

*Previous findings on Tractin and LeechCAM*

As discussed before, mAb Lan3-2, specifically identifies all sensillar neurons and their
central projections. Several lines of experimental evidences suggest that Lan3-2 antigens are
involved in neuronal pathway formation of sensillar neurons (Peinado et al., 1987; Zipser et
al., 1989; Johansen et al., 1992). Biochemical characterization with the Lan3-2 mAb shows
that it recognizes a glycoepitope on several protein bands in western blot analysis (Mckay et
al., 1983; Johansen et al., 1992). Among these antigens, the glycoprotein band of 130 kD is
the prime candidate for mediating the common fasciculation exhibited by the sensillar
neurons, since only this version of band is detectable when the Lan3-2 positive fascicles are
being formed in E8-10 embryos. By immunoaffinity chromatography with the Lan3-2 mAb,
two novel Ig-superfamily members, Tractin and LeechCAM, have been cloned as the Lan3-2
antigens of 130kD (Huang et al., 1997).

Tractin is a putative transmembrane protein with a short cytoplasmic tail. Its predicted
molecular weight is 197kD after cleavage of the signal peptide. The extracellular part of
Tractin contains multiple tandemly arranged domains, beginning with 6 immunoglobulin (Ig)-like domains. The Ig-like domains are followed by four fibronectin type III (FNIII) domains and a unique block of 64 acidic amino acids. After the acidic block, there exists 12 repeats of a 30 amino acid motif rich in prolines and glycines. The PG/YG repeating unit of this proline- and glycine rich motif, can be alternatively read as G-X1-X2. The cytoplasmic tail contains a stretch of amino acid residues, which conforms to the consensus sequence for an ankyrin binding motif. Tractin also possesses an XS/TXV motif at the extreme carboxyl end and a tripeptide sequence of R-G-D in the second FNIII domain. Furthermore, sequence alignment shows Tractin possesses several putative cleavage sites. Additionally, it is also suggested by western and northern blot analysis that Tractin may be co-translationally processed (Huang et al., 1997).

LeechCAM is a protein of 95 kD with the signal peptide excluded. It has sequence homology to the N-CAM subfamily of Ig molecules in the range from 26-30%. From the N-terminus to the C-terminus, it has a domain organization of five Ig-like domains, two FNIII like domains, a putative transmembrane domain and a cytoplasmic tail. Northern blot analysis also shows that LeechCAM has only a single transcript (Huang et al., 1997).

Both Tractin and LeechCAM possess sites for potential post-translational modification, such as N-glycosylation. It is interesting that Tractin and LeechCAM are shown to be expressed by both peripheral and central neurons. But only in the peripheral sensory neurons and their axonal projections, they are differentially glycosylated with the Lan3-2 and Lan4-2 glycoepitopes (Huang et al., 1997).

*Significance of the study*

CAMs have been suggested to regulate many neuronal activities. Their significance in neuronal development has been underscored by several functional studies. For example,
some types of human mental retardation and malfunctions are attributed to mutations in LI (Wong et al., 1995). Previous studies have suggested that Lan3-2 antigens of 130kD are involved in the pathway formation of the sensillar neurons in leech (Peinado et al., 1987; Zipser et al., 1989; Johansen et al., 1992). The accomplishment of cloning the corresponding Lan3-2 antigens (Huang et al., 1997), Tractin and LeechCAM, promises to provide further valuable insights into the molecular guidance mechanisms of nerve pathway formation. Leech embryos possess a relatively simple and well defined nerve system, which can be assessed for functional studies in multiple ways, such as perturbation and dye injection. Combined with these advantageous features of the leech nervous system, the study of Tractin and LeechCAM provides a unique opportunity for examining the underlying molecular mechanisms of axonal guidance in this system. Thus, a continuing extensive characterization of Tractin and LeechCAM will lay the cornerstone for further investigation of their functional roles in the nerve pathway formation. Moreover, it has been demonstrated that many important protein characteristics are functionally conserved throughout evolution. Therefore, the study in this dissertation should also advance our basic understanding of axonal guidance mechanisms and may facilitate the thorough resolution of the underlying causes of aberrant neural connections and abnormal brain development.
POST-TRANSLATIONAL PROCESSING AND DIFFERENTIAL GLYCOSYLATION OF TRACTIN, AN Ig-SUPERFAMILY MEMBER INVOLVED IN REGULATION OF AXONAL OUTGROWTH

A paper to be submitted to the Journal of Neurobiology

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Kristen M. Johansen* and Jørgen Johansen*

ABSTRACT

Tractin is a novel member of the Ig-superfamily which has a highly unusual structure. It contains 6 Ig-domains, 4 FNIII-like domains, an acidic domain, 12 repeats of a novel proline- and glycine-rich motif with sequence similarity to collagen, a transmembrane domain, and an intracellular tail with an ankyrin and a PDZ-domain binding motif. By generating domain-specific antibodies we show that Tractin is proteolytically processed at two cleavage sites, one located in the third FNIII-domain, and a second located just proximal to the transmembrane domain. The alternative processing results in the formation of four fragments. The most NH₂-terminal fragment, which is glycosylated with the Lan3-2, Lan4-2, and Laz2-369 glycoepitopes is secreted, and we propose a model in which the remaining fragments combine to form a secreted homodimer as well as a transmembrane heterodimer. The extracellular domain of the dimers is mostly made up of the collagen-like PG/YG-repeat

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domain but also contains 11/2 FNIII-domains and the acidic domain. The collagen-like PG/YG-repeat domain can be selectively digested by collagenase and we show by yeast two-hybrid analysis that the intracellular domain of Tractin can interact with ankyrin. Thus, the transmembrane heterodimer of Tractin constitutes a novel protein domain configuration where sequence that has properties similar to that of extracellular matrix molecules is directly linked to the cytoskeleton through interactions with ankyrin.

INTRODUCTION

During tissue formation, growth cone extension, and synapse formation neurons exhibit selective recognition and adhesive interactions which are mediated by families of glycosylated cell adhesion molecules (CAMs). A defining feature of the molecular structure of the neural CAMs of the Ig-superfamily is the variability and complexity of their extracellular regions, which in most cases contain multiple tandemly arranged domains (Tessier-Lavigne and Goodman, 1996; Walsh and Doherty, 1997). Some of the CAMs are attached to the membrane by glycosyl-phosphatidylinositol anchors whereas others have transmembrane and intracellular domains providing a potential direct link to signal transduction pathways and/or interactions with the cytoskeleton (Doherty and Walsh, 1994; Hortsch, 1996). In addition, the diversity in the structure of neural CAMs is amplified with the existence of many splice variants and various post-translational modifications such as differential glycosylation and proteolytic processing (Johansen and Johansen, 1997; Hooper et al., 1997).

We have recently cloned and identified a new member of the Ig-superfamily in the nervous system of the leech, Tractin, which has a highly unusual structure (Huang et al., 1997). It contains 6 Ig-domains, 4 FNIII-like domains, an acidic domain, 12 repeats of a
novel proline- and glycine-rich motif with sequence similarity to type IV collagen, a transmembrane domain, and an intracellular tail with an ankyrin and a PDZ-domain binding motif. The core protein of Tractin is expressed by all neurons but is differentially glycosylated with the mAb Lan3-2 and Lan4-2 glycoepitopes only in sets and subsets of peripheral sensory neurons that form distinct fascicles in the CNS (Johansen et al., 1992). In addition, at least four other mAbs (Lan2-3, Laz6-212, Laz2-369, Laz7-79) that recognize different glycoepitopes specific to distinct subsets of these neurons have been identified (McKay et al., 1983; Peinado et al., 1987; Zipser et al., 1994). In vivo and in vitro antibody perturbation of some of these glycoepitopes have demonstrated that they can selectively regulate axonal outgrowth. For example, perturbation with Lan3-2 antibody leads to an inhibition of filopodial extension, truncated fascicle formation, and a decrease in synaptogenesis (Zipser et al., 1989; Huang et al., 1997; Tai and Zipser, 1998). In contrast, perturbation with Laz2-369 antibody leads to enhanced neurite and filopodial sprouting as well as an increase in synapse formation (Song and Zipser, 1995b; Tai and Zipser, 1999). In this study we demonstrate that the Laz2-369 glycoepitope, in addition to the previously reported Lan3-2 and Lan4-2 glycoepitopes, also represents differential glycosylation of the Tractin protein.

The predicted molecular weight of Tractin is 197 kD. However, antibodies to NH₂-terminal sequence of Tractin do not recognize a full length version of the protein on immunoblots, but only a 130 kD glycosylated fragment suggesting that Tractin may be co-translationally cleaved (Huang et al., 1997). Here we further characterize the post-translational modifications of Tractin by making antibodies to different regions of the COOH-terminal region. Our findings suggest that Tractin possesses an additional cleavage site just amino to the transmembrane domain and that the alternative processing results in the formation of four fragments. The most NH₂-terminal fragment, which is glycosylated with
the Lan3-2, Lan4-2, and Laz2-369 glycoepitopes, is secreted, and we propose a model in which the remaining fragments combine to form a secreted homodimer as well as a transmembrane heterodimer.

MATERIALS AND METHODS

Experimental preparations

For the present experiments we used the two hirudinid leech species *Hirudo medicinalis* and *Haenioips 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* embryos at 22-25°C were as previously described (Fernández and Stent, 1982; Jellies et al., 1987). Embryonic day 10 (E10) was characterized by the first sign of a tail sucker, while E30 is the termination of embryogenesis.

Antibodies and antibody production to domain specific GST-fusion proteins

Three previously reported mAbs, Lan3-2 (IgG₁), 4G5 (IgG₁), and Laz2-369 (IgG₃) (Zipser and McKay, 1981; McKay et al., 1983; Hogg et al., 1983; Huang et al., 1997) were used in these studies. In addition new antibodies were made to GST-fusion proteins of three different Tractin (accession # U92813) domains in the pGEX vector system (Pharmacia): the first from A₉¹¹₉-D¹¹₅⁰ encompassed the 4th FNIII- and the acidic domain, the second from K₉⁸⁴-G¹¹⁹₆ covered the acidic domain, and the third from S¹⁷₄⁷-V¹⁸₈⁰ was made up of the cytoplasmic domain. The correct orientation and frame of all the constructs were verified by
sequencing the inserts. Automated sequencing was performed at Iowa State University DNA Sequencing Facility. The fusion proteins were expressed in XL1-Blue cells (Stratagene), harvested, and purified over glutathione agarose (Sigma) columns according to standard protocols (Pharmacia). For polyclonal antibody production two rabbits were injected with from 200 µg to 400 µg of each of the purified fusion proteins in Freund's complete adjuvant (Gibco/BRL), and then boosted at 21 day intervals using Freund's incomplete adjuvant 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 fusion protein and with GST-protein only. Specific antisera named Ming, Yuan, and Qing, were obtained for all three fusion proteins respectively 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, two mAbs, 3A11 and 3A12, were generated against the A^919-D^1150 and the S^1747-V^1880 amino acid sequences, respectively, by injecting Balb C mice with 50 µg of the purified fusion proteins at 21 day intervals. After the third boost spleen cells of the mice were fused with Sp2 myeloma cells and monospecific hybridoma lines established using standard procedures (Harlow and Lane, 1988). The 3A11 mAb was raised to the A^919-D^1150 sequence but did not recognize the K^984-G^1196 fusion protein in dot blot analysis. The mAb 3A11 epitope is therefore likely to be located within the A^919-K^984 region of Tractin. 3A11 and 3A12 ascites was obtained by injecting mice intraperitoneally with antibody producing hybridoma cells. The 3A11 mAb is of the IgG1 subtype whereas mAb 3A12 is of the IgG2B subtype. All procedures for monoclonal antibody production were performed by the Iowa State University Hybridoma Facility.
**Biochemical analysis**

*SDS-PAGE and immunoblotting.* SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with 1X buffer containing 20% methanol and in most cases including 0.1% SDS. 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 DAB (0.1 mg/ml) and H2O2 (0.03%) and enhanced with 0.008% NiCl₂. The immunoblots 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).

*Immunoprecipitation.* Immunoprecipitations with Laz2-369 antibody were performed at 4°C. Dissected *Haemopis* leech nerve cord were homogenized in 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 the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and Aprotinin (Sigma)) and the homogenate (20 μl) incubated with the nonspecific mouse IgG conjugated to protein A Sepharose matrix for 2 hr. After spinning, the resulting supernatant was then incubated with Laz2-369 antibody conjugated to protein A Sepharose matrix (10 μl) overnight. After a brief spin for 20 sec at 2000 rpm the supernatant was discarded and the immunoaffinity matrix resuspended and washed 3 times with 400 μl of extraction buffer for 15 min. 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.

*Deglycosylation.* Enrichment for the NH₂-terminal fragment of Tractin was achieved by selecting for the glycoprotein fraction using a lentil lectin-Sepharose column (Pharmacia).
Dissected nerve cords were homogenized in lentil lectin-column binding buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM CaCl₂, 0.2% NP-40, 0.2% Triton X-100, pH 7.4) containing protease inhibitors. This homogenate was then batch-incubated overnight at 4°C with lentil lectin-Sepharose beads. The beads were poured into a column, the column washed with lentil lectin binding buffer, and the bound fraction competed off with two 15 min incubations with 25 mM Tris-HCl, 10% methyl α-D-mannopyranoside, 0.15% SDS, pH 7.4.

The eluted glycoprotein fraction was mixed with 2X reaction buffer containing 100 mM sodium phosphate, pH 7.5 with 12.5 mU of the N-glycosidase PNGase F according to the manufacturer's protocols (GLYKO). The deglycosylation was performed with or without sample denaturation prior to the addition of enzyme. For deglycosylation with sample denaturation, half of the mixture (glycoprotein fraction + 2X reaction buffer) was denatured by heating in 0.005% SDS and 2.5 mM β-mercaptoethanol, at 100°C for 5 min and then cooled on ice. NP-40 and glycosidase enzyme were then sequentially added to the mixture to complete the components of the reaction samples. The reaction sample mixture was incubated for 3 hr 45 min at 37°C with shaking, and then processed for SDS-PAGE and immunoblotting analysis. The other half of the mixture (glycoprotein fraction + 2X reaction buffer) was treated identically as a negative control, except enzyme was replaced by H₂O. For deglycosylation without sample denaturation by SDS/β-mercaptoethanol, half of the mixture (the eluted glycoprotein fraction + 2X reaction buffer) was digested by glycosidase enzyme for 24 hr at 37°C with shaking, and then processed for SDS-PAGE and immunoblot analysis. The other half of the mixture (glycoprotein fraction + 2X reaction buffer) was treated identically as a negative control, except enzyme was replaced by H₂O. The protease inhibitors PMSF and Aprotinin were maintained in the buffers throughout the whole process.

Triton X-114 phase separation. The phase separation of proteins was performed according to Bordier (1981) and Bajt et al. (1990). Haemopis nerve cords were
homogenized in TBS (150mM NaCl, 10mM Tris-HCl, pH 7.4) containing 1% Triton X-114 (Sigma) and protease inhibitors (PMSF and Aprotinin). Debris was removed by centrifugation at 2000 rpm at 4°C, and 200 μl of the resulting supernatant was overlaid onto a cushion of 6% (wt/vol) sucrose and 0.06% Triton X-114 in TBS in a 0.5 ml eppendorf tube. Phase separation was then introduced by incubating the samples at 37°C for 3 min. After clouding indicated completion of the phase separation, the samples were centrifuged for 10 min at 2000 rpm. The aqueous phase was transferred to a separate ice cold tube without sucrose cushion and adjusted to the volume of 200 μl by adding fresh TBS buffer. Subsequently, 2 μl fresh Triton X-114 was added to the samples for a final concentration of 1% and its dissolution was achieved by bathing the samples on ice for 10 min and pipeting back and forth occasionally. The 200 μl samples with dissolved Triton X-114 was overlaid back to the lower detergent phase obtained from the previous centrifugation for two further rounds of phase separation. At the end of the third round of phase separation, the upper aqueous phase was taken to another ice cold tube without sucrose cushion, and washed by the addition of Triton X-114 to a final concentration of 2%. The dissolution of 2% Triton X-114 was followed by induction of phase separation and centrifugation as before. The resulting detergent phase from this washing process was discarded and the washing procedure was repeated once more. At the end of the second wash, proteins in the aqueous phase were precipitated by the addition of ethanol to the final concentration of 66% (Pohl, 1990). For analysis of the detergent phase derived from the first three rounds of phase separation, the detergent phase was rinsed twice with 300 μl of fresh TBS. At the end of the second rinse, the detergent phase was dissolved in 200 μl of fresh TBS and 66% ethanol was used to precipitate proteins after the dissolution on ice. Proteins concentrated from both phases by precipitation were vacuum dried, boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis.
**Collagenase digestion.** Collagenase digestion was performed with highly purified bacterial collagenase form III (Advance Biomanufactures Corporation, Lynbrook, NY) from *Clostridium histolyticum*. The powdered collagenase was reconstituted with 1.0 ml of storage buffer (0.05M Tris, 0.01M calcium acetate, pH 7.2) for a final concentration of 42 μg/ml as recommended by the manufacturer. *Haemopis* nerve cords were homogenized in 2X reaction buffer (0.08 M NaCl, 0.10 mM Tris, 0.02 M CaCl₂, pH 7.5), according to the methods by Peterkofsky (1982) and Bond et al. (1984). Debris was removed by centrifugation. Half of the homogenate supernatant was then incubated with an equal volume of collagenase in the presence of proteinase inhibitors (PMSF and Aprotinin). For partial digestions, the incubation was stopped 4 hr after shaking at 37°C by addition of SDS-PAGE sample buffer and the samples processed for SDS-PAGE and immunoblot analysis. For complete digestions, the incubation was stopped with addition of SDS-PAGE sample buffer after incubation at 37°C for 17 hr. For controls of the partially or completely digested samples, the other half of the homogenate supernatant was identically treated except that the collagenase was replaced by an equal volume of 2X storage buffer.

**Yeast two-hybrid analysis**

A 110 amino acid fragment (C¹⁷⁷¹ - V¹⁸⁸⁰) containing the cytoplasmic region of Tractin was PCR amplified and subcloned into the pBD-GAL4 Cam phagemid vector (Tractin¹¹⁰-BD) using standard methods (Sambrook et al., 1989). The fidelity of the construct was verified by sequencing. Tractin¹¹⁰-BD was used to screen a *Drosophila* 0-2 hr embryonic yeast two-hybrid library (the generous gift of Dr. L. Ambrosio, Iowa State University) in yeast YRG-2 cells following previously described methods (Bartel et al., 1993; Durfee et al., 1993). Positively interacting clones were identified using two criteria: growth of the yeast cells on His-, Leu-, Trp- medium as well as by induction of a blue
reaction product with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The positive clones were isolated and re-transformed with the bait construct to verify the interaction. The identity of the interacting clones were determined by sequencing.

The interaction of Tractin¹¹⁰-BD with previously characterized Drosophila ankyrin and neuroglian cytoplasmic domain was also characterized. These constructs, pACTII-ankyrin (ankyrin-AD), pACTII-nrg¹⁶⁷-cyto (nrg¹⁶⁷-AD), and pAS1-CYH2-nrg¹⁶⁷-cyto (nrg¹⁶⁷-BD) (Dubreuil et al., 1996; Hortsch et al., 1998a; 1998b) were the generous gift of Dr. M. Hortsch (University of Michigan). As above co-transfected YRG-2 cells were grown on His-, Leu-, Trp- medium and positive interactions identified by a blue reaction product in the X-gal assay.

**Immunohistochemistry**

Dissected Hirudo embryos grown at 22-25°C were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The embryos were incubated overnight at room temperature with diluted antibody (Lan3-2, 1:75; Laz2-369, 1:10; 4G5, 3A11, or 3A12 ascites 1:1000; Ming, Yuan, or Qing rabbit antiserum, 1:1000) in PBS containing 0.5% Triton X-100 and 0.005% 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 DAB (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. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T 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.

Double labeled preparations were obtained by a subsequent incubation in the other primary antibody and by using fluorescently conjugated subtype-specific secondary antibodies. A rabbit anti-mouse IgG Texas Red-conjugated secondary antibody (Cappel) was used for Laz2-369 and a rabbit anti-mouse IgG\(_i\) FITC-conjugated secondary antibody (Cappel) for the Lan3-2 mAb. Fluorescently labeled preparations were mounted in glycerol with 5% \(n\)-propyl gallate. A separate confocal series of images for each fluophor of the labeled preparations were obtained simultaneously with the Leica confocal TCS NT microscope at 1 \(\mu m\) intervals using the krypton and argon laser lines and the appropriate filter sets. An average projection image for each of the image stacks were obtained using the NIH-Image software. These were subsequently imported into Photoshop where they were pseudocolored, image processed, and merged.

RESULTS

**Domain specific Tractin antibodies and N-linked glycosylation**

Figure 1A shows a diagram of the domain structure of Tractin. It was previously suggested that Tractin was cleaved at a site in the third FNIII-domain, based on labeling of a 130 kD band on immunoblots by an NH\(_2\)-terminal antibody raised to a peptide sequence from the second Ig-domain (Fig. 1A,B) (Huang et al., 1997). To examine the properties and distribution of Tractin sequences carboxylic to this potential cleavage site we made polyclonal (Ming, Yuan, and Qing) and monoclonal (3A11 and 3A12) antibodies to three domain specific GST-fusion proteins: one encompassing the fourth FNIII- and the acidic-domains, one covering the acidic domain only, and one containing the entire intracellular
domain (Fig. 1A). mAb 3A11 does not recognize the acidic domain fusion protein on dot blots and its epitope is therefore located proximally to this domain (data not shown). Immunocytochemically, the five new antibodies towards domains distal to the first cleavage site all label the soma and projections of all central and peripheral neurons as illustrated in Fig. 1D for mAb 3A11. This pattern is identical to that previously reported for Tractin antibody to the NH2-terminal fragment (Huang et al., 1997; 1998). On immunoblots antibodies to the fourth FNIII- and acidic domain (Ming, Yuan, mAb 3A11) all recognize a doublet of bands of approximately 165 and 185 kD whereas antibodies to the intracellular domain (Qing, mAb 3A12) only recognize the higher 185 kD band (Fig. 1C). These findings suggest that there are two versions of this part of the Tractin protein; one version containing the intracellular domain and one without it indicating that a second cleavage site is present in the Tractin protein. The sequence of Tractin contains several putative protease cleavage motifs just proximal to the transmembrane domain. If Tractin is cleaved in this region the predicted molecular mass of the transmembrane fragment would be 98 kD whereas the cleaved fragment containing the acidic domain and PG/YG-repeat region would be about 76 kD (Fig. 1A). These predicted molecular masses are much lower than those observed experimentally on immunoblots (Fig. 1C) suggesting that the fragments may form dimers or multimers.

To assess the contribution of glycosylation to the molecular mass of the Tractin fragments we deglycosylated lentil lectin purified CNS protein homogenate with N-glycosidase F, which cleaves N-linked glycosylation. The extracellular portion of Tractin possesses 19 potential N-glycosylation sites (Huang et al., 1997). For all of the three Tractin bands detectable on immunoblots N-glycosidase F treatment resulted in faster migration on SDS-PAGE (Fig. 2). The NH2-terminal fragment recognized by mAb 4G5 was most heavily glycosylated with an estimated 30 kD of oligosaccharides (Fig. 2A) whereas glycosylation
contributed about 10-15 kD to the mass of each of the bands in the doublet recognized by mAb 3A11 (Fig. 2B).

**Tractin is processed into both secreted and transmembrane forms**

The labeling pattern of domain specific antibodies on immunoblots suggested that Tractin may be processed into peripherally membrane attached as well as into integral transmembrane forms. To further test this hypothesis we conducted phase separation experiments of homogenized CNS proteins with Triton X-114. A homogeneous Triton X-114 solution at 0°C segregates into detergent and aqueous phases after the solution temperature is raised above 20°C. This phase separation can be used to distinguish loosely associated membrane proteins, which will partition into the aqueous phase, from transmembrane proteins which will mainly partition into the detergent phase (Bordier 1981; Bajt et al., 1990). As shown on the immunoblots in Fig. 3 the NH$_2$-terminal fragment as detected by mAb 4G5 is found exclusively in the aqueous phase. This is also the case for the lower band recognized by mAb 3A11 whereas the higher band partitions equally into the detergent and aqueous phases (Fig. 3). Although some of the higher band recognized by mAb 3A11 is found in the aqueous phase the fact that at least half is found in the detergent phase whereas none is detectable in this phase from the other Tractin bands strongly indicates that the higher band represents a transmembrane version of Tractin. That some partitions to the aqueous phase could be due to the highly polar nature of the acidic domain where 43 of 64 residues are charged.

**The PG/YG-repeat region is digested by collagenase**

The secreted fragment and the extracellular domain of the transmembrane fragment of the COOH-terminal part of Tractin would largely be made up of the PG/YG-repeat region.
The 12 repeat segments are connected by short linker sequences (Huang et al., 1997). Thus, the sequence of this region, although considerably more structured, is reminiscent of that of type IV 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). Type IV collagen is a major constituent of extracellular matrix and basal lamina and can form dimers and polymers through stable covalent nonreducible cross-links (Eyre et al., 1984). 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 suggesting that the PG/YG-repeat region of Tractin may have some of the same structural and functional properties as type IV collagen (Huang et al., 1997). To test this hypothesis we treated extracted leech CNS protein with a highly purified collagenase (Schäcke et al., 1998) with the expectation that collagen-like regions of Tractin would be digested whereas non-collagenous stretches would stay intact. Figure 4 shows the result of such an experiment where the collagenase treated samples were immunoblotted after SDS-PAGE and labeled with Tractin domain specific antibodies. After collagenase treatment for 17 hr the 165 and 185 kD doublet of bands recognized by mAb 3A11 was reduced to a single band of 47 kD. This would be the expected size for the fragment from cleavage site I to the beginning of the PG/YG-repeat region recognized by mAb 3A11 strongly suggesting that collagenase digested the PG/YG-repeats. During intermediate collagenase incubation times numerous mAb 3A11 positive fragments were observed indicating the presence of multiple cleavage sites (Fig. 4A). The intracellular domain specific antibody Qing recognized a smaller 29 kD band after collagenase treatment which corresponds to the expected size of the fragment from the end of the PG/YG-repeat region and to the COOH-terminal. Control experiments demonstrated that the NH₂-terminal fragment recognized by mAb 4G5 which does not contain collagen-like sequence was unaffected by collagenase treatment indicating that incubation with the enzyme...
did not affect non-collagenous protein sequences (Fig. 4B). These experiments suggest that the PG/YG-repeat region is sensitive to collagenase and that consequently its structure and properties may be collagen-like.

The Tractin intracellular domain interacts with ankyrin

The cytoplasmic tail of Tractin has a stretch of residues 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 (Huang et al., 1997). This ankyrin motif is also found in many members of the L1 subfamily of CAMs and is highly conserved (Hortsch, 1996; Brümmendorf et al., 1998). For example, it has been demonstrated that both Drosophila neuroglian and its human L1 homolog can interact with Drosophila ankyrin in a yeast two hybrid interaction assay (Hortsch et al., 1998a). In the absence of a suitable leech two-hybrid library we decided to take advantage of this conservation by using a yeast two hybrid system to screen an embryonic Drosophila cDNA library with a bait-construct containing the entire intracellular Tractin domain (Tractin\textsuperscript{110}-BD) in order to establish whether the ankyrin binding motif in Tractin is likely to be functional. From this screen we obtained four positive clones; however, only one of these was positive after re-transformation and re-screening on selective medium. Both ends of this clone were sequenced with the resulting sequences having 100% identity to Drosophila ankyrin (Dubreuil and Yu, 1994). The identified Drosophila ankyrin insert contained the sequence from A\textsuperscript{25} to G\textsuperscript{443}. We also examined the interaction of Tractin\textsuperscript{110}-BD with a previously characterized Drosophila ankyrin construct fused to a GAL4 activation domain (ankyrin-AD) (Dubreuil et al., 1996) in the yeast two hybrid system. The Drosophila neuroglian cytoplasmic domain construct (nrg\textsuperscript{167}) either fused to the GAL4 activation domain or the GAL4 DNA-binding domain (Hortsch et al., 1998a; 1998b) were used as controls. As
shown in Fig. 5 *Drosophila* ankyrin-AD interacts with Tractin\(^{110}\)-BD and nrg\(^{167}\)-BD whereas Tractin\(^{110}\)-BD in control co-transfections with nrg\(^{167}\)-AD did not show any interaction. These experiments demonstrate that the cytoplasmic domain of Tractin can interact with *Drosophila* ankyrin in the yeast two-hybrid interaction assay and strongly indicate that Tractin's ankyrin binding domain is functional.

**Tractin is glycosylated with the Laz2-369 glycoepitope**

We have previously shown that Tractin is differentially glycosylated by the Lan3-2 and Lan4-2 glycoepitopes (Huang et al., 1997). However, an additional glycoepitope recognized by the mAb Laz2-369 and found on 130 kD proteins was also a candidate to represent an additional glycomodification of the Tractin protein (Bajt et al., 1990; Song and Zipser, 1995b). Tractin is glycosylated with the Lan3-2 epitope in all peripheral sensory neurons (Jellies et al., 1994; Huang et al., 1997) and it has been estimated that a subset constituting about 45% of these neurons are also Laz2-369 positive (Zipser et al., 1994). In contrast to the Lan3-2 epitope, the antibody labeling of which is greatly reduced by mannose-BSA, the labeling of the Laz2-369 epitope is reduced by galactose-BSA and not by mannose-BSA (Song and Zipser, 1995b). Thus, the two glycoepitopes are likely to have different oligosaccharide compositions. To test whether the Laz2-369 epitope is present on Tractin we immunoprecipitated leech CNS proteins with Laz2-369 antibody coupled to protein A-Sepharose beads. The Laz2-369 immunoprecipitate was then washed, boiled, separated by SDS-PAGE and immunoblotted. Figure 6 shows that the same 130 kD band was recognized by Laz2-369, Lan3-2, and by the Tractin specific mAb 4G5. No higher molecular weight bands were observed suggesting that only the NH\(_2\)-terminal fragment of Tractin is glycosylated with the Laz2-369 epitope.
We examined the developmental expression of the Laz2-369 epitope in relation to the Lan3-2 epitope in peripheral sensory neurons by double labeling E10 *Hirudo* embryos with the respective antibodies and using isotype-specific, fluorescently labeled second antibodies. During development the peripheral sensory neurons send axons to the CNS where they bifurcate and segregate into specific axon fascicles (Jellies et al., 1994). In leech the formation of both the central and peripheral nervous systems proceeds in a rostro-caudal sequence with each posterior segment approximately 3 hr later in development than the more anterior one (Jellies and Kristan, 1991). Consequently, since there are 32 segments, an embryo exhibits segments in different stages of development spanning a period of about 2-3 days, which greatly facilitates the analysis of neuronal differentiation. Figure 7 shows the relative degree of expression of the Laz2-369 and Lan3-2 epitopes in three ganglia at different developmental stages in the same embryo. While the Lan3-2 epitope is detectable from the earliest onset of growth cone extension (Jellies et al., 1994; 1995; 1996) the Laz2-369 epitope is clearly only detectable with a delay of 12-24 hr compared to the Lan3-2 epitope. Furthermore, the Laz2-369 epitope does not appear to be expressed on the axons before they have reached and bifurcated in the CNS. Thus, the temporal expression of the Laz2-369 glycoepitope is different from that of the Lan3-2 glycoepitope and may be developmentally regulated depending on interactions within the CNS.

**DISCUSSION**

In this study we have used domain specific antibodies to characterize the post-translational processing and glycosylation of Tractin. We show that NH2-terminal antibody recognizes a 130 kD fragment that is found in the aqueous phase of phase partitioning experiments and therefore is likely to be released by shedding. Antibodies specific to the
fourth FNIII-like domain and the acidic domain recognize a doublet of bands on immunoblots of about equal intensity of 165 and 185 kD; however, only the latter band is recognized by antibodies specific to the cytoplasmic domain of Tractin. The higher band partitions partially in the detergent phase suggesting it is transmembrane whereas the lower band is found only in the aqueous phase. Interestingly, the molecular weights of the doublet of bands recognized by Tractin COOH-domain specific antibodies are much higher than the 76 and 98 kD predicted for these bands based on their amino acid sequence. Thus, to account for these data we propose a model for the post-translational processing of Tractin which is illustrated in Fig. 8. In this model Tractin contains two proteolytic cleavage sites as indicated in Fig. 1A. All Tractin proteins are cleaved at site I whereas only half of the molecules are cleaved at site II as suggested by the equal intensity of antibody labeling of both the high and the low band. This gives rise to a secreted glycosylated 130 kD fragment which may be anchored to the cell surface by interactions with integrins through the RGD integrin binding motif in the second FNIII-domain (Fig. 8A). While we have no direct evidence for this linkage such an interaction has been demonstrated for the Tractin related CAM L1 (Ruppert et al., 1995; Montgomery et al., 1996). The remaining fragments combine to form a secreted homodimer (Fig. 8B) made up of the sequence between the two cleavage sites and a transmembrane heterodimer (Fig. 8C) made up of sequence between the cleavage sites and the intact transmembrane fragment. The predicted molecular mass of the homodimer would be approximately 152 kD which with glycosylation of 10-15 kD taken into account would be close to the observed relative molecular weight of the lower band on immunoblots of 165 kD. The predicted molecular mass of the heterodimer would be 174 kD which with glycosylation would be close to the observed relative molecular weight on immunoblots of 185 kD. In this model the proposed dimers are linked by SDS-resistant and non-reducible covalent bonds. Furthermore, the transmembrane heterodimer is likely to be
linked to the cytoskeleton through interactions with ankyrin (Fig. 8C) as demonstrated in the yeast two-hybrid assay where the cytoplasmic chain of Tractin interact with Drosophila ankyrin.

One of the main features of the model for Tractin processing is homo- and heterodimer formation through covalent bonds. While we cannot directly rule out that the discrepancy in predicted and observed molecular masses of the Tractin fragments is due to anomalous gel migration or to linkage to unrelated molecules which happen to form complexes of the same observed sizes with Tractin, collagen-like sequences are known to form extensive cross-links (Eyre et al., 1984). These cross-links are mainly formed through lysine side chains although the covalent cross-links of type IV collagen, which is most similar to the PG/YG-repeat domain, have not been well characterized (Eyre et al., 1984). Thus, the observation that digesting the PG/YG-repeat region with collagenase eliminates cross-linking and reduces the mAb 3A11 positive doublet on immunoblots to the predicted sizes for monomers of the flanking proteinaceous sequences supports the proposed model. It further provides evidence that the PG/YG-repeat region is collagenous and that the Tractin COOH-terminal fragment in effect forms an integral transmembrane collagen. While this would be the first example of a type I transmembrane collagen, two type II transmembrane collagens, collagen XIII (Pihlajaniemi and Tamminen, 1990) and collagen XVII (Li et al., 1993; Shäcke et al., 1998), have been previously described. Collagen XVII is found in human keratinocytes and it contains a sizable cytoplasmic domain of 51 kD, a transmembrane domain, and an extracellular domain composed of 15 collagenous subdomains which combine to form triple-helical proteins (Shäcke et al., 1998). It is thought that the interrupted collagenous ectodomain is able to maintain flexibility of the protein for efficient ligand interactions and that it may play a role in hemidesmosomes of maintaining linkage between intracellular and extracellular structural elements (Shäcke et al., 1998).
very similar organization of the Tractin transmembrane heterodimer and the demonstrated interaction with ankyrin of its cytoplasmic domain suggest that it may have a comparable function in neurons. Interestingly, collagen XVII is specifically cleaved just proximally to the transmembrane domain resulting in a secreted ectodomain (Shäcke et al., 1998) as is also the case for the secreted Tractin homodimer.

There is a growing body of evidence that secreted forms of both type I and type II integral membrane proteins including CAMs, growth factor and cytokine receptors, and receptor ligands are derived from selective post-translational proteolysis from the cell surface (Hooper et al., 1997; Blobel et al., 1997). The cleavage generally occurs close to the extracellular face of the membrane and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. Many of these enzymes are metallo- and/or serine proteases and are themselves integral membrane proteins localized at the cell surface. The biological function of the proteolytic cleavage of transmembrane proteins are still not well characterized and may vary. In some cases it may be a process for rapidly down-regulating the protein from the cell surface, in others it may be to generate a soluble form of the protein that has properties somewhat different from those of the membrane bound form (Hooper et al., 1997). In some cases the processing may be necessary for biological activity. For example, in order to generate a functional Notch receptor in Drosophila the protein is cleaved by the disintegrin metallo-protease Kuzbanian to form a disulfide linked heterodimer (Pan and Rubin, 1997; Blaumueller et al., 1997). Furthermore, loss-of-function mutations in the kuzbanian gene in Drosophila embryos show that its proteolytic activity is required for axonal extension (Fambrough et al., 1996). Similarly, we propose that the proteolytic cleavage of Tractin by such proteases is likely to be a prerequisite for its proper function in axonal growth regulation.
An important feature of the secreted NH₂-terminal fragment of Tractin is its glycosylation with specific glycoepitopes expressed by subsets of neurons fasciculating in distinct axonal tracts. For example, while Tractin is present in all neurons of both the CNS and the PNS only peripheral sensory neurons are glycosylated with the Lan3-2 glycoepitope (Huang et al., 1997). Here we present evidence that a subset of these peripheral sensory neurons in addition is glycosylated with the Laz2-369 glycoepitope as illustrated in the diagram in Fig. 9. Furthermore, we show that, as previously reported (Song and Zipser, 1995b; Tai and Zipser, 1999), the Laz2-369 glycomodification is temporally regulated and does not occur before the peripheral sensory neuron axons have reached the CNS. This is in contrast to the Lan3-2 glycoepitope which is expressed on Tractin at the earliest extension of axons (Johansen et al., 1992; Huang et al., 1997). Antibody perturbation experiments have shown that these glycoepitopes are involved in regulating axon outgrowth and synapse formation in a way that may be correlated with their relative temporal expression. The Lan3-2 glycoepitope promotes filopodial extension and synapse formation at the initial stages of rapid axonal growth and synaptic target exploration (Zipser et al., 1989; Song and Zipser, 1995a; Huang et al., 1997; Tai and Zipser, 1998). However, at the time when synaptogenesis is likely to occur upregulation of the Laz2-369 glycoepitope which inhibits filopodial sprouting may function to promote stable synapse formation (Song and Zipser, 1995b; Tai and Zipser, 1999). Thus, these findings suggest that differential glycosylation of a widely expressed neural CAM can functionally regulate neuronal outgrowth and synapse formation of distinct neuronal subpopulations.

The importance of specific glycomodifications for protein function and their tissue specific expression patterns have been most clearly demonstrated in the process of lymphocyte homing, which is mediated by selectins that are capable of recognizing and binding ligands expressing specific oligosaccharide structures (Springer, 1994; Lasky, 1995).
In the nervous system a striking example of how the developmental regulation of glycosylation can affect neural pathway formation is provided by the modulation of the polysialic acid content of NCAM in the plexus region of the chick limb bud where its up-regulation allows the axons to defasciculate into their proper pathways (Rutishauser and Landmesser, 1996). 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). These findings together with the results of the present study suggest that specific carbohydrate structures on neural proteins are promising candidates for playing a prominent role in establishing neural connections during development.

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REFERENCES


FIGURE LEGENDS

*Figure 1.* Domain specific Tractin antibodies. A, Diagram of the Tractin protein. The protein sequence is organized into six Ig-domains, four FNIII-domains, an acidic domain, a PG/YG-repeat containing domain which is collagen-like, a transmembrane domain (TM), and a cytoplasmic domain with an ankyrin-binding and a PDZ-binding (SxV) motif. An RGD integrin-binding motif is located in the second FNIII-domain. The two putative proteolytic cleavage sites in the third FNIII-domain (cleavage site I) and between the PG/YG-repeat and transmembrane domains are indicated by arrows. Monoclonal (3A11, 3A12) and polyclonal (Ming, Yuan, Qing) antibodies were made to fusion proteins of Tractin sequences as indicated by the black horizontal bars. In addition, the mAb 4G5 was
previously made to a peptide sequence from the second Ig-domain (Huang et al., 1997). The predicted molecular mass of the fragments generated by proteolytic cleavage at site I and II is shown on the line below the diagram. B. Immunoblot of *Haemopis* nerve cord proteins labeled with mAb 4G5. C. Immunoblots of *Haemopis* nerve cord proteins labeled with Tractin domain specific antibodies. Antibodies (Ming, Yuan, mAb 3A11) to the fourth FNIII and/or acidic domain recognize a doublet of bands whereas antibodies to the cytoplasmic domain (Qing, mAb 3A12) only recognize the high band of the doublet. D. mAb 3A11 labels all central and peripheral neurons and their projections in a *Hirudo* E13 embryo. Two ganglia (g) as well as the four main nerve tracks (arrows) are indicated. Scale bar: 100 µm.

**Figure 2.** NH$_2$- and COOH-terminal fragments of Tractin are N-glycosylated. Extracts of *Haemopis* nerve cord proteins were digested with N-Glycosidase F (deglycosylation) or mock digested (control), separated by SDS-PAGE, and immunoblotted. N-glycosidase F treatment of both the NH$_2$-terminal fragment as detected by mAb 4G5 (A) and of the doublet of COOH-terminal fragments as detected by mAb 3A11 (B) leads to faster gel migration. The migration of 200, 116, and 97 kD markers is indicated.

**Figure 3.** Triton-X-114 phase separation of proteolytically cleaved Tractin fragments. The top panel shows the partitioning of the NH$_2$-terminal fragment of Tractin (arrow) into the aqueous phase as detected by the mAb 4G5 on immunoblots. The detergent phase is devoid of immunoreactivity. The bottom panel shows partitioning of the COOH-terminal fragments of Tractin (arrows) as detected by the mAb 3A11 on immunoblots. The lower band partitions exclusively to the aqueous phase whereas the higher band is found approximately equally in both the detergent and the aqueous phase. Control lanes in both
panels show immunoblots of the extracted *Haemopis* nerve cord proteins before phase separation.

*Figure 4.* Collagenase digestion of the COOH-terminal Tractin fragments. Protein extracts from *Haemopis* nerve cords were digested with collagenase for 4 or 17 hr, separated by 15% SDS-PAGE, and immunoblotted. A, After complete collagenase digestion for 17 hr the doublet (control, lane 1) recognized by mAb 3A11 was reduced to a single band of 47 kD (lane 3, top arrow). A smaller band of 29 kD was detected by the cytoplasmic domain specific antibody Qing (lane 4, bottom arrow). During incomplete collagenase digestion (4 hr) multiple bands of intermediate sizes were detected by the 3A11 antibody (lane 2) suggesting the presence of several collagenase cleavage sites. B, The migration of the NH2-terminal fragment of Tractin as detected by mAb 4G5 was unaltered by collagenase digestion for 17 hr (lane 2).

*Figure 5.* The cytoplasmic domain of Tractin binds to *Drosophila* ankyrin in the yeast two-hybrid interaction assay. The figure shows a qualitative yeast two-hybrid analysis between the cytoplasmic domain of Tractin (Tractin110-BD) and neuroglian (nrg167-BD/AD) and *Drosophila* ankyrin (Ankyrin-AD). Blue colonies (dark reaction product) result from the induction of β-galactosidase activity and indicate an interaction between two GAL4 fusion proteins. BD-constructs were fusion proteins containing the GAL4 DNA-binding domain whereas AD-constructs were fusion proteins containing the GAL4 activation domain. In this assay both Tractin and neuroglian cytoplasmic domains interacted with *Drosophila* ankyrin whereas there was no interaction between the Tractin and neuroglian cytoplasmic domains.
Figure 6. The NH2-terminal fragment of Tractin is glycosylated with the Laz2-369 glycoepitope. Immunoblots of Laz2-369 immunoprecipitated \textit{Haemopis} nerve cord proteins. The Laz2-369 immunoprecipitate (Laz2-369 ip) was recognized by both mAb Laz2-369, Lan3-2, and the NH2-terminal fragment of Tractin specific mAb 4G5 as a single band of 130 kD.

Figure 7. Temporal expression of the Laz2-369 glycoepitope. E10 \textit{Hirudo} embryo grown at 22-25°C double labeled with mAb Laz2-369 (green, top row) and Lan3-2 (red, middle row). The bottom row shows the merged images. The labeling of peripheral neuron projections in three of the 21 midbody ganglia (g3, g12, and g16) are shown. Development in leech embryos proceeds in a rostro-caudal gradient such that g16 represents the earliest and g3 the latest developmental stage shown. The Laz2-369 glycoepitope was upregulated with a temporal delay of about 12-24 hr in comparison to the Lan3-2 glycoepitope which is expressed at the earliest outgrowth of peripheral neuron axons. Scale bar: 25 µm.

Figure 8. Model for the proteolytic processing of Tractin. A. All Tractin proteins are co-translationally cleaved in the second FNIII-domain giving rise to an NH2-terminal fragment containing the six Ig-domains and 21/2 FNIII-domain which is released by shedding. This fragment of Tractin may be tethered to the cell surface through interactions with integrins at the RGD integrin-binding motif located in the second FNIII-domain. B, C, In addition to being cleaved in the third FNIII-domain a portion of the Tractin molecules are proteolytically cleaved just proximally to the transmembrane domain. This gives rise to a secreted homodimer (B) and a transmembrane heterodimer (C). We propose that the dimers are formed by covalent cross-links in the collagenous PG/YG-repeat domain. While the secreted homodimer may be localized to the cell surface through interactions with
extracellular matrix molecules, the transmembrane heterodimer is likely to be linked to the cytoskeleton through interactions with ankyrin \((C)\).

**Figure 9.** The NH\(_2\)-terminal fragment of Tractin is differentially glycosylated with the Lan3-2 and Laz2-369 glycoepitopes. The NH\(_2\)-terminal fragment of Tractin is expressed by and present on all neurons in the CNS and PNS \((A)\). However, in a subset of these neurons, the peripheral sensory neurons, Tractin is differentially glycosylated with the Lan3-2 glycoepitope \((B)\). In addition, some of the peripheral sensory neurons are glycosylated with the Laz2-369 glycoepitope \((C)\). In this model the Lan3-2 and Laz2-369 glycoepitopes are depicted at different glycosylation sites since proteolytic digestion has shown that the Lan3-2 and Laz2-369 antibodies recognize different peptide fragments (Bajt et al., 1990).
Fig. 1
Fig. 2

Fig. 3
Fig. 4

Fig. 5
Fig. 6

Laz2-369 ip

2-369  3-2  4G5

130 kDa
Fig. 8

A. Integrin released by shedding

B. Integrin released by shedding

C. Integrin released by shedding

Fig. 9

A. All neurons

B. All peripheral sensory neurons

C. Subset of peripheral sensory neurons

Fig. 9
DIFFERENTIAL GLYCOSYLATION AND PROTEOLYTICAL PROCESSING OF LEECHCAM IN CENTRAL AND PERIPHERAL LEECH NEURONS

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ABSTRACT

LeechCAM is a recently described member of the Ig-superfamily which has five Ig-domains, two FNIII-domains, a transmembrane domain, and a cytoplasmic domain. Phylogenetic analysis indicated that LeechCAM is the leech homolog of apCAM, FasII, and vertebrate NCAM. Using a leechCAM specific monoclonal antibody we show by immunoblot analysis and by Triton X-114 phase separation experiments that LeechCAM is likely to be proteolytically cleaved into a secreted form without the transmembrane domain and the intracellular tail in addition to the transmembrane version. Furthermore, by immunoprecipitation we demonstrate that LeechCAM is glycosylated with the Laz2-369 glycoepitope that has been specifically implicated in regulation of axonal outgrowth and synapse formation.

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INTRODUCTION

Glycosylated cell adhesion molecules (CAMs) are expressed on the surface of axons and growth cones and fall into several structural classes, most notably the immunoglobulin (Ig)-superfamily, the cadherins, and the integrins. The combined activities of these molecules are required to facilitate growth cone extension and synapse formation and to maintain the complexity of mature neural structures (Walsh and Doherty, 1997). They have also been implicated in higher order processes such as synaptic plasticity underlying learning and memory (Martin and Kandel, 1996; Hagler and Goda, 1998). An important feature of the molecular structure of the neural CAMs of the Ig-superfamily is the variability of their extracellular regions which in most cases contain multiple tandemly arranged domains (Tessier-Lavigne and Goodman, 1996). This kind of modular composition allows them to interact with an array of different proteins. In addition, the diversity in the structure of neural CAMs is amplified with the existence of many splice variants and various post-translational modifications such as differential glycosylation and proteolytic processing (Johansen and Johansen, 1997; Blobel, 1997).

We have recently cloned and identified a new member of the Ig-superfamily in the nervous system of the leech, LeechCAM, which contains five Ig- and two FNIII-domains, a transmembrane domain, and an intracellular tail (Huang et al., 1997). LeechCAM is one of two different members of the Ig-superfamily in leech, the other being Tractin, which were identified based on their expression of the Lan3-2 glycoepitope (Huang et al., 1997). Whereas the core protein of LeechCAM is expressed by all neurons it is differentially glycosylated with the mAb Lan3-2 and Lan4-2 glycoepitopes only in sets and subsets of peripheral sensory neurons that form distinct fascicles in the CNS (Johansen et al., 1992; Huang et al., 1997). In addition, at least four other mAbs (Lan2-3, Laz6-212, Laz2-369,
Laz7-79) which recognize different glycoepitopes specific to distinct subsets of these neurons have been identified (McKay et al., 1983; Peinado et al., 1987; Bajt et al., 1990; Zipser et al., 1994). Antibody perturbation experiments have shown that these glycoepitopes are involved in regulating axon outgrowth and synapse formation in a way which may be correlated with their relative temporal expression. For example, the Lan3-2 glycoepitope promotes filopodial extension and synapse formation at the initial stages of rapid axonal growth and synaptic target exploration (Zipser et al., 1989; Song and Zipser, 1995a; Huang et al., 1997; Tai and Zipser, 1998). In contrast, at the time when synaptogenesis is likely to occur upregulation of the Laz2-369 glycoepitope, which inhibits filopodial sprouting, may function to promote stable synapse formation (Song and Zipser, 1995b; Tai and Zipser, 1999). Thus, these findings suggest that differential glycosylation of a widely expressed neural CAM can functionally regulate neuronal outgrowth and synapse formation of distinct neuronal subpopulations. In this study we demonstrate that the Laz2-369 glycoepitope, in addition to the previously reported Lan3-2 and Lan4-2 glycoepitopes, also represents differential glycosylation of the LeechCAM protein. Furthermore, we present evidence that LeechCAM is proteolytically processed into a secreted as well as a transmembrane form.

MATERIALS AND METHODS

Experimental preparations

For the present experiments we used the two hirudinid leech species *Hirudo medicinalis* and *Haemopis marmorata*. The leeches were either captured in the wild or purchased from commercial sources. Dissections of nervous tissue and embryos were performed in leech saline solutions with the following composition (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.

**Antibodies and immunocytochemistry**

Three previously reported mAbs, P6FN (IgG₁), 4G5 (IgG₁), and Laz2-369 (IgG₃) (Hogg et al., 1983; Huang et al., 1997) in addition to a P6FN mouse antiserum were used in these studies. P6FN ascites were obtained by injecting four mice intraperitoneally with antibody producing hybridoma cells. All procedures for monoclonal antibody ascites production were performed by the Iowa State University Hybridoma Facility.

For immunocytochemistry dissected *Hirudo* embryos grown at 22-25°C were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The embryos were incubated overnight at room temperature with diluted P6FN antiserum (1:500) in PBS containing 0.5% Triton X-100 and 0.005% sodium azide, washed in PBS with 0.4% Triton X-100, and incubated with HRP-conjugated goat anti-mouse antibody (Bio-Rad, 1:200 dilution). After washing in PBS the HRP-conjugated antibody complex was visualized by reaction in DAB (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. The labeled preparations were photographed on a Zeiss Axioskop using Ektachrome 64T 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.
Biochemical analysis

SDS-PAGE and immunoblotting. SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with 1X buffer containing 20% methanol and in most cases including 0.1% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse 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 DAB (0.1 mg/ml) and H₂O₂ (0.03%) and enhanced with 0.008% NiCl₂. The immunoblots 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).

Immunoprecipitation. Immunoprecipitations with Laz2-369 antibody were performed at 4°C. Dissected Haemopsis leech nerve cord were homogenized in 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 the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and Aprotinin from Sigma) and the homogenate (20 μl) incubated with the nonspecific mouse IgG conjugated to protein A Sepharose matrix for 2 hr. The resulting supernatant was then incubated with Laz2-369 antibody conjugated to protein A Sepharose matrix (10 μl) overnight. After a brief spin for 20 sec at 2000 rpm the supernatant was discarded and the immunoaffinity matrix resuspended and washed 3 times with 400 μl of extraction buffer for 15 min. 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.

Deglycosylation. Enrichment for the LeechCAM protein was achieved by selecting for the glycoprotein fraction using a lentil lectin-Sepharose column (Pharmacia). Dissected
nerve cords were homogenized in lentil lectin-column binding buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM CaCl₂, 0.2% NP-40, 0.2% Triton X-100, pH 7.4) containing protease inhibitors. This homogenate was then batch-incubated overnight at 4°C with lentil lectin-Sepharose beads. The beads were poured into a column, the column washed with lentil lectin binding buffer, and the bound fraction competed off with two 15 min incubations with 25 mM Tris-HCl, 10% methyl α-D-mannopyranoside, 0.15% SDS, pH 7.4.

The eluted glycoprotein fraction was mixed with 2X reaction buffer containing 100 mM sodium phosphate, pH 7.5 with 12.5 mU of the N-glycosidase PNGase F according to the manufacturer's protocols (GLYKO). The deglycosylation was performed with or without sample denaturation prior to the addition of enzyme. For deglycosylation with sample denaturation, half of the mixture (glycoprotein fraction + 2X reaction buffer) was denatured by 0.005% SDS and by 2.5 mM β-mercaptoethanol at 100°C for 5 min and then cooled on ice. NP-40 and glycosidase enzyme were then sequentially added to the mixture to complete the components of the reaction samples. The reaction sample mixture was incubated for 3 hr 45 min at 37°C with shaking, and then processed for SDS-PAGE and immunoblotting analysis. The other half of the mixture (glycoprotein fraction + 2X reaction buffer) was treated identically as a negative control, except enzyme was replaced by H₂O. For deglycosylation without sample denaturation by SDS/β-mercaptoethanol, half of the mixture (the eluted glycoprotein fraction + 2X reaction buffer) was digested by glycosidase enzyme for 24 hr at 37°C with shaking, and then processed for SDS-PAGE and immunoblot analysis. The other half of the mixture (glycoprotein fraction + 2X reaction buffer) was treated identically as a negative control, except enzyme was replaced by H₂O. The protease inhibitors PMSF and Aprotinin were maintained in the buffers throughout the whole process.

*Triton X-114 phase separation.* The phase separation of proteins was performed according to Bordier (1981) and Bajt et al. (1990). *Haemopis* nerve cords were
homogenized in TBS (150mM NaCl, 10mM Tris-HCl, pH 7.4) containing 1% Triton X-114 (Sigma) and proteinase inhibitors (PMSF and Aprotinin). Debris was removed by centrifugation at 2000 rpm at 4°C, and 200 μl of the resulting supernatant was overlaid onto a cushion of 6% (wt/vol) sucrose and 0.06% Triton X-114 in TBS in a 0.5 ml eppendorf tube. Phase separation was then introduced by incubating the samples at 37°C for 3 min. After clouding indicated completion of the phase separation, the samples were centrifuged for 10 min at 2000 rpm. The aqueous phase was transferred to a separate ice cold tube without sucrose cushion and adjusted to the volume of 200 μl by adding fresh TBS buffer. Subsequently, 2 μl fresh Triton X-114 was added to the samples for a final concentration of 1% and its dissolution was achieved by bathing the samples on ice for 10 min and pipeting back and forth occasionally. The 200 μl samples with dissolved Triton X-114 was overlaid back to the lower detergent phase obtained from the previous centrifugation for two further rounds of phase separation. At the end of the third round of phase separation, the upper aqueous phase was taken to another ice cold tube without sucrose cushion, and washed by the addition of Triton X-114 to a final concentration of 2%. The dissolution of 2% Triton X-114 was followed by induction of phase separation and centrifugation as before. The resulting detergent phase from this washing process was discarded and the washing procedure was repeated once more. At the end of the second wash, proteins in the aqueous phase were precipitated by the addition of ethanol to the final concentration of 66% (Pohl, 1990). For analysis of the detergent phase derived from the first three rounds of phase separation, the detergent phase was rinsed twice with 300 μl of fresh TBS. At the end of the second rinse, the detergent phase was dissolved in 200 μl of fresh TBS and 66% ethanol was used to precipitate proteins after the dissolution on ice. Proteins concentrated from both phases by precipitation were vacuum dried, boiled in SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis.
**Phylogenetic analysis**

LeechCAM sequence was compared with known and predicted sequences using the National Center for Biotechnology Information BLAST e-mail server. Phylogenetic analysis was performed by first generating alignments of CAM sequences with the computer program Clustalw version 1.7. Gaps in the resulting alignments were removed by deleting residues corresponding to the gaps. Trees were constructed by maximum parsimony using the PAUP program version 3.1.1 on a Power Macintosh G3. All trees were generated by heuristic searches and bootstrap values in percent of 1000 replications are indicated on the bootstrap 50% majority rule consensus tree.

**RESULTS**

**LeechCAM is a glycosylated homolog of apCAM and FasII.**

Figure 1A shows a diagram of the domain structure of LeechCAM. The monoclonal antibody (mAb) P6FN used in this study was made to a synthetic peptide from the second FNIII-domain (Fig. 1A). On immunoblots this antibody recognizes a doublet of protein bands of approximately 138 kDa and 120 kDa, respectively (Fig. 1B). The difference between these bands of 18 kDa suggests that LeechCAM may be proteolytically cleaved proximally to the transmembrane domain, but distally to the second FNIII-domain and thus that there are two versions of the LeechCAM protein; one version containing the intracellular domain and one without it. Immunocytochemically, the mAb P6FN labels the soma and projections of all central and peripheral neurons as illustrated in Fig. 1C.

To assess the contribution of glycosylation to the molecular mass of the LeechCAM protein we deglycosylated lentil lectin purified CNS protein homogenate with N-glycosidase F, which cleaves N-linked glycosylation. The extracellular portion of LeechCAM possesses
10 potential N-glycosylation sites (Huang et al., 1997). For both of the LeechCAM bands detectable on immunoblots N-glycosidase F treatment resulted in faster migration on SDS-PAGE (Fig. 2). Each of the two bands recognized by mAb P6FN was heavily glycosylated with an estimated 20-30 kDa of oligosaccharides (Fig. 2).

The domain organization of LeechCAM of five Ig- and two FNIII-domains (Fig. 1A) is similar to that of the NCAM subfamily of CAMs to which it has sequence homology in the range from 26-30%, which suggests it is evolutionarily related to these proteins (Huang et al., 1997). To further determine the relationship between LeechCAM and other CAMs we constructed phylogenetic trees based on maximum parsimony (Swofford, 1993). Figure 3 shows an unrooted consensus tree based on sequences from CAMs that had the highest sequence identity with LeechCAM in database searches. The phylogenetic analysis indicates that LeechCAM is grouped together in a monophyletic clade with 100% bootstrap support consisting of apCAM and Drosophila and grasshopper FasII. Thus, LeechCAM is likely to be the leech homolog of these proteins which have been shown to be directly involved in regulating growth and remodeling of synaptic connections in Drosophila (Schuster et al., 1996a; 1996b; Davis et al., 1997) as well as in Aplysia (Mayford et al., 1992; Zhu et al., 1994; 1995).

**LeechCAM is processed into both a secreted and a transmembrane form**

The labeling by mAb P6FN of two bands on immunoblots suggested that LeechCAM may be processed into a peripherally membrane attached as well as into an integral transmembrane form. To further test this hypothesis we conducted phase separation experiments of homogenized CNS proteins with Triton X-114. A homogeneous Triton X-114 solution at 0°C segregates into detergent and aqueous phases after the solution temperature is raised above 20°C. This phase separation can be used to distinguish loosley
associated membrane proteins, which will partition into the aqueous phase, from transmembrane proteins which will mainly partition into the detergent phase (Bordier 1981; Bajt et al., 1990). As shown on the immunoblots in Fig. 4 the lower band detected by mAb P6FN is found exclusively in the aqueous phase whereas the higher band partitions entirely into the detergent phase (Fig. 4). These results strongly indicate that the higher band represents a transmembrane version of LeechCAM whereas the lower band indicates a proteolytically cleaved and secreted form of LeechCAM.

**LeechCAM is glycosylated with the Laz2-369 glycoepitope**

We have previously shown that LeechCAM is differentially glycosylated by the Lan3-2 and Lan4-2 glycoepitopes in peripheral sensory neurons but not in central neurons (Huang et al., 1997). However, an additional glycoepitope recognized by the mAb Laz2-369 and found on 130 kD proteins was also a candidate to represent an additional glycomodification of the LeechCAM protein (Bajt et al., 1990; Song and Zipser, 1995b). LeechCAM is glycosylated with the Lan3-2 epitope in all peripheral sensory neurons (Jellies et al., 1994; Huang et al., 1997) and it has been estimated that a subset constituting about 45% of these neurons are also Laz2-369 positive (Zipser et al., 1994). In contrast to the Lan3-2 epitope, the antibody labeling of which is greatly reduced by mannose-BSA, the labeling of the Laz2-369 epitope is reduced by galactose-BSA and not by mannose-BSA (Song and Zipser, 1995b). Thus, the two glycoepitopes are likely to have different oligosaccharide compositions. We have recently shown that the Laz2-369 glycoepitope is also present on the secreted NH2-terminal fragment of the Ig-superfamily member Tractin in leech (Jie et al., 1999). To test whether the Laz2-369 epitope also is present on LeechCAM we immunoprecipitated leech CNS proteins with Laz2-369 antibody coupled to protein A-Sepharose beads. The Laz2-369 immunoprecipitate was then washed, boiled, separated by SDS-PAGE and immunoblotted.
Figure 5 (lane 1) shows that the Laz2-369 immunoprecipitate forms a broad protein band centered around 130 kD and that the LeechCAM specific mAb P6FN recognizes the top and bottom part of this band (Fig. 5; lane 2). In contrast, the Tractin specific mAb 4G5 recognizes the middle portion of the Laz2-369 immunoprecipitated protein band. This indicates that the Laz2-369 immunoprecipitate is made up of both Tractin and LeechCAM proteins and that the Laz2-369 glycoepitope is present on LeechCAM. Thus, while LeechCAM is present on all neurons of both the CNS and PNS only peripheral sensory neurons are glycosylated with the Lan3-2 glycoepitope and a subset of these peripheral sensory neurons in addition is glycosylated with the Laz2-369 glycoepitope as illustrated in the diagram in Fig. 6.

DISCUSSION

In this study we have used a monoclonal antibody to characterize the post-translational processing and glycosylation of the Ig-superfamily member LeechCAM. We show that the mAb P6FN on immunoblots recognizes two fragments of 138 and 120 kDa, respectively. The high band in phase partitioning experiments was found in the detergent phase whereas the low band was found in the aqueous phase. This suggest that there are two versions of LeechCAM: a transmembrane version and a secreted version consisting of the five Ig-domains and the two FNIII-domains, but without the transmembrane domain and the intracellular tail. In contrast to its mammalian homolog, NCAM, which is alternatively spliced from multiple transcripts (Cunningham et al., 1987), LeechCAM mRNA was identified as a single transcript on Northern blots (Huang et al., 1997) indicating that LeechCAM is likely to be post-translationally processed by proteolytic cleavage.
There is increasing evidence that secreted forms of integral membrane proteins including CAMs are released by selective post-translational proteolysis from the cell surface (Hooper et al., 1997; Blobel et al., 1997). The cleavage generally occurs close to the extracellular face of the membrane and is catalyzed by a group of enzymes collectively referred to as secretases or sheddases. The biological function of the proteolytic cleavage of transmembrane proteins are still not well characterized and may vary. In some cases it may be a process for rapidly down-regulating the protein from the cell surface, in others it may be to generate a soluble form of the protein that has different properties from the membrane bound form (Hooper et al., 1997). In some cases the processing may be necessary for biological activity. For example, in order to generate a functional Notch receptor in Drosophila the protein is cleaved by the disintegrin metallo-protease Kuzbanian to form a disulfide linked heterodimer (Pan and Rubin, 1997; Blaumueller et al., 1997). In leech, it has recently been shown that the Ig-superfamily member Tractin is proteolytically processed at two cleavage sites giving rise to a secreted NH2-terminal fragment in addition to a secreted homodimer and a transmembrane heterodimer constituted by the remaining fragments (Jie et al., 1999). Thus, proteolytic cleavage of both Tractin and LeechCAM may be a prerequisite for their proper function in axonal growth regulation.

The most NH2-terminal fragment of Tractin is glycosylated with the Lan3-2, Lan4-2, and Laz2-369 glycoepitopes (Jie et al., 1999). Here by performing immunoprecipitation experiments with the Laz2-369 antibody we show that this is also the case for LeechCAM. In vivo and in vitro antibody perturbation of some of these glycoepitopes have demonstrated that they can functionally assist in regulating axonal outgrowth. For example, perturbation with Lan3-2 antibody leads to an inhibition of filopodial extension, truncated fascicle formation, and a decrease in synaptogenesis (Zipser et al., 1989; Huang et al., 1997; Tai and Zipser, 1998). In contrast, perturbation with Laz2-369 antibody leads to enhanced neurite
and filopodial sprouting as well as an increase in synapse formation (Song and Zipser, 1995b; Tai and Zipser, 1999). Consequently, these findings suggest the interesting possibility that although Tractin and LeechCAM have different protein sequences that they may have partially overlapping functions due to their shared glycoepitopes.

It has long been recognized that the structural diversity of cell surface carbohydrates make them ideal candidates for mediating cell specific recognition processes (Roseman, 1971; Lis and Sharon, 1993; Dwek, 1995). That this is the case has been most clearly demonstrated by the process of lymphocyte homing, which is mediated by selectins that are capable of recognizing and binding ligands expressing specific oligosaccharide structures (Springer, 1994; Lasky, 1995). In the nervous system distinct carbohydrate epitopes such as that recognized by anti-HRP antibody in insects and the HNK-1/L2 epitope in vertebrates have been demonstrated to be widely expressed on glycoproteins (Jessell et al., 1990). In addition, several carbohydrate epitopes of more restricted expression and distribution have been identified. For example, in the vertebrate olfactory system, stage- and position-specific carbohydrate antigens were found to be topographic markers for selective projection patterns of olfactory axons (Key and Akeson, 1991; Schwarting et al., 1992). A striking example of how the developmental regulation of glycosylation can affect neural pathway formation is provided by the modulation of the polysialic acid content of NCAM in the plexus region of the chick limb bud where its up-regulation allows the axons to defasciculate into their proper pathways (Rutishauser and Landmesser, 1996). Thus, specific carbohydrate structures on neural proteins are promising candidates for assisting in patterning neural connections during development.
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REFERENCES


FIGURE LEGENDS

**Figure 1.**  
A. Diagram of the LeechCAM protein. The protein sequence is organized into five Ig-domains, two FNIII-domains, a transmembrane domain (TM), and a cytoplasmic domain. The putative proteolytic cleavage site between the second FNIII-domain and the transmembrane domain is indicated by an arrow. The mAb P6FN was made to a LeechCAM peptide sequence located in the second FNIII-domain as indicated by the black horizontal bar.  
B. Immunoblot of *Haemopis* nerve cord proteins labeled with mAb P6FN. The migration of molecular markers are indicated in kDa.  
C. mAb P6FN labels all central and peripheral neurons and their projections in a *Hirudo* E12 embryo. Two ganglia (g) as well as the four main nerve tracks (arrowheads) are indicated. Scale bar: 100 μm.

**Figure 2.** LeechCAM is N-glycosylated. Extracts of *Haemopis* nerve cord proteins were digested with N-Glycosidase F (deglycosylation) or mock digested (control), separated by SDS-PAGE, and immunoblotted. N-glycosidase F treatment of both LeechCAM fragments as detected by mAb P6FN leads to faster gel migration. The migration of 200, 116, 97, and 66 kD markers is indicated.

**Figure 3.** Phylogenetic relationship of LeechCAM with other CAMs. Consensus maximum parsimony tree derived from an alignment with all the gaps removed of LeechCAM and members from the most closely related CAMs. The tree is unrooted. The bootstrap 50% majority rule consensus of 1000 maximum parsimony trees is depicted with associated bootstrap support values.
**Figure 4.** Triton-X-114 phase separation of the proteolytically cleaved LeechCAM fragment. The lower band partitions exclusively to the aqueous phase (lane 3) whereas the higher band is found entirely in the detergent phase (lane 2). The control lane (lane 1) shows an immunoblot of the extracted *Haemopis* nerve cord proteins before phase separation. The migration of molecular markers are indicated in kDa.

**Figure 5.** LeechCAM is glycosylated with the Laz2-369 glycoepitope. Immunoblots of Laz2-369 immunoprecipitated *Haemopis* nerve cord proteins. The Laz2-369 immunoprecipitate (Laz2-369 ip) was recognized by both mAb Laz2-369, mAb P6FN, and the NH$_2$-terminal fragment of Tractin specific mAb 4G5. The position of the 130 kD molecular weight marker is indicated.

**Figure 6.** LeechCAM is differentially glycosylated with the Lan3-2 and Laz2-369 glycoepitopes. LeechCAM is expressed by and present on all neurons in the CNS and PNS (A). However, in a subset of these neurons, the peripheral sensory neurons, LeechCAM is differentially glycosylated with the Lan3-2 glycoepitope (B). In addition, some of the peripheral sensory neurons are glycosylated with the Laz2-369 glycoepitope (C). In this model the Lan3-2 and Laz2-369 glycoepitopes are depicted at different glycosylation sites since proteolytic digestion has shown that the Lan3-2 and Laz2-369 antibodies recognize different peptide fragments (Bajt et al., 1990).
Fig. 5

Fig. 6
GENERAL CONCLUSIONS

Some novel characteristics of Tractin and LeechCAM are presented in this dissertation. Since it has been demonstrated that many important protein motifs and features have been functionally conserved throughout evolution, these characteristics may suggest some potential functional significance of Tractin and LeechCAM in axonal outgrowth.

Both Tractin and LeechCAM were shown to undergo post-translational cleavage. Our data suggest that Tractin is first cleaved at site I in the third FN-llI domain, which gives rise to an N-terminal secreted polypeptide and a C-terminal long transmembrane fragment. Half of the C-terminal long transmembrane fragment is apparently further cleaved at site II, which may be similar to the cleavage of collagen XVII and the removal of the C-propeptide of pro-α1(V) collagen (Imamura et al., 1998; Schäcke et al., 1998). For LeechCAM, it is suggested to exist in two versions due to post-translational processing: a transmembrane version and a secreted version consisting of the five Ig-domains and the two FN-III domains, but without the transmembrane domain and the intracellular tail. The multiple cleavage products of Tractin and LeechCAM may play diverse functional roles in axonal pathway formation of the sensillar neurons. This would suggest an efficient model for how the complex information needed to specify precise and complicated patterns of neuronal innervation may arise given a limited gene pool, namely, the processed products of a precursor protein encoded by a single gene play different but coordinated functional roles in pathway formation.

An interesting phenomenon is that both Tractin and LeechCAM are shown to possess the Lan3-2, Lan4-2 and Laz2-369 glycoepitopes when expressed in the sensillar neurons. Taking into account the molecular features of these two proteins, one hypothesis would be that Tractin and LeechCAM may interact. Sequence alignment shows that LeechCAM is
homologous to the NCAM subfamily while the Ig domains of Tractin have homology to L1 subfamily. The Lan3-2 glycoepitope has been shown to contain the mannoside sugar (Bajt et al., 1990). It has been suggested that the oligo-mannosidic carbohydrates expressed on L1 determine the interaction between NCAM and L1, and modulate neurite outgrowth (Horstkorte et al., 1993). Thus, it is possible that a similar interaction mechanism to that between L1 and NCAM may exist for Tractin and LeechCAM. An alternative explanation to the phenomenon would be that Tractin and LeechCAM have some overlapping function. But the physiological significance of this alternative needs to be further examined. In the context of the first hypothesis, the interaction between Tractin and LeechCAM potentially induce interaction between the cytoskeleton and the cytoplasmic domains of integrins. Tractin contains the tripeptide of R-G-D, which features the ligands of certain integrins. Integrins are capable of linking the proteins on the extracellular face of the cell membrane to the intracellular responses. Certain integrin ligands can cross-link integrins by binding to adjacent integrin molecules on the cell surface. Both the ligand occupancy and the cross-linking of integrins are critical to the activation of intracellular integrin-mediated responses (Clark and Brugge, 1995). The cytoplasmic domains of integrins are believed to interact with cytoskeleton proteins and other signaling molecules (Horwitz et al., 1986; Tapley et al., 1989; Shattil and Brugge, 1991; Clark and Brugge, 1995). Interestingly, a recent study suggests that the R-G-D motif in L1 promotes neurite outgrowth via interaction with the α5β3 integrin (Yip et al., 1998). Therefore, the potential interaction between Tractin and LeechCAM may trigger the association of Tractin with some integrin(s), which may subsequently cause some cytoskeletal changes in axonal outgrowth.

Our data show that the cytoplasmic domain of Tractin can interact with Drosophila ankyrin in the yeast two-hybrid interaction assay, suggesting that Tractin’s ankyrin binding motif is functionally conserved. This motif potentially interacts with the membrane-
associated cytoskeleton network, which is attached to the plasma membrane through the protein ankyrin (Bennett and Gilligan, 1993). Ankyrin, in turn, interacts with the cytoplasmic domains of several membrane integral proteins, including Ig molecules such as the Neurofascin/L1/NrCAM subfamily (Davis et al., 1994). A detailed biochemical study showed that L1 and NrCAM associate with the membrane-binding domain of ankyrin through a highly conserved ankyrin-binding motif contained within their cytoplasmic domains (Davis et al., 1994). The functional significance of this kind of association was addressed by a study with neuroglian, a *Drosophila* homolog of L1 and Neurofascin. It was shown that the neuroglian-mediated cell adhesion induces assembly of the membrane-associated skeleton at cell contact sites and that the ankyrin-binding motif of neuroglian is indispensable for this process (Dubreuil et al., 1996). Therefore, it is imaginable that Tractin may induce membrane-associated skeleton changes of the sensillar neurons through its ankyrin binding motif during axonal outgrowth.

The PG/YG repeating unit of the proline- and glycine rich region in Tractin can be alternatively read as G-X1-X2, which is reminiscent of the characteristic sequence of collagens. Our experimental data show that this region is sensitive to collagenase treatment, suggesting that it may possess collagen-like structure and properties. As discussed before, collagens have been shown to be involved in assembling extracellular matrix (ECM) and activating signal transduction events (Vogel et al., 1997). Accordingly, it is possible that Tractin interacts with other ECM proteins and/or membrane integral molecules via the PG/YG region. For example, integrins have been shown to bind collagen I and IV in vitro (Calderwood et al., 1997; Kern et al., 1998). Thus, the PG/YG repeating region of Tractin may be a ligand for integrins, which potentially interact with the cytoskeleton upon activation.
Other features of Tractin and LeechCAM may also contribute additional functions in nerve pathway formation. For example, at the extreme carboxyl end of Tractin, there exists an XS/TXV motif, which is suggested to interact with the PDZ domains of the PSD-95/Dlg/ZO-1 family (Doyle et al., 1996; Zito et al., 1997). A physiological function of the PDZ domains has been proposed to be membrane protein localization and clustering, e.g. that of FasII and Shaker-type K⁺ channel. Moreover, the PDZ domains of one protein can form specific homophilic associations with the PDZ domains of another protein (Brenman et al., 1996). Thus, it is possible that Tractin is localized to specific regions of the sensillar neurons through the interaction between its XS/TXV motif and the PDZ domains of members of the PSD95/Dlg/ZO-1 family. The PG/YG repeats of Tractin, which potentially interact with the extracellular matrix molecules, and this potential specialization in localization, may work together to stabilize the established projections of the sensillar neurons.

The various features of Tractin and LeechCAM provide opportunities to interact with ECM proteins, membrane integral molecules and/or the cytoskeleton directly or indirectly. During these possible processes, the different domains/motifs and cleavage products of Tractin and/or LeechCAM may act in concert to transduce various molecular signals readily across the membrane in a two way fashion. As a result, the physiological consequences may be involved in regulating the pathway formation of the sensillar neurons. Further studies will provide insights into the molecular mechanisms of how Tractin and LeechCAM may be involved in nerve pathway formation.
LITERATURE CITED


Ishiko A, Shimizu H, Kikuchi A, Ebihara T, Hashimoto T and Nishikawa T (1993) Human autoantibodies against the 230-kD bullous pemphigoid antigen (BPAG1) bind only to the intracellular domain of the hemidesmosome, whereas those against the 180-kD bullous pemphigoid antigen (BPAG2) bind along the plasma membrane of the hemidesmosome in normal human and swine skin. J Clin Invest 91(4):1608-15


Kayyem JF, Rman JM, de la Rosa EJ, Schwarz U, and Dreyer WJ (1992) Bravo/Nr-CAM is closely related to the cell adhesion molecules L1 and Ng-CAM and has a similar heterodimer structure. J Cell Biol 118(5): 1259-1270


Schuster CM, Davis GW, Fetter RF and Goodman CS (1996b) Genetic dissection of structural and functional components of synaptic plasticity: fasciclin II controls
structural plasticity. Neuron 17:655-667

Schwarting GA, Deutsch G, Gatley DM and Crandall JE (1992) Glycoconjugates are
stage- and position-specific cell surface molecules in the developing olfactory
system. II. Unique carbohydrate antigens are topographic markets for

Shattil, SJ and Brugge JS (1991) Integrins as signaling molecules and targets for tumor

protein tyrosine kinase receptor required for photoreceptor development in
Drosophila. Proc Natl Acad Sci 86, 8333-8337

Song J and Zipser B (1995a) Kinetics of the inhibition of axonal defasciculation and
arborization mediated by carbohydrate markers in the embryonic leech. Dev Biol
168:319-331.

Song J and Zipser B (1995b) Targeting of neuronal subsets by their sequentially expressed
carbohydrate markers. Neuron 14:537-547

Springer TA (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration:
the multistep paradigm. Cell 76:301-314

adhesion molecule, apCAM, mediates growth cone steering by substrate-

Tang J, Landmesser L, Rutishauser U (1992) Polysialic acid influences specific
pathfinding by avian motoneurons. Neuron 8(6): 1031-44

Tang J, Rutishauser U and Landmesser L (1994) Polysialic acid regulates growth cone
behavior during sorting of motor axons in the plexus region. Neuron 13:405-414

Tapley P, Horwitz AF, Buck, CA, Burridge K, Duggan K, Hirst R, and Rohes Schneider


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