Spatial and Temporal Regulation of Tenascin-R Glycosylation in the Cerebellum*

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The cellular adhesion molecule tenascin-R is a multifunctional extracellular matrix component expressed exclusively in the central nervous system. The expression of tenascin-R by oligodendrocytes and small interneurons in the hippocampus and cerebellum is highly regulated during development of these regions. This complex glycoprotein displays both adhesive and anti-adhesive properties that contribute to the formation and maintenance of synapses. We have determined that tenascin-R associated with Purkinje cell bodies and their dendrites in the molecular layer of the cerebellum bears N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-SO₄, whereas tenasin-R in other regions of the cerebellum does not bear this modification. Expression of this unique sulfated carbohydrate structure is also temporally regulated, increasing throughout cerebellar development. The most dramatic increase in GalNAc-4-SO₄ occurs between postnatal days 14 and 21, corresponding to a period of Purkinje cell dendrite extension and synaptogenesis. The spatially and temporally regulated addition of this unique sulfated carbohydrate to tenasin-R may serve to modulate its adhesive/anti-adhesive or other biological properties in vivo.

The central nervous system is both the most complex and dynamic biological structure in vertebrates. Formation and maintenance of the vast array of synapses and other structural features that is required for neurological function depends on specific interactions between extracellular matrix (ECM) and cell membrane components. Carbohydrates in the form of glycoproteins, proteoglycans, and glycolipids play a critical role in a number of these interactions by enhancing either the adhesive or anti-adhesive properties of cellular adhesion molecules (CAMs), often through interactions with carbohydrate-specific binding proteins or receptors.

Several lines of evidence suggest that addition of unique N-linked carbohydrates to CAMs in the nervous system is essential for their in vivo function. For example, the addition of α2,8-linked polysialic acid (PSA) to the N-linked oligosaccharides on the neural adhesion molecule NCAM reduces its adhesive properties and promotes neuronal migration, neurite outgrowth, and dendritic arborization during development and regeneration following injury (1, 2). Another unique carbohydrate epitope, HNK-1 (SO₄-3-GlcUAβ1,3Galβ1,4GlcNAc) is found on glycolipids as well as on a number of CAMs, including NCAM, L1, myelin-associated glycoprotein, tenascin-R (TN-R), and tenasin-C. HNK-1 is thought to play a role in modulation of neurite outgrowth, adhesion between neurons and glial cells and/or the ECM, and synaptic plasticity (1, 3–6). HNK-1 may fulfill these roles by binding to specific proteins and receptors such as SBF-1 in the cerebellum (7).

The addition of PSA and HNK-1 to CAMs in the brain is both temporally and spatially regulated (1, 8). As a result, only a fraction of any CAM is modified with either of these carbohydrate structures, indicating the synthesis of these glycoproteins and the carbohydrate structures that modify them are regulated independently. Thus, the extent to which a CAM is modified with different carbohydrate structures likely reflects its functional role in vivo. Structural and behavioral changes that are observed when glycosyltransferases responsible for the synthesis of PSA (9) and HNK-1 (10) are ablated in mice suggest that the resulting deficits in carbohydrate modifications alter synaptic plasticity and disrupt modulation of complex neuronal networks.

The specific interactions required for the formation and maintenance of synapses and other structures in the nervous system make it likely that the synthesis of the unique carbohydrate structures that contribute to these interactions is highly regulated. These unique carbohydrate structures are added to a select group of key recognition molecules, indicating that their addition is protein-specific. For example, the limited number of glycoproteins in the nervous system that bear either PSA or HNK-1 indicates that the glycosyltransferases involved in their synthesis are protein-specific (1, 11). We previously identified and characterized a protein-specific β1,4-N-acetylgalactosaminyltransferase (β1,4GalNAcT) that recognizes a peptide determinant in the α- and β-subunits of the glycoprotein hormone lutropin (LH) (12–14). This enzyme in the pituitary accounts for the protein-specific addition of β1,4-linked GalNAc to N-linked oligosaccharides on LH and other glycoproteins (14). The β1,4-linked GalNAc is subsequently modified with SO₄ by a GalNAc-4-sulfotransferase (GalNAc-4-ST1) (14, 15) to produce the unique terminal sequence SO₄-4-GalNAcβ1,4GlcNAc. The terminal GalNAc-4-SO₄ on LH is critical for embryo implantation (16), because it is recognized by a receptor expressed in hepatic endothelial cells, the Man/GalNAc-4-SO₄-receptor, that determines LH circulatory half-life following release from the pituitary into the blood (17–19). In addition to pituitary, we have observed high levels of GalNAc-4-ST1 mRNA and activity in the cerebellum and other regions of the brain (1, 5).

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regions of the brain (15, 20). The presence of GalNAc-4-ST1 as well as protein-specific β1,4GalNAcT activity (20) in cerebellum indicates that specific glycoproteins in this region of the brain are modified with terminal GalNAc-4-SO4. We report here that terminal GalNAc-4-SO4 is present on N-linked oligosaccharides of cerebellar TN-R, an extracellular matrix CAM. TN-R that is modified with GalNAc-4-SO4 is specifically associated with Purkinje cell bodies and dendrites. In addition, we found that the expression of these structures increases dramatically during the synaptic phase of cerebellar development. The temporal and spatial regulation of expression of this unique carbohydrate structure strongly supports the possibility that terminal GalNAc-4-SO4 on TN-R is important for cerebellar development and function in vivo.

MATERIALS AND METHODS

Preparation of the Cys-Fc Chimera—The chimeric protein Cys-Fc consisting of the cysteine-rich domain of the Man/GalNAc-4-SO4-receptor and the Fc domain of human IgG1 was obtained from CHO-Tag 30A cells that were selected for stable expression of Fc-Fc following transfection with pG1-Man54GnM(Nu11) (18). CHO-Tag 30A cells were adapted to low serum/low protein media, Ultra CHO (BioWhittaker), and propagated in a Cell Max artificial capillary system (Spectrum Labs). Cys-Fc was purified from Ultra CHO media by incubation with Protein A-Sepharose and eluted with 100 mM glycine, pH 3.0. The eluted protein was immediately neutralized with Tris, pH 7.8, and dialyzed against 20 mM NaPO4, 150 mM NaCl, pH 7.4. Biotinylated Cys-Fc was prepared by adding 12% (v/v) aminohexanoylbiotin-N-hydroxysuccinimide ester (2.5 mg/ml in Me2SO) to 100 μg of Cys-Fc (1 mg/ml) in 100 mM sodium carbonate, pH 8.4, and incubated for 4–24 h at 4°C. Free biotin was removed by gel filtration on Sephadex G-25 in 20 mM Tris-HCl, pH 7.5.

Immunohistochemistry—Rat cerebella were removed and immediately postfixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1 h at 4°C. Sagittal 10-μm sections were cut at −20°C with a cryostat. Frozen sections were fixed in methanol for 6 min at −20°C. Sections were incubated in 5% goat serum in TNP (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% casein) for 20 min at 25°C to inhibit nonspecific binding. Cys-Fc biotin, 5–10 ng/ml, in TNP was incubated with sections for 4–16 h at 4°C. Slides were washed with TNT (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and then incubated with Cy3-conjugated Streptavidin (Jackson Labs) for 1 h at 4°C. Sections were washed, and nuclei were counterstained with Hoechst 33258 at 10 μg/ml. For inhibition studies, Cys-Fc biotin was incubated with 500 μM GalNAc-4-SO4 or GalNAc-6-SO4 for 15 min prior to staining sections.

Enzymatic Removal of N-linked Oligosaccharides—Protein in the RCS fraction were denatured by boiling in 0.1% SDS, 50 mM β-mercaptoethanol. Samples were cooled, adjusted to 0.35% Nonidet P-40, 50 mM NaPO4 buffer, pH 8.6, 10 mM EDTA. The denatured proteins were then treated with 1–2 units of PNGase-F (Glyko) or incubated in buffer alone for 4–20 h at 37°C. LDS sample buffer was then added, and samples were analyzed by SDS-PAGE as described above.

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RESULTS

Cys-Fc-reactive Saccharides Are Associated with Purkinje Cells in Developing and Adult Cerebella—Mouse and rat cerebellum express significant levels of a protein-specific GalNAc-transferase and a GalNAc-4-sulfotransferase (GalNAc-4-ST1) (15, 20), both of which are required to synthesize N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-SO4 (14). We used the Cys-Fc chimera, which binds terminal GalNAc-4-SO4, to probe rat and mouse brains for the presence of glycoproteins bearing terminal GalNAc-4-SO4.

The Purkinje cell and molecular layers of adult rat cerebellum are intensely stained by biotinylated Cys-Fc followed by Cy3-Streptavidin (Fig. 1A). No staining was observed when sections were incubated with biotinylated human IgG1 (not shown). Staining with Cys-Fc was inhibited by incubation with 500 μM GalNAc-4-SO4 (Fig. 1B) but not by incubation with 500 μM GalNAc-4-SO4.
μm GalNAc-6-SO₄ (Fig. 1C), consistent with the specificity of the Cys-rich domain of the Man/GalNAc-4-SO₄-receptor for GalNAc-4-SO₄ (18, 21). A GalNAc-4-SO₄-specific antibody, monoclonal antibody 6.3 (17) also stained the Purkinje cell and molecular layers (not shown). Taken together with the presence of both the protein-specific GalNAc-transferase and GalNAc-4-ST1 in the cerebellum, this suggested that one or more of the GalNAc-transferase makes it likely that only a limited number of glycoproteins in the cerebellum are selectively modified with terminal β1,4-linked GalNAc-4-SO₄. Identification of the glycoproteins bearing terminal GalNAc-4-SO₄ may potentially provide insight into the function of this unique modification in the cerebellum. We therefore established conditions for the isolation and characterization of Cys-Fc-reactive glycoproteins as summarized in Table I. The amount of material present in the different fractions obtained following solubilization of the cerebellum was estimated by monitoring ligand binding to immobilized Cys-Fc using surface plasmon resonance.

The major fraction of Cys-Fc-reactive material, roughly 60% of the total, was present in the RCS fraction (Table I, Fraction 2). Additional washing steps did not release significant additional protein (Table I, Fraction 4). The RCM fraction ac-

![Fig. 1. Cys-Fc-reactive material is associated with the cell bodies and dendrites of Purkinje cells in the cerebellum of the adult and developing rat. Cryostat sections of rat cerebella were incubated with biotinylated Cys-Fc in the presence or absence of 500 μm GalNAc-4-SO₄ or GalNAc-6-SO₄. Bound Cys-Fc was visualized using Cy3-streptavidin. Nuclei were stained with Hoescht dye #33258. A, adult cerebellum stained with Cys-Fc. B, adult cerebellum stained with Cys-Fc in the presence of 500 μm GalNAc-4-SO₄. C, adult cerebellum stained with Cys-Fc in the presence of 500 μm GalNAc-6-SO₄. D, adult cerebellum stained with Cys-Fc. E, adult cerebellum stained with Cys-Fc; F, adult cerebellum stained with Cys-Fc and anti-Cys-Fc monoclonal antibody 6.3. G, regions of the cerebellum are indicated as: molecular layer (M), Purkinje cell layer (P), granular layer (G), external granular layer (EGL), and internal granular layer (IGL).](image)

![Fig. 2. Analysis of affinity-purified Cys-Fc-reactive proteins from rat cerebellum. Proteins obtained during the purification described in Table I were analyzed by SDS-PAGE and visualized by silver staining (A) or by ligand blotting with the biotinylated Cys-Fc chimera (B). A: lane 1, 0.1% of the soluble fraction following dialysis (RCSD in Table I); lane 2, 0.1% of the rat cerebellar proteins not bound to Cys-Fc-agarose; lanes 3–5, 1% of three successive washes from the affinity column with TBS-TX; lanes 6–7, 1% of fractions eluted from the Cys-Fc affinity column with 500 μm GalNAc-4-SO₄; lanes 8–9, 1% of fractions eluted from the Cys-Fc affinity column with 500 μm GalNAc-4-SO₄ and 1 M NaCl. Arrows indicate the major protein band with 160-kDa molecular mass that was visualized by silver staining and excised for analysis by mass spectrometry following tryptic digestion.](image)

### Table I

**Distribution and purification of Cys-Fc-reactive glycoproteins from rat cerebellum**

The relative amount of glycoprotein(s) bearing oligosaccharides with terminal β1,4-linked GalNAc-4-SO₄ was determined by monitoring the amount of glycoprotein binding to the immobilized Cys-Fc chimeric protein using surface plasmon resonance (RU). Because glycoproteins with multiple GalNAc-4-SO₄ termini dissociate at a negligible rate, the rate of increase in RU values with respect to time, ΔRU, was used to estimate the amount of ligand as described under “Materials and Methods.” Values represent the mean of three independent purifications. ND, not determined.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Volume</th>
<th>ΔRU/μl</th>
<th>Cys-Fc binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2. Soluble and PM proteins (RCS)</td>
<td>29.55 μg/ml</td>
<td>53,000 μl</td>
<td>18.12</td>
<td>924 × 10⁶</td>
</tr>
<tr>
<td>3. RCS-dialysed (RCSD)</td>
<td>11.53 μg/ml</td>
<td>50,000 μl</td>
<td>16.04</td>
<td>802 × 10⁵</td>
</tr>
<tr>
<td>3a. Cys-Fc-agarose unbound</td>
<td>4.01 μg/ml</td>
<td>50,000 μl</td>
<td>5.70</td>
<td>285 × 10⁵</td>
</tr>
<tr>
<td>3b. Cys-Fc-agarose bound</td>
<td>0.47 μg/ml</td>
<td>7,100 μl</td>
<td>24.04</td>
<td>170 × 10⁵</td>
</tr>
<tr>
<td>4. Salt wash (RCW)</td>
<td>8.96 μg/ml</td>
<td>45,000 μl</td>
<td>2.14</td>
<td>96 × 10⁵</td>
</tr>
<tr>
<td>5. IM proteins (RCM)</td>
<td>33.70 μg/ml</td>
<td>35,000 μl</td>
<td>13.00</td>
<td>455 × 10⁵</td>
</tr>
</tbody>
</table>

*PM, peripheral membrane; IM, Triton X-100 solubilized integral membrane.*
Regulation of Tenascin-R Glycosylation in the Cerebellum

Table II

Peptides identified by MALDI-TOF following digestion of the 160-kDa protein with trypsin

<table>
<thead>
<tr>
<th>No.</th>
<th>Amino acid sequence</th>
<th>TN-R domain</th>
<th>Mass calculated (m/z Da)</th>
<th>Mass measured (MH⁺ Da)</th>
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<tbody>
<tr>
<td>1</td>
<td>MRPYIHR</td>
<td>FBG</td>
<td>972.55</td>
<td>972.52</td>
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<tr>
<td>2</td>
<td>LILNYSPR</td>
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<td>3</td>
<td>ASGPIDHYR</td>
<td>FNIII-5</td>
<td>1015.47</td>
<td>1015.50</td>
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<tr>
<td>4</td>
<td>YEVISIAYR</td>
<td>FNIII-3</td>
<td>1023.50</td>
<td>1023.55</td>
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<tr>
<td>5</td>
<td>GIEGETVLK</td>
<td>Cys-rich</td>
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<td>1086.60</td>
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<tr>
<td>6</td>
<td>QNGQTDPFRR</td>
<td>FBG</td>
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<td>7</td>
<td>QSANISQWPPR</td>
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<td>VATHLSTPQGLOFK</td>
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<td>15</td>
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<td>LQPLLPSYSQVQLRPGSR</td>
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<tr>
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<td>FIG</td>
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<tr>
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<td>FNIII-8</td>
<td>2422.25</td>
<td>2422.21</td>
</tr>
<tr>
<td>20</td>
<td>TSYTLTDLGPAEYISITAE</td>
<td>FNIII-5</td>
<td>2443.22</td>
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</table>

Fig. 4. TN-R is one of the glycoproteins bound by immobilized Cys-Fc. The fractions generated during the affinity purification of Cys-Fc-reactive glycoproteins from rat cerebellum as summarized in Table I were analyzed as in Fig. 2 by SDS-PAGE, electrophoretically transferred to PVDF, and probed with biotinylated Cys-Fc (A) or anti-TN-R (anti-TNR) (B). Lane 1, RCSD; lane 2, proteins not bound to Cys-Fc-agarose; lanes 3–5, three successive wash fractions from the affinity column; lanes 6–7, proteins eluted from Cys-Fc-agarose with 500 μM GalNAc-4-SO₄; lanes 8–9, proteins subsequently eluted from Cys-Fc-agarose with 1 x NaCl with 500 μM GalNAc-4-SO₄. The arrows indicate the locations of four species of TN-R; from top to bottom these are trimer, dimer, 180-kDa monomer, and 160-kDa monomer. The arrowheads indicate the locations of unidentified Cys-Fc-reactive glycoproteins that do not react with anti-TN-R.

Characterization of the Cys-Fc-reactive Glycoproteins from Rat Cerebellum.—The most abundant affinity-purified protein, migrating with Mᵣ 160,000 (Fig. 2A, arrow), was digested with trypsin and analyzed by mass spectrometry. Twenty peptides were obtained that had masses corresponding to predicted tryptic fragments for rat TN-R (Table II). The peptides identified correspond to different regions distributed across the entire length of the protein (see Fig. 3). TN-R is a large, multidomain protein that contains an N-terminal cysteine-rich region, four complete and one partial EGF-like repeats, eight fibronectin type III repeats, one alternatively spliced fibronectin type III repeat, and a C-terminal fibrinogen-like domain (22). TN-R is a soluble, extracellular matrix CAM that is expressed only in the central nervous system and is highly modified with both N- and O-linked oligosaccharides (Fig. 3). Based on both in situ analyses and immunohistological stains, TN-R expression is characteristic of the hippocampus and the cerebellum (23, 24).
Fig. 5. Immunoprecipitated TN-R is recognized by Cys-Fc. TN-R was immunoprecipitated from RCSD fractions using anti-TN-R, separated by SDS-PAGE, and blotted with biotinylated Cys-Fc (A) or anti-TN-R (B). Lane 1, 1% of the RCSD; lane 2, immunoprecipitated TN-R from rat cerebellum; lane 3, normal goat serum control. Arrows indicate the species of TN-R.

Analysis of aliquots of the input, unbound, and bound fractions generated during the affinity purification on immobilized Cys-Fc by Western blot analysis using biotinylated Cys-Fc (Fig. 4A) indicated that the affinity isolation of glycoproteins using the Cys-Fc chimera is highly efficient. Virtually all of the Cys-Fc-reactive material present in the soluble fraction prepared from rat cerebellum was bound by the affinity column and selectively eluted with GalNAc-4-4-SO₄. Western analysis of the same blot with an antibody specific for TN-R revealed the presence of four protein species (Fig. 4B, arrows) corresponding to the two monomeric species that are generated by alternative splicing of 160 and 180 kDa, the dimeric, and the trimeric forms of TN-R (25). The immobilized Cys-Fc chimera bound the majority of TN-R present in the extract, 50–75% (Fig. 4B). Notably, although the monomeric and oligomeric forms of TN-R retained by the Cys-Fc chimera affinity column reacted with biotinylated Cys-Fc (arrows in Fig. 4B), other Cys-Fc-reactive proteins were also present (arrowheads in Fig. 4A). The protein most intensely reactive with the Cys-Fc chimera migrated ahead of the TN-R with M₉, 140,000 (middle arrowhead). Two additional glycoproteins migrating with molecular weights of 120,000 and 200,000 also reacted with Cys-Fc (lower and upper arrowheads). The material migrating with M₉, 130,000 in Fractions 1–4 and 6–9 reacted with the secondary antibody alone and was not considered Cys-Fc-reactive. Subsequent tryptic digestion and MALDI analysis of the Cys-Fc-reactive proteins migrating at the positions of dimeric and trimeric TN-R confirmed that they were TN-R. Thus, of the seven protein species that bear carbohydrate moieties that are recognized by the Cys-rich domain of the Man/GalNAc-4-4-SO₄-receptor, four represent forms of TN-R.

The presence of Cys-Fc-reactive carbohydrate on TN-R was further confirmed by examining immunoprecipitated TN-R (Fig. 5). Cerebellar TN-R, which had been immunoprecipitated from the RCSD, yielded two species of 160- and 180-kDa molecular mass as well as two species of >200 kDa when blotted with anti-TN-R (Fig. 5B). This indicates that the two forms of TN-R that arise by alternative splicing both react with Cys-Fc; however, the 160-kDa form of TN-R was significantly more reactive than the 180-kDa form (Fig. 5A).

TN-R bears multiple N- and O-linked oligosaccharides, including O-linked chondroitin chains (25). TN-R was digested with PNGase-F (Fig. 6) to determine if the carbohydrate epitope that is recognized by the Cys-Fc chimera is located on N- or O-linked oligosaccharides. Digestion with PNGase-F resulted in a complete loss of reactivity with Cys-Fc for monomeric and trimeric forms of TN-R (arrows) as well as the unknown glycoprotein migrating at 140 kDa (arrowhead) (Fig. 6A). The dimeric forms of TN-R did, however, display some reactivity with Cys-Fc following PNGase-F digestion. We have not yet determined the basis for the retention of residual Cys-Fc reactivity by a fraction of the material migrating at the position of the dimeric form of TN-R following PNGase-F digestion. The loss of Cys-Fc reactivity did not reflect a loss of TN-R protein, because the TN-R remained reactive with anti-TN-R but migrated with a lower apparent molecular weight than the material incubated with buffer in the absence of PNGase-F (Fig. 6B). Furthermore, digestion with chondroitinase ABC did not reduce the reactivity with Cys-Fc (not shown). Thus, the Cys-Fc-reactive carbohydrate is confined to one or more N-linked oligosaccharides on TN-R. The chondroitin chains on TN-R that are O-glycosidically linked to the peptide do not account for the reactivity with Cys-Fc even though they do have the potential to terminate in β1,4-linked GalNAc-4-4-SO₄.

DISCUSSION

The presence of protein-specific GalNAc-transferase activity, GalNAc-4-sulfotransferase activity (20), and GalNAc-4-ST1 mRNA in the cerebellum (15) indicated that glycoproteins bearing N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-4-SO₄ are present in this region of the brain. The studies described in this report demonstrate that these unique sulfated structures are present predominantly on TN-R and one or two additional glycoproteins in rat cerebellum and that the expression of these structures is highly regulated. Although clusters of basic amino acids in proximity to glycosylation sites can be found in TN-R that could act as recognition determinants for the protein-specific GalNAc-transferase, additional studies will be required to identify the actual sequences that are recognized.

The Cys-Fc chimera stains both Purkinje cell bodies and dendrites in the molecular layer of the rat and mouse cerebellum. The staining associated with the cell body of the Purkinje cell is, however, more intense than that associated with the dendrites. At birth, Purkinje cells are numerous and chaotically dispersed throughout the rodent cerebellum. By P4, the majority of the Purkinje cells have migrated to the region between the molecular and internal granular layer. Initial growth of dendrites by P5 produces a strict monolayer of Purkinje cells (26) that is associated with the expression of low levels of Cys-Fc-reactive carbohydrate on the Purkinje cell body. At P7 developing primary dendrites of Purkinje cells increase the depth of the molecular layer and the first synapses with parallel fibers are seen. Growth of Purkinje cell dendrites continues through P21 as does synapse formation with parallel fibers and small interneurons known as basket and stellate cells (26). During this time there is a dramatic increase in expression of Cys-Fc-reactive material. The addition of β1,4-linked terminal GalNAc-4-4-SO₄ to oligosaccharides on TN-R and other glycoproteins that are synthesized between P14 and P21 may be critical for some aspects of Purkinje cell growth, synapse formation, and/or synapse maintenance.

The temporal and spatial distribution of a number of unique carbohydrate structures in the nervous system is highly regulated, supporting a role for these carbohydrates on glycoproteins, glycolipids, and proteoglycans in regulating various aspects of development. In the case of carbohydrates such as HNK-1 (SO₄-3-GlcUAβ1,3Galβ1,4GlcNAc) (1, 27) and polysialic acid (2), these structures, like those terminating with Gal-
N-acetylglucosamine (GlcNAc)-4-sulfate (GalNAc-4-SO₄) are only found on specific glycoproteins. In each instance, changes in the amount of carbohydrate present in the brain may reflect either a change in the level of synthesis of the glycoprotein and/or the extent to which it is modified with a particular structure.

TN-R is one member of a family of five multidomain adhesion molecules that are components of the ECM (Fig. 3). In contrast to other tenascin family members, TN-R is expressed exclusively in the central nervous system. Oligodendrocytes and inhibitory interneurons in the cerebellum, motor neurons in the spinal cord and brain, and horizontal cells of retina all express TN-R. It is thought to play roles in the regulation of dendrite formation, outgrowth, and synaptogenesis as well as neural cell adhesion to glial cells and/or the ECM. Notably, TN-R was originally identified as an HNK-1-bearing glycoprotein and was subsequently described as also containing another sulfated carbohydrate, chondroitin sulfate (28, 29).

The pattern of terminal glycosylation of TN-R is complex with three distinct sulfated carbohydrate structures potentially present. The sulfated structures that are added to TN-R (GalNAc-4-SO₄, HNK-1, and/or chondroitin) will reflect the repertoire of transferases expressed in the specific cells responsible for the synthesis of TN-R. Based on the proportion that is bound by the Cys-Fc affinity matrix, a major portion (50–75%) of the TN-R in the cerebellum bears one or more oligosaccharides terminating with GalNAc-4-SO₄.

Alternative splicing results in the expression of two major isoforms of TN-R of 160 kDa (TN-R 160) and 180 kDa (TN-R 180) that are found predominantly in the form of disulfide linked dimers (TN-R 160) and trimers (TN-R 180) (23, 25). TN-R 160 and TN-R 180 both are modified with GalNAc-4-SO₄; however, TN-R 160 is more intensely reactive with Cys-Fc on Western blots than TN-R 180. Dimeric and trimeric forms of TN-R were also modified indicating that both TN-R 160 and TN-R 180 in their oligomeric forms bear Cys-Fc-reactive structures.

Cys-Fc does not interact with the HNK-1 or chondroitin sulfate on TN-R. BSA that has been conjugated with SO₄-4-GalNAcβ1,4GlcNAcβ1,2Man (SAGGnM-BSA) reacts strongly with Cys-Fc, but not with an anti-HNK-1 antibody in Western blot analyses (not shown). Furthermore, BSA conjugated with HNK-1 (HNK-1-BSA) reacts intensely with the anti-HNK-1 monoclonal but not with Cys-Fc (not shown). TN-R that has been affinity-purified on Cys-Fc does, however, react with anti-HNK-1 suggesting that at least some fraction of TN-R in the cerebellum may bear both structures. Although chondroitin chains, which may terminate in GalNAc-4-SO₄, are present on TN-R, they do not contribute to recognition by the Cys-Fc chimera, because digestion with PNGase-F but not chondroitinase abolishes the reactivity with Cys-Fc on Western blot analysis.

The distribution of Cys-Fc-reactive glycoproteins and the HNK-1 epitope in cerebellum upon immunostaining also differs. Whereas anti-HNK-1 stains predominantly the dendrites of the Purkinje cells with little staining of the Purkinje cell body itself (5, 30), Cys-Fc stains predominantly the Purkinje cell’s body and to a lesser extent the dendrites (Fig. 1). In addition, HNK-1-bearing structures that are associated with glycoproteins in the developing cerebellum decline after P14 (27), whereas the amounts of Cys-Fc-reactive glycoproteins increase after P14 (Fig. 1, E and F).

TN-R is both structurally and functionally complex. It can display either adhesive or anti-adhesive properties and can either enhance or inhibit neurite outgrowth in vitro (25). It is not known, however, whether the pattern of terminal glycosylation on TN-R contributes positively or negatively to these functions. Between 25 and 50% of the TN-R isolated from the adult cerebellum does not appear to bear terminal GalNAc-4-SO₄ and may correspond to TN-R that is present in the white matter and granular layer. During development TN-R is initially expressed predominantly in the white matter by oligodendrocytes. TN-R expression expands to include the molecular and granular cell layers later in development. In adult rats and mice TN-R expression is down-regulated in oligodendrocytes, whereas it continues to be expressed in the molecular layer (25). Because no Cys-Fc staining of the white matter is seen in either the developing or the adult cerebellum, it is likely that TN-R synthesized by oligodendrocytes in this region of the cerebellum is devoid of terminal GalNAc-4-SO₄. After P7, TN-R mRNA is found in small interneurons, stellate and basket cells, that reside in the molecular layer (24). This suggests that basket and stellate cells synthesize the GalNAc-4-SO₄-bearing forms of TN-R seen in the molecular layer and that these cells express both the protein-specific GalNAc-transferase and GalNAc-4-ST1 that we have previously determined are present in the cerebellum. Immunostains using antibodies that are specific for the 160- and 180-kDa isoforms of TN-R show that TN-R 160 is found predominantly in the molecular layer, whereas TN-R 180 is found predominantly in the white matter and granular layer of the cerebellum (23). The presence of the TN-R 160 predominantly in the molecular layer agrees with our observation that TN-R 160 is more reactive with Cys-Fc than TN-R 180 and further supports the conclusion that interneurons express GalNAc-transferase and GalNAc-4-ST1.

The presence of multiple unique sulfated carbohydrate structures on a major component of the ECM suggests that one or more of these structures in the brain is recognized by specific receptors. The Man/GalNAc-4-SO₄-receptor, which binds terminal GalNAc-4-SO₄ and mediates the clearance of lutropin (LH) from the blood (18, 19), is also expressed in the brain, specifically in perivascular microglia.² Perivascular microglia do not reside near either the molecular or Purkinje cell layers, but the Man/GalNAc-4-SO₄-receptor may have access to GalNAc-4-SO₄-bearing glycoproteins in the cerebellum during various forms of brain trauma when they migrate to the site of injury. Recent evidence suggests that TN-R possesses anti-adhesive properties when incubated with activated microglia (31). This could be mediated through a signaling interaction between GalNAc-4-SO₄ on TN-R and its receptor on perivascular microglia. It is also possible that there are additional GalNAc-4-SO₄-specific receptors in the cerebellum that remain to be identified.

We have shown that N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-SO₄ are added to specific glycoproteins that are closely associated with the cell bodies and dendrites of Purkinje cells in the cerebellum. One of these proteins is the cellular adhesion molecule TN-R, a multifunctional matrix protein that contributes to the development and/or maintenance of synapses, dendrites, and axons in the cerebellum. Thus modification of TN-R with GalNAc-4-SO₄ and other sulfated carbohydrate moieties, at specific times by different cells during axon extension and synaptogenesis, could represent the mechanism that generates one or more of the different functional forms of TN-R seen in vitro.

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