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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 in addition to existing in a transmembrane version LeechCAM is likely to be proteolytically cleaved into a secreted form without the transmembrane domain and the intracellular tail. Furthermore, by immunoprecipitation we demonstrate that LeechCAM is glycosylated with the Laz2-369 glycoepitope, an epitope that has been specifically implicated in regulation of axonal outgrowth and synapse formation.

Keywords: LeechCAM; Differential glycosylation; Proteolytic processing; Neuron; (Leech)

1. 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 [1]. They have also been implicated in higher order processes such as synaptic plasticity underlying learning and memory [2,3]. 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 [4]. 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 [5,6].

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 [7]. LeechCAM is one of two different members of the Ig-superfamily in leech, the other being Tractin, which were identified based

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on their expression of the Lan3-2 glycoepitope [7]. 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 [7,8]. 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 [9–12]. 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 [7,13–15]. 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 [16,17]. 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.

2. Materials and methods

2.1. 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 $CaCl_2$, 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.

2.2. Antibodies and immunocytochemistry

Three previously reported mAbs, P6FN (IgG_1), 4G5 (IgG_1), and Laz2-369 (IgG_3) [7,18] 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 H_2O_2 (0.01%) for 10 min. The final preparations were dehydrated in alcohol, cleared in xylene, and embedded as whole mounts in Depex mountant. 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.

2.3. Biochemical analysis

2.3.1. SDS–PAGE and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to standard procedures [19]. Electroblot transfer was performed as in Towbin et al. [20] with 1× 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 H_2O_2 (0.03%) and enhanced with 0.008% $NiCl_2$. The im-

munoblots were digitized using the NIH-image software, a cooled high-resolution CCD-camera (Paultek), and a PixelBuffer framegrabber (Perceptics) or an Arcus II scanner (AGFA).

2.3.2. Immunoprecipitation

Immunoprecipitations with Laz2-369 antibody were performed at 4°C. Dissected *Haemopis* leech nerve cords were homogenized in extraction buffer (20 mM Tris–HCl, 200 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% NP-40, 0.2% Triton X-100 (pH 7.4), containing 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 h. 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 s at 2000 rpm the supernatant was discarded and the immunoaffinity matrix resuspended and washed three 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.

2.3.3. 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 $CaCl_2$, 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 2×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 +2×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 h 45 min at 37°C with shaking, and then processed for SDS–PAGE and immunoblotting analysis. The other half of the mixture (glycoprotein fraction +2×reaction buffer) was treated identically as a negative control, except enzyme was replaced by H_2O. For deglycosylation without sample denaturation by SDS/β-mercaptoethanol, half of the mixture (the eluted glycoprotein fraction+2×reaction buffer) was digested by glycosidase enzyme for 24 h at 37°C with shaking, and then processed for SDS–PAGE and immunoblot analysis. The other half of the mixture was treated identically as a negative control, except enzyme was replaced by H_2O. The protease inhibitors PMSF and Aprotinin were maintained in the buffers throughout the whole process.

2.3.4. Triton X-114 phase separation

The phase separation of proteins was performed according to Bordier [21] and Bajt et al. [11]. *Haemopis* nerve cords were homogenized in TBS (150 mM NaCl, 10 mM 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% (w/v) 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 pipetting back and forth occasionally. The 200-μl samples with dissolved Triton X-114 were overlaid back to the lower detergent phase obtained from the previous

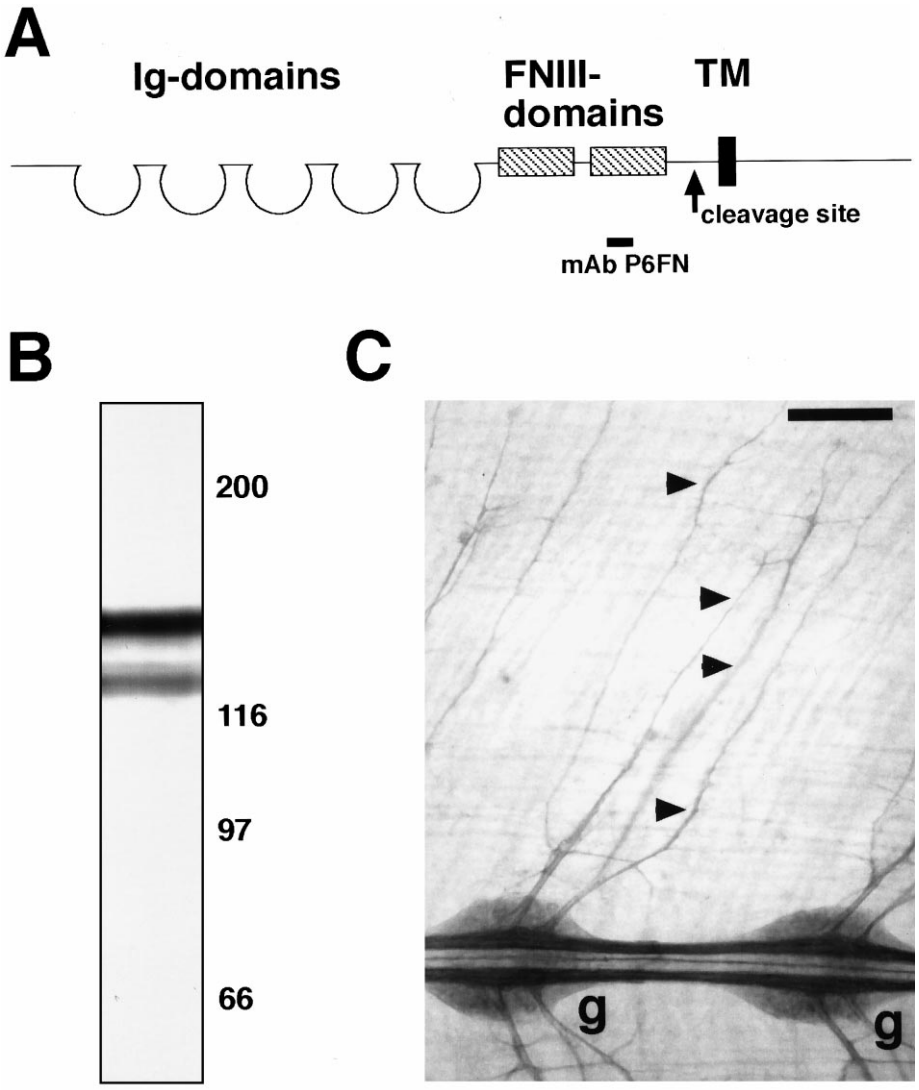

Fig. 1. (A) Diagram of the LeechCAM protein. The protein sequence is organized into five Ig-domains, two FNIII-domains, a transmembrane domain (TM), and a cytoplasmic domain. The putative proteolytic cleavage site between the second FNIII-domain and the transmembrane domain is indicated by an arrow. 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 mass markers is 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.

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, ethanol was added to the aqueous phase to precipitate proteins with the final concentration of ethanol to be 66% [22]. 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.

2.4. 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 [23] 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 majority rule consensus tree.

3. Results

3.1. LeechCAM is a glycosylated homolog of apCAM and FasII

Fig. 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 [7]. 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).

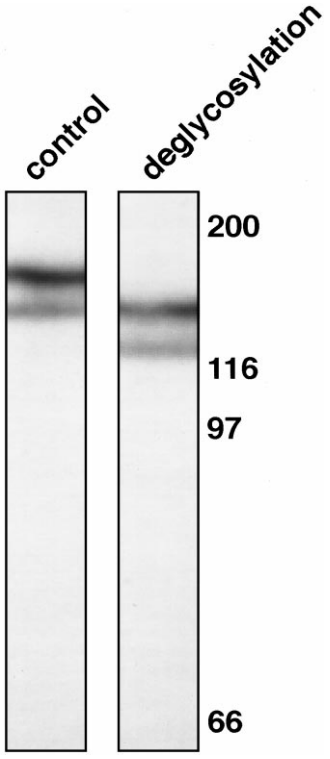

Fig. 2. LeechCAM is *N*-glycosylated. Extracts of *Haemopis* nerve cord proteins were digested with *N*-glycosidase F (deglycosylation) or mock digested (control), separated by SDS–PAGE, and immunoblotted. *N*-Glycosidase F treatment of both LeechCAM fragments as detected by mAb P6FN leads to faster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

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% to 30%, which suggests it is evolutionarily related to these proteins [7]. To further determine the relationship between LeechCAM and other CAMs we con-

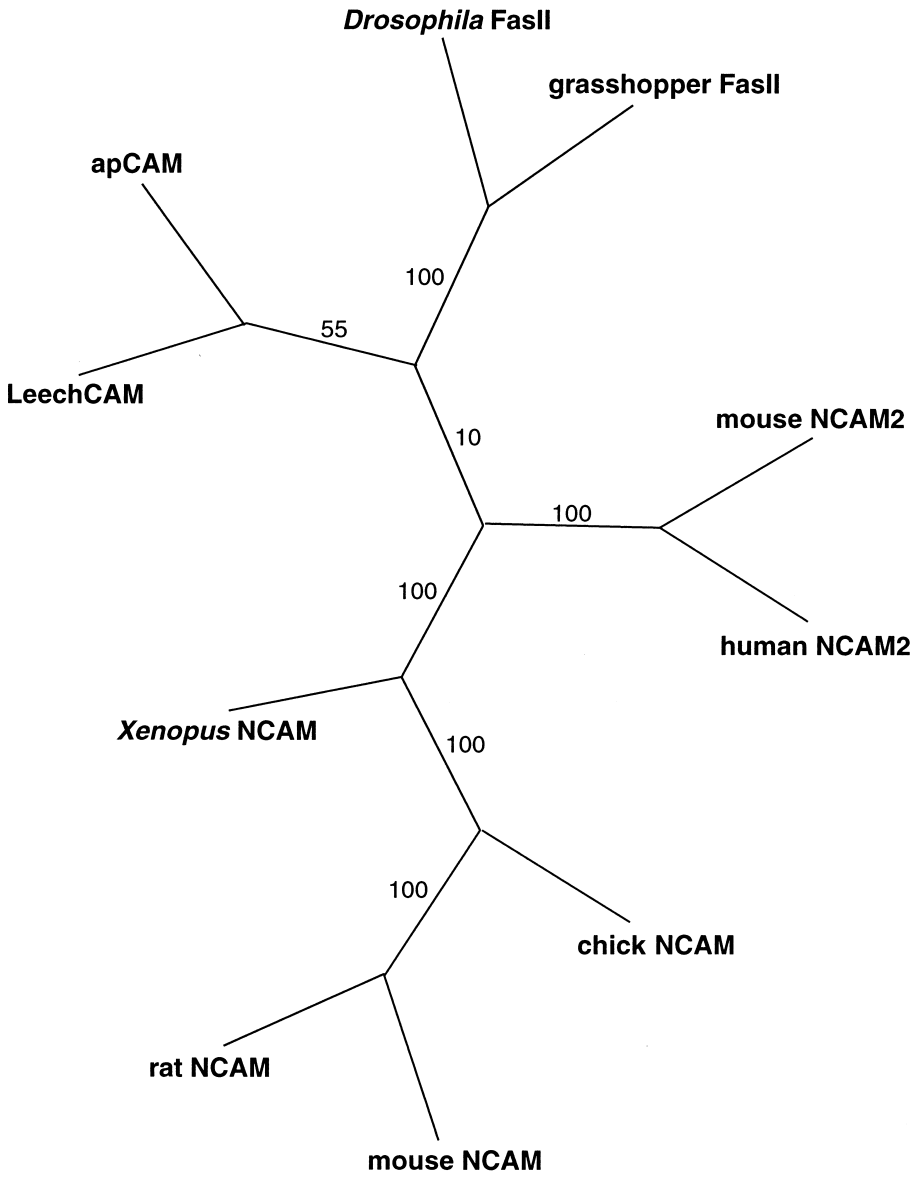

Fig. 3. Phylogenetic relationship of LeechCAM with other CAMs. Consensus maximum parsimony tree derived from an alignment with all the gaps removed of LeechCAM and members from the most closely related CAMs. The tree is unrooted. The bootstrap 50% majority rule consensus of 1000 maximum parsimony trees is depicted with associated bootstrap support values.

structed phylogenetic trees based on maximum parsimony [23]. Fig. 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* [24–26] as well as in *Aplysia* [27–29].

3.2. *LeechCAM is processed into both secreted and transmembrane forms*

The labeling by mAb P6FN of two bands on im-

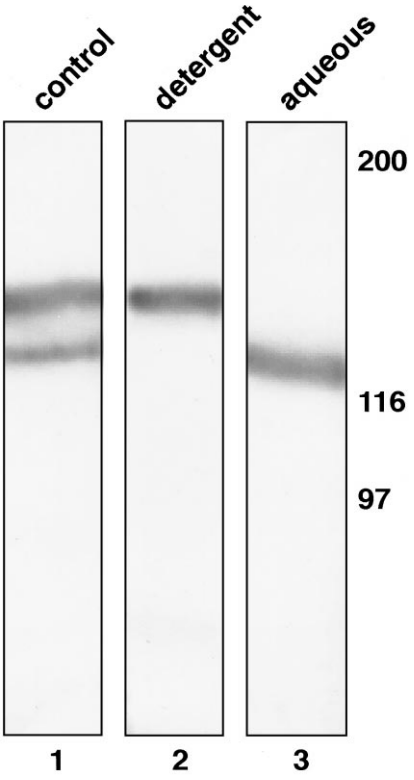

Fig. 4. Triton X-114 phase separation of the proteolytically cleaved LeechCAM fragment. The lower band partitions exclusively to the aqueous phase (lane 3) whereas the higher band is found entirely in the detergent phase (lane 2). The control lane (lane 1) shows an immunoblot of the extracted *Haemopis* nerve cord proteins before phase separation. The migration of molecular mass markers is indicated in kDa.

munoblots 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 loosely associated membrane proteins, which will partition into the aqueous phase, from transmembrane proteins which will mainly partition into the detergent phase [11,21]. As shown on the immunoblots in Fig. 4, the low band as detected by mAb P6FN is found exclusively in the aqueous phase whereas the high band partitions entirely into the detergent phase (Fig. 4). These results strongly indicate that the high band represents a transmembrane version of LeechCAM whereas the low band indicates a proteolytically cleaved and secreted form of LeechCAM.

3.3. *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 [7]. However, an additional glycoepitope recognized by the mAb Laz2-369 and found on 130 kDa proteins was also a candidate to represent an additional glycomodification of the LeechCAM protein [11,16]. LeechCAM is glycosylated with the Lan3-2 epitope in all peripheral sensory

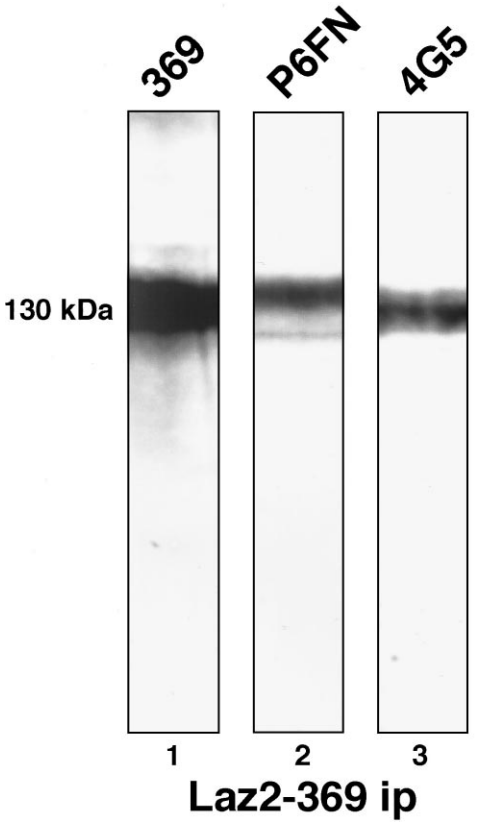

Fig. 5. LeechCAM is glycosylated with the Laz2-369 glycoepitope. Immunoblots of Laz2-369 immunoprecipitated *Haemopis* nerve cord proteins. The Laz2-369 immunoprecipitate (Laz2-369 ip) was recognized by both mAb Laz2-369, mAb P6FN, and the NH_2-terminal fragment of Tractin-specific mAb 4G5. The position of the 130 kDa molecular mass marker is indicated.

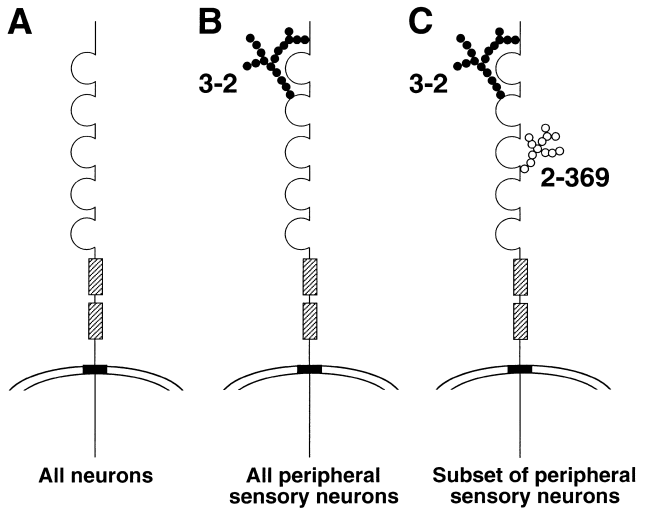

Fig. 6. LeechCAM is differentially glycosylated with the Lan3-2 and Laz2-369 glycoepitopes. LeechCAM is expressed by and is present on all neurons in the CNS and PNS (A). However, in a subset of these neurons, the peripheral sensory neurons, LeechCAM is differentially glycosylated with the Lan3-2 glycoepitope (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 glycopeptide fragments [11].

neurons [7,30] and it has been estimated that a subset constituting about 45% of these neurons are also Laz2-369 positive [12]. 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 [16]. 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 NH_2-terminal fragment of the Ig-superfamily member Tractin in leech [31]. 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. Fig. 5 (lane 1) shows that the Laz2-369 immunoprecipitate forms a broad protein band centered around 130 kDa 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.

4. 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 suggests 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 [32], LeechCAM mRNA was identified as a single transcript on Northern blots [7], 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 [6,33]. 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 properties somewhat different from those of the membrane bound form [33]. 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 [34,35]. 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 NH_2-terminal fragment in addition to a secreted homodimer and a transmembrane heterodimer constituted by the remaining fragments [31]. Thus, proteolytic cleavage of both Tractin and LeechCAM may be a prerequisite for their proper function in axonal growth regulation.

The most NH_2-terminal fragment of Tractin is glycosylated with the Lan3-2, Lan4-2, and Laz2-369 glycoepitopes [31] and 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 [7,13,15]. In contrast, perturbation with Laz2-369 antibody leads to enhanced neurite and filopodial sprouting as well as an increase in synapse formation [16,17]. Consequently, these findings suggest the interesting possibility that although Tractin and LeechCAM have different protein sequences, 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 [36–38]. 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 [39,40]. 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 [41]. 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 [42,43]. 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 [44]. Thus, specific carbohydrate structures on neural proteins are promising candidates for assisting in patterning neural connections during development.

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