Neuronal-specific Synthesis and Glycosylation of Tenascin-R*

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Tenascin-R (TN-R) is a member of the tenasin family of multidomain matrix glycoproteins that is expressed exclusively in the central nervous system by oligodendrocytes and small neurons during postnatal development and in the adult. TN-R contributes to the regulation of axon extension and regeneration, neurite formation and synaptogenesis, and neuronal growth and migration. TN-R can be modified with three distinct sulfated oligosaccharide structures: HNK-1 (SO4-3-GlcUA1,3Galβ1,4GlcNAc), GalNAc-4-SO4, and chondroitin sulfate. We have determined that TN-R expressed in dendrite-rich regions of the rat cerebellum, hippocampus, and cerebral cortex is one of the major matrix glycoproteins that bears N-linked carbohydrates terminating with β1,4-linked GalNAc-4-SO4. The synthesis of these unique sulfated structures on TN-R are differentially regulated. Levels of HNK-1 on TN-R rise and fall in parallel to the levels of TN-R during postnatal development of the cerebellum. In contrast, levels of GalNAc-4-SO4 are regulated independently from those of TN-R, rising late in cerebellar development and continuing into adulthood. As a result, the pattern of TN-R modification with distinct sulfated carbohydrate structures changes dramatically over the course of postnatal cerebellar development in the rat. Because TN-R interacts with a number of different matrix components and, depending on the circumstances, can either activate or inhibit neurite outgrowth, the highly regulated addition of these unique sulfated structures may modulate the adhesive properties of TN-R over the course of development and during synapse maintenance. In addition, the 160-kDa form of TN-R is particularly enriched for terminal GalNAc-4-SO4 later in development and in the adult, suggesting additional levels of regulation.

Neural pattern formation in the central nervous system (CNS) involves initial interactions between cell surfaces and components of the extracellular matrix (ECM) that direct cells to proliferate, migrate, and finally differentiate. Early in CNS development the ECM contains components such as fibronectin, laminin, and collagen, whereas chondroitin sulfate proteoglycans and multimeric glycoproteins such as the tenascins appear later in maturation. Changes in the composition of the ECM play a critical role in generating the positive and negative signals necessary for normal pattern formation during development and tissue repair following injury (1, 2).

Tenascin-R (TN-R) is a member of the tenasin family of multidomain, matrix glycoproteins that are expressed exclusively in the central nervous system by oligodendrocytes and small neurons during postnatal development and in the adult. It has been implicated in the regulation of axon extension and regeneration, neurite formation and synaptogenesis, and neuronal growth and migration (3–6). TN-R binds different cell surface receptors and/or ECM components depending upon the local environment and is able to either enhance or inhibit adhesion depending on the specific circumstances (3). The adhesive properties of TN-R reflect the cell type that expresses TN-R, the time in development it is synthesized, its post-translational modifications, the repertoire of receptors on adjacent cell surfaces, and intracellular signaling mechanisms (3, 7). Consequently the synthesis and processing of TN-R and its receptors are highly regulated throughout the development of the CNS and in the adult to facilitate neural pattern formation and tissue repair.

TN-R has a highly conserved domain structure among different species as well as within the tenasin family of proteins (3, 8). It consists of an NH2-terminal cysteine-rich domain, 4.5 epidermal growth factor-type repeats, 9 fibronectin type III (FN-III) repeats, and a COOH-terminal fibronogen domain (9). Synthesis of TN-R by oligodendrocytes commences early in postnatal development, peaks during times of active myelination, and is down-regulated at later developmental stages. In contrast, neurons synthesize TN-R continuously beginning in late development and throughout adulthood (10, 11). Two molecular isoforms of TN-R of 160 (TN-R 160) and 180 kDa (TN-R 180) have been identified. TN-R 160 has been reported to be an alternatively spliced form of TN-R that is missing the sixth FN-III repeat (3); however, the difference between the two forms has not been determined at the protein level. TN-R forms disulfide-linked dimeric and trimeric species through its NH2-terminal cysteine-rich region (11). The differing patterns of expression observed for the two isoforms of TN-R in vivo have led to the suggestion that they may have different roles (3, 11).

TN-R is extensively modified with both N- and O-linked oligosaccharides, 10–20% by weight (10, 11). Three distinct sulfated oligosaccharide structures have thus far been described on TN-R: HNK-1 (SO4-3-GlcUA1,3Galβ1,4GlcNAc) (12), O-linked chondroitin sulfate glycosaminoglycans (13), and SO4-4-GalNAcβ1,4GlcNAc (14). Each may contribute to TN-R function. For example, the O-linked chondroitin sulfate glycosaminoglycans may mediate TN-R interactions with fibronectin and tenasin-C, which induces inhibition of cell adhesion and neurite outgrowth in vitro (13, 15). There is evidence that HNK-1, present on all known isoforms of TN-R During neuronal development, modulates perisomatic inhibition of synapse...
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formation and synaptic plasticity (16, 17). The sulfated carbohydrate may regulate these functions through interaction with an HNK-1-specific receptor on the surface of neuronal cells, SBP-1 (18). Although modification of glycolipids and other adhesion molecules in the CNS with HNK-1 is highly regulated, the developmental and tissue-specific pattern of HNK-1 addition to TN-R has not been determined (19, 20). Terminal GalNAc-4-SO4 is prominent in the molecular layer of the rat cerebellum late in cerebellar development and persists into adulthood (14). GalNAc-4-SO4 on N-linked oligosaccharides is synthesized by the sequential action of a protein-specific GalNAc transferase and a GalNAc-4-sulfotransferase that are coordinately expressed in cerebellum and other regions of the brain such as the hippocampus, cerebrum, and pituitary (21). The protein specificity of the GalNAc transferase ensures that this structure is confined to a limited number of glycoproteins bearing an appropriate recognition determinant. We have previously shown that TN-R is one of the glycoproteins in the cerebellum that bears N-linked sugars with terminal GalNAc-4-SO4. Furthermore, with HNK-1, the appearance of the GalNAc-4-SO4 epitope in the cerebellum is regulated and confined to the molecular and Purkinje cell layers. We initially described terminal GalNAc-4-SO4 on the pituitary glycoprotein hormones lutropin (LH) and thyrotropin (TSH) (22). GalNAc-4-SO4 on glycoprotein lutropin hormone is essential for regulating its circulatory half-life, and in turn regulating ovum implantation (23, 24). Lutropin hormone is essential for regulating its circulatory half-life, lactoprotein hormone is essential for regulating its circulatory half-life, and in turn regulating ovum implantation (23, 24).

We report here that TN-R is a major target for GalNAc-4-SO4 modification in the rat cerebellum, hippocampus, and cortex, and that TN-R isoforms are differentially modified in the cerebellum during postnatal development and adulthood. Furthermore, modification of TN-R with GalNAc-4-SO4 versus HNK-1 is regulated differently during development. We have also determined that an established neuroblastoma cell line synthesizes TN-R that is modified with both GalNAc-4-SO4 and HNK-1. The highly regulated addition of different sulfated carbohydrate structures to TN-R may be important for recognition by carbohydrate-specific cell surface receptors and/or ECM molecules or for modulating adhesiveness or signaling potential of TN-R during normal CNS development and upon injury.

EXPERIMENTAL PROCEDURES

Materials—Rat tissues were obtained from Pel Freez. Cys-Fc, biotinylated Cys-Fc, and Cys-Fc-agarose were prepared as previously described (14). Polyclonal goat anti-TN-R (SC-9874) was raised against the N terminus of TN-R was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-TN-R (BD) was obtained from BD Transduction Laboratories. Polyclonal (596) and monoclonal (596) anti-TN-R antibodies were prepared as previously described (4).

Preparation of Cell and Tissue Extracts—Developing and adult rat cerebellar, hippocampal, and cortical fractions were prepared as previously described (14). SH-SY5Y cells were prepared as follows. Media were harvested and cell debris was removed by centrifugation. Cells were washed in phosphate-buffered saline, 2% Triton X-100, 10 mM EDTA, 1 mM Pefabloc SC, and 2 mM γ-glutamyl aprotinin. Unbroken cells, nuclei, and Triton-insoluble proteins were removed by sedimentation.

Cys-Fc Affinity Chromatography—Rat cerebellar fractions were affinity purified by incubation with Cys-Fc-agarose as previously described (14). SH-SY5Y cell extracts or [35S]SO4-labeled SH-SY5Y cell extracts were incubated with Cys-Fc-agarose overnight at 4 °C in batch and unbound proteins were removed. Subsequently, the Cys-Fc-agarose was washed with Tris-buffered saline, plus 0.2% hydrogenated Triton X-100 and eluted with 500 μM GalNAc-4-SO4 in Tris-buffered saline, plus 0.2% hydrogenated Triton X-100.

Surface Plasmon Resonance—Cys-Fc was immobilized on a Biacore CM5 sensor surface as previously described (14). The concentration of the GalNAc-4-SO4-bearing ligands present in SH-SY5Y media and cell extracts was determined as with the cerebellar extracts (14).

Western Blots, Ligand Blots, and Autoradiography—Samples were incubated in NuPAGE lithium dodecyl sulfate sample buffer with 0.05 M dithiothreitol, heated for 10 min at 70 °C, and separated on 7.0% Tris-acetate acrylamide gels (Novex/Invitrogen). Proteins were visualized by autoradiography (below) or were electrophoretically transferred to polyvinylidene difluoride membranes. Following transfer, polyvinylidene difluoride membranes were treated with 1% Casein prior to incubation with a polyclonal or monoclonal anti-TN-R antibody, anti-HNK-1 (Sigma), or Cys-Fc biotin in TBS (20 mM Tris-Cl, pH 7.5, 250 mM NaCl), containing 0.05% Tween 20 for 0.5 h at 4 °C. After removing excess primary reagent, the blots were incubated with donkey anti-goat IgG-horseradish peroxidase (Santa Cruz), goat anti-rabbit IgG-horseradish peroxidase (BIO SOURCE), goat anti-mouse IgM-horseradish peroxidase (Jackson Labs), or horseradish peroxidase-streptavidin (Jackson Labs) for 1 h at 4 °C. Western and ligand blots were visualized using chemiluminescence as described by the manufacturer (PerkinElmer Life Sciences). Gels containing [35S]SO4-labeled proteins were fixed in 40% methanol, 25% acetic acid for 30 min at room temperature, incubated in Amplify (Amersham Biosciences) for 1 h, dried, and exposed to film at 80 °C overnight to 1 week.

Peptide Analysis—One-dimensional protein gel bands were visualized with colloidal Coomassie Blue G-250. The relevant bands were excised, using a Proteome Works Spot-cutter (Bio-Rad). The gel pieces were digested with sequencing grade trypsin (Promega). The recovered tryptic peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE-PRO mass spectrometer (Applied Biosystems). Automated data acquisition and automated data base searching were performed with applied Biosystems Proteomics Solution 1 (PSI) software.

Immunoprecipitations—Prior to immunoprecipitation with specific antisera, the soluble and peripheral membrane proteins from adult and developing rat cerebella or SH-SY5Y tissue culture media were diluted 1:2 in IP buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% SDS), incubated with rabbit serum for 1 h at 4 °C, and then with protein A-Sepharose for an additional 1 h. The protein A-Sepharose was washed by sedimentation. pTN-R was added and the sample was incubated overnight at 4 °C. Protein A-Sepharose was added for 1 h and washed extensively with IP buffer. Bound proteins were eluted by heating in lithium dodecyl sulfate sample buffer for 10 min at 70 °C and analyzed by SDS-PAGE as described above.

Immunohistochemistry and Immunocytochemistry—Rat and mouse brains were removed and 20-μm cryostat sections of hippocampus, cortex, and cerebellum were prepared as described (14). SH-SY5Y cells were cultured (as described below) overnight on coverslips. After washing with phosphate-buffered saline, cells were fixed in methanol at −20 °C for 6 min or 4% paraformaldehyde for 8 h. After fixation, coverslips were blocked with 5% goat serum in phosphate-buffered saline. After blocking, frozen brain sections and fixed cells were incubated with biotinylated Cys-Fc (10 ng/ml) overnight at 4 °C. Unbound fusion protein was removed prior to incubation with Cy3-conjugated streptavidin (Jackson Labs) and counterstaining with Hoechst number 33258 (Sigma).

Tissue Culture—SH-SY5Y neuroblastoma cells were grown in a 1:1 mixture of Ham’s F-12 and minimal essential Earle’s media with 15% fetal bovine serum and 1× non-essential amino acids. For [35S]SO4 labeling, 3 × 106 SH-SY5Y cells were plated in 100-mm dishes and allowed to grow overnight at 37 °C. Cells were incubated with minimal essential Earle’s media (without MgCl2, SO4, Cys, and Met) for 1 h at 37 °C. Media was removed and the cells were labeled in the above media supplemented with [35S]SO4 (ICN) at 100 μCi/ml for 6 h. Media and cells were collected for further analysis.

Enzymatic Removal of N-Linked Oligosaccharides—For affinity purification [35S]SO4-labeled SH-SY5Y cell extracts, PNGase-F digests were prepared using the CSV buffer (ICN). For labeling PNGase-F media, 2 ml of media was immunoprecipitated with pTN-R as described above, except after washes, immunoprecipitated proteins were denatured and removed from protein A-Sepharose by heating in 50 μl of 1% β-mercaptoethanol, 0.5% SDS for 10 min at 100 °C, sedimented, and the supernatant was collected. The Sepharose was resuspended in 50 μl of CSV buffer (ICN) and the supernatant was added. The two supernatants were combined and half was treated with 1 unit of PNGase-F (New England Biolabs) and the other half incubated in buffer alone for 1 h at 37 °C. The samples were separated by SDS-PAGE and visualized by autoradiography.

RESULTS

TN-R Bears a Major Fraction of the GalNAc-4-SO4 Residues Present in Rat Cerebellum—We previously used a polyclonal goat antibody (anti-TN-R/SC) from Santa Cruz Biotechnology
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raised to a synthetic peptide included within the amino-terminal 50 amino acids of TN-R, to identify TN-R as one of the proteins from mouse and rat cerebellum that is bound by immobilized Cys-Fc because it is modified with terminal GalNAc-4-SO_4 (Fig. 1C) (14). However, the most abundant protein band bearing terminal GalNAc-4-SO_4 (Fig. 1A) did not react with anti-TN-R(SC) and remained to be identified. This glycoprotein did react with anti-TN-R(BD) (Fig. 1B), a monoclonal raised to a tryptic peptide that included the entire NH_2-terminal cysteine-rich region of TN-R, as well as with two other antibodies, pTN-R and monoclonal 596 (not shown), raised to other regions of TN-R (Fig. 1D). This suggested that the most abundant Cys-Fc reactive protein (Fig. 1A), which migrates more rapidly than the forms of TN-R previously identified using anti-TN-R(SC), is also a form of TN-R. This form of TN-R appears to correspond to TN-R 160, whereas the slower migrating band previously identified as TN-R 160 corresponds to TN-R 180 and the band previously identified as TN-R 180 corresponds to a form of TN-R 180 bearing additional carbohydrate that we have designated TN-R 180(g).

The affinity purified, Cys-Fc reactive glycoprotein (Fig. 1A, arrow marked 160) that also reacted with anti-TN-R(BD) (Fig. 1B) but not with anti-TN-R(SC) (Fig. 1C) was examined by MALDI-TOF (Fig. 1F). The analysis confirmed that the glycoprotein was a form of TN-R. In contrast to the results obtained with anti-TN-R(SC), the relative intensities of the bands corresponding to TN-R were similar when probed with anti-TN-R(BD) and Cys-Fc (Fig. 1). The same pattern of staining was observed with pTN-R raised to immunopurified mouse TN-R 160 and with monoclonal antibody 596 raised to an HNK-1 positive fraction from chick brain that both react with rat TN-R (11) (data not shown). The regions recognized by each of the TN-R-specific antibodies are indicated in Fig. 1D. Because the most abundant form of TN-R did not react with the anti-TN-R(SC), this suggested that in the adult rat cerebellum this TN-R isoform is missing a portion of its amino terminus including the 50-amino acid region containing the cysteine peptide used to generate anti-TN-R(SC). In support of this conclusion, no peptides corresponding to the amino-terminal region of TN-R were present in the MALDI-TOF analysis of the protein with the highest mobility (Fig. 1A, arrow marked 160 and Panel F), whereas a peptide corresponding to this region was present in analyses of the monomeric and multimeric forms of TN-R reactive with anti-TN-R(SC) (Fig. 1, arrows marked 180, d, and f and Ref. 14).

The disulfide bonds resulting in covalent association of monomeric TN-R into dimeric and trimeric species are formed between cysteine located in the amino-terminal cysteine-rich region of TN-R (11). The monomeric form of TN-R that does not react with the anti-TN-R(SC) is present following SDS-PAGE analysis in either the presence or absence of reducing agents (not shown). In contrast, monomeric forms of TN-R that are recognized by the amino terminus specific antibody are only observed following reduction. Our results suggest that monomeric forms of TN-R that do not contain the amino-terminal region arise by proteolytic cleavage of multimeric forms of TN-R and loss of covalent association through disulfide bonds (see Fig. 1E). To determine whether the forms of TN-R that do and do not contain the amino-terminal region correspond to the forms of TN-R that have previously been designated as TN-R 180 and TN-R 160 (11, 25), TN-R 160 and TN-R 180 were purified from the brains of adult Wistar rats and NMRI mice by sequential immunoaffinity chromatography on monoclonal TN-R1 (clone 597) and TN-R2 (clone 596) antibody columns as previously described (11). Following separation by SDS-PAGE the immunoaffinity purified TN-R 160/180 preparations were visualized by Western blotting with anti-TN-R(SC) and anti-TN-R monoclonal antibody 596 as shown in Fig. 2. As predicted, amino terminus-specific antibody TN-R(SC) recognized the doublet at 180 kDa (equivalent to 180 and 180g in Fig. 1) and TN-R dimers but did not recognize the 160-kDa band (Fig. 2).

Previous studies based on PCR analyses indicated that TN-R 160 might arise by alternative mRNA splicing resulting in the loss of the sixth FN-III domain (10). Whereas MALDI-TOF analysis of the band migrating at the position of TN-R 160 did not yield any peptide fragments from the amino-terminal region of TN-R, it did reveal the presence of two peptides fragments from the sixth FN-III repeat (Fig. 1D). Both fragments from the sixth FN-III repeat and a fragment from the amino-terminal region of TN-R were observed in the MALDI-TOF analysis of TN-R 180. Thus, TN-R 160 contains the sixth FN-III repeat but is missing a portion of its amino-terminal region.

TN-R represents a major glycoprotein in the soluble and peripheral membrane protein fraction from rat cerebellum that is modified with terminal GalNAc-4-SO_4. Whereas the majority of Cys-Fc reactive material is retained by Cys-Fc immobilized on agarose (Fig. 1A and Ref. 14) only a portion of TN-R is retained (Fig. 1B) indicating that not all cerebellar TN-R bears N-linked structures terminating with β1,4-linked GalNAc-4-SO_4.

TN-R from Hippocampus and Cerebral Cortex Bears Oligosaccharides That Are Reactive with Cys-Fc—We have shown that the enzyme activities required for the addition of terminal β1,4-linked GalNAc-4-SO_4 to N-linked oligosaccharides are present in extracts of rat brain cortex, midbrain, and cerebellum, as well as in human neuroblastoma cells, SH-SY5Y (21). Furthermore, GalNAc-4-sulfotransferase-1 mRNA is present in human hippocampus, hippocampal cerebral cortex, spinal cord, and pituitary (26). We used the Cys-Fc chimera to probe histologic sections of mouse brain for the presence of glycoproteins bearing terminal GalNAc-4-SO_4. As was seen in the rat, the Purkinje cell and molecular layers of adult mouse cerebellum were intensely stained by the Cys-Fc chimera (Fig. 3B). No staining was observed when sections were incubated with biotinylated human IgG1 (not shown). The mouse hippocampus, like the cerebellum, was intensely stained with Cys-Fc (Fig. 3A), especially in regions rich in dendrites and extracellular matrix components such as CA1, CA2, CA3, and the dentate gyrus. In contrast, the granular layer was largely devoid of Cys-Fc reactive material. The cellular layers of the cerebral cortex, layers II-V (Fig. 3, C and D), were reactive with Cys-Fc. Perineuronal nets that form “rings” around neurons in the cortex and hippocampus (Fig. 3, A, C, and D, arrows) were also stained. The Cys-Fc reactivity along with the presence of protein-specific β1,4-GalNAc transferase and GalNAc-4-sulfotransferase activities in the matrix-rich areas of the hippocampus and cortex suggested that one or more glycoproteins are present in these regions that bear N-linked terminal GalNAc-4-SO_4.

Extracts of the hippocampus and cortex were examined for the presence of TN-R and other glycoproteins that might bear terminal GalNAc-4-SO_4. Monomeric, dimeric, and trimeric forms of TN-R were present in both regions of the brain (Fig. 4A) and reacted with the Cys-Fc chimera (Fig. 4B). Thus, TN-R bearing terminal GalNAc-4-SO_4 is present in at least three regions of the brain.

TN-R Glycosylation Is Temporarily Regulated in Rat Cerebellum—N-Linked oligosaccharides on TN-R can also be modified with the HNK-1 epitope that consists of the terminal sequence SO_4-3-GlcUAβ1,3Galβ1,4GlcNAc (12). TN-R in the rat cerebellar extracts that was immunoprecipitated with pTN-R (Fig. 5A, lanes 1 and 2) reacted with both biotinylated Cys-Fc (Fig. 5B)
FIG. 1. Analysis of Cys-Fc reactive proteins isolated from rat cerebellum. Soluble and peripheral membrane proteins from rat cerebellum were affinity purified on immobilized Cys-Fc. Affinity purified proteins were reduced, separated by SDS-PAGE, and visualized by ligand blotting with Cys-Fc (panel A) or Western blotting with two different anti-TN-R antibodies (panels B and C). Panel A, ligand blot with biotinylated Cys-Fc: lane 1, 1% of rat cerebellar soluble and peripheral membrane proteins following dialysis (RCSD); lane 2, 1% of RCSD proteins not bound by Cys-Fc-agarose; lane 3, 10% of RCSD proteins bound and eluted from Cys-Fc-agarose with 500 μM GalNAc-4-SO₄. Panel B, samples from panel A blotted with anti-TN-R(BD). Panel C, samples from panel A blotted with anti-TN-R(SC). Arrows indicate isoforms of TN-R, 160, 180, 180g, dimer (d), and trimer (t). The 180g form differs from the 180 form in its glycosylation. Panel D, schematic showing the multidomain structure of TN-R. The regions of TN-R used as immunogens to generate anti-TN-R (SC), pTN-R, anti-TN-R (BD), and monoclonal 596 are indicated below the structure. EGF, epidermal growth factor. Panel E, schematic showing the location of the disulfide bonds in trimeric TN-R and the proposed site of proteolysis that would result in dimeric and monomeric forms of TN-R in the absence of reduction. Panel F, table presenting the unique peptides obtained by MALDI-TOF analysis following trypsin digestion of the 180 kDa obtained by SDS-PAGE following reduction (see panel B above). The sequence, position in TN-R, calculated mass value, and measured mass value are indicated.

| # | Amino Acid Sequence | TN-R Domain | Mass Calculated (M/Z Da) | Mass Measured (M/Z Da) |
|---|------------------|-------------|-------------------------|-----------------------|
| 1 | LILNYSPR         | FNIII-AS    | 975.56                  | 975.56                |
| 2 | QNGQTDFFR        | FBG         | 1095.48                 | 1095.49               |
| 3 | QSALISWQPPR      | FNIII-8     | 1265.68                 | 1265.66               |
| 4 | SSLTSTIFTTGGR    | FNIII-8     | 1327.70                 | 1327.69               |
| 5 | ELIIVADEDTWIR    | FNIII-8     | 1459.75                 | 1459.74               |
| 6 | VATHLSTPQLGLQFK  | FNIII-2     | 1526.83                 | 1526.83               |
| 7 | DVSDTVAFVEWTPPR  | FNIII-3     | 1719.01                 | 1917.97               |
| 8 | ITFTPSSGSSETVTPR | FNIII-5     | 1777.98                 | 1777.93               |
| 9 | LDSSVVPNTTEFTITR | FNIII-6     | 1879.05                 | 1878.98               |
| 10 | DEEEEMMEVLLDATKR | FNIII-AS    | 1937.99                 | 1937.88               |
| 11 | LQPLLQSVQALRPGSR | FNIII-3     | 1997.15                 | 1997.09               |
| 12 | LEGLSENDDTVLLQAAAESATR | FNIII-8 | 2422.25 | 2422.21 |
| 13 | TSYLTDLEPAGAYIISITAE | FNIII-5 | 2443.32 | 2443.22 |
and anti-HNK-1 (Fig. 5C). Thus, TN-R from adult rat cerebellum can be modified with terminal GalNAc-4-\(\text{SO}_4\) and/or HNK-1. The distribution is similar for the GalNAc-4-\(\text{SO}_4\) and GluUA-3-\(\text{SO}_4\) (HNK-1) epitopes on the immunoprecipitated TN-R from rat cerebellum; however, biotinylated Cys-Fc and anti-HNK-1 do not cross-react with HNK-1 and GalNAc-4-\(\text{SO}_4\), respectively (data not shown).

Expression of the HNK-1 epitope is down-regulated during postnatal CNS development (19, 20). In contrast, we observed an increase in Cys-Fc staining of the molecular layer of the rat cerebellum on postnatal day 21 as compared with postnatal day 14 suggesting that GalNAc-4-\(\text{SO}_4\) expression increases during postnatal development of the cerebellum (14). Because the addition of GalNAc-4-\(\text{SO}_4\), and likely GluUA-3-\(\text{SO}_4\), to N-linked oligosaccharides is protein-specific and highly regulated, the pattern of TN-R glycosylation with sulfated oligosaccharides may change during cerebellar development.

TN-R was immunoprecipitated from cerebellar extracts at various times during postnatal development beginning with postnatal day 5 using pTN-R and probed with monoclonal anti-TN-R (BD), biotinylated Cys-Fc, and anti-HNK-1 (Fig. 6). As was seen previously (3), the 180-kDa monomeric form of TN-R appeared early in postnatal development (Fig. 6A, 6B, and 6C) along with smaller amounts of the 160-kDa form. TN-R expression increased throughout postnatal development when it is synthesized by both oligodendrocytes and neurons and declined in the adult when it is synthesized exclusively by neurons (3). TN-R did not react with Cys-Fc until postnatal day 15 (Fig. 6B). The amount of terminal GalNAc-4-\(\text{SO}_4\) increased between postnatal days 15 and 20 but then remained relatively constant at postnatal day 25 and in the adult. Notably, only the 180-kDa band reacted with Cys-Fc at postnatal day 15, whereas both the 180- and 160-kDa forms reacted later in development and in the adult. HNK-1 is present on immunoprecipitated TN-R throughout postnatal development (Fig. 6C). The levels of HNK-1 expression correspond closely to TN-R protein expression (compare Fig. 6, A and C), increasing over the course of postnatal development and declining in the adult. A similar pattern of HNK-1 expression has been reported for the cerebellar glycoproteins L1 and NCAM (20). Thus, there are at least two populations of TN-R in the cerebellum that differ in the extent to which they are modified with terminal GalNAc-4-\(\text{SO}_4\) and GluUA-3-\(\text{SO}_4\), and when they are modified with the sulfated carbohydrates.

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**Fig. 2. Analysis of immunopurified TN-R 160/180.** Immunopurified TN-R 160/180 preparations were separated by SDS-PAGE and visualized by Western blotting with the polyclonal antibody anti-TN-R (lanes 1–4) and the monoclonal antibody anti-TN-R 596 (lanes 5–8). Lanes 1 and 5, TN-R 180 from adult mouse brain. Lanes 2 and 6, TN-R 160/180 from adult mouse brain (immunopurified on 596 antibody column). Lanes 3 and 7, TN-R 160 preparation from adult mouse brain containing low amounts of TN-R 180. Lanes 4 and 8, TN-R 160 from adult rat brain. Arrows indicate isoforms of TN-R, dimer (d), 180-kDa monomer (m 180), and 160-kDa monomer (m 160).

**Fig. 3. Cys-Fc reactive material is present in mouse hippocampus and cortex.** Cryostat sections of adult mouse brain were incubated with Cys-Fc biotin and visualized using Cy3-conjugated streptavidin. Nuclei were stained with Hoechst dye 33258. Panel A, mouse hippocampus (×10). Panel B, mouse cerebellum (×10). Panel C, mouse cerebral cortex (×10). Panel D, mouse cortex (×20). Perineuronal nets are indicated with arrows. Roman numerals designate the layer of the cerebral cortex. Abbreviations: dg, dentate gyrus; M, molecular layer; G, granular layer; P, Purkinje cell layer.

**Fig. 4. TN-R from rat cerebellum, hippocampus, and cerebral cortex is modified with N-linked oligosaccharides terminating in GalNAc-4-\(\text{SO}_4\).** Rat cerebellar (RCS) or rat hippocampal (RHS) and cortical soluble and peripheral membrane proteins were immunoprecipitated with pTN-R, separated by SDS-PAGE, and visualized by ligand blotting with biotinylated Cys-Fc (panel B) or Western blotting with monoclonal anti-TN-R (BD) (panel A). Panel A: lane 1, 50% of the RCS, lane 2, immunoprecipitated TN-R from rat cerebellum, lane 3, 50% of the RHS, lane 4, immunoprecipitated TN-R from rat hippocampus and cortex. Panel B: identical samples as panel A blotted with biotinylated Cys-Fc. Arrows indicate isoforms of TN-R, 160, 180, dimer (d), and trimer (t).

**Fig. 5. Adult rat cerebellar TN-R is modified with GalNAc-4-\(\text{SO}_4\) and HNK-1.** RCSD proteins were immunoprecipitated with pTN-R, separated by SDS-PAGE, and visualized by blotting with monoclonal anti-TN-R (BD) (panel A), biotinylated Cys-Fc (panel B), or anti-HNK-1 (panel C). Lane 1, 50% RCSD; lane 2, 100% RCSD immunoprecipitated by pTN-R. Arrows indicate isoforms of TN-R, 160, 180, dimer (d), and trimer (t).
presence of glycoproteins terminating with GalNAc-4-SO₄. The surface and surrounding matrix of SH-SY5Y cells fixed in paraformaldehyde were intensely stained by Cys-Fc (Fig. 7A). SH-SY5Y cells fixed in methanol displayed intracellular, as well as surface and matrix staining with Cys-Fc (Fig. 7B). Similar results were obtained when SH-SY5Y cells were stained with a GalNAc-4-SO₄-specific monoclonal antibody, 6.3 (23) (not shown). Thus, SH-SY5Y cells synthesize glycoproteins that are modified with terminal GalNAc-4-SO₄.

Isolation and Identification of Glycoproteins Bearing Terminal GalNAc-4-SO₄ from SH-SY5Y Cells—The amount of Cys-Fc reactive material present in the culture media and Triton X-100 extracts of SH-SY5Y cells was determined using surface plasmon resonance to monitor binding to immobilized Cys-Fc (14). Whereas the specific activity (change in surface resonance units (RU)/µg) of the Triton X-100-solubilized SH-SY5Y cell extract (41.0 RU/µg) was 23-fold greater than that of the medium (1.8 RU/µg), the total amount of activity (total RU) present in the cell extract and medium was similar. The specific activity of the SH-SY5Y cell extract was 30-fold greater than that obtained from extracts of cerebellum (0.4–1.4 RU/µg).

Triton X-100 extracts prepared from SH-SY5Y cells contained multiple glycoproteins that were reactive with biotinylated Cys-Fc (Fig. 8A, lane 1) and could be affinity purified on immobilized Cys-Fc (Fig. 8A). Five major bands ranging in size from 60 to >200 kDa were specifically eluted from the Cys-Fc column with GalNAc-4-SO₄ (Fig. 8A, lane 5, arrows). Affinity purification of a Triton X-100 extract of SH-SY5Y cells metabolically labeled with [³⁵S]SO₄ yielded four major bands when examined by autoradiography following separation by SDS-PAGE (Fig. 8B, lane 4). Digestion of the [³⁵S]SO₄-labeled, Cys-Fc affinity-purified glycoproteins with protein N-glycosidase F released all of the label indicating that the [³⁵S]SO₄ is located on N-linked oligosaccharides (Fig. 8C). SH-SY5Y cells therefore synthesize a limited number of glycoproteins that bear N-linked oligosaccharides terminating with β1,4-linked GalNAc-4-SO₄.

SH-SY5Y Neuroblastoma Cells Synthesize TN-R Bearing N-Linked Oligosaccharides Terminating with β1,4-Linked GalNAc-4-SO₄—One of the glycoproteins affinity purified using Cys-Fc–agarose has a molecular weight that corresponds to that of TN-R. pTN-R was used to immunoprecipitate TN-R from both the culture media and Triton X-100 extracts of SH-SY5Y cells. A single species of TN-R was detected in both the media (Fig. 9A, arrow) and Triton X-100 extract (not shown) of SH-SY5Y cells using the monoclonal anti-TN-R (BD) following SDS-PAGE. The immunoprecipitated TN-R also reacted with biotinylated Cys-Fc (Fig. 9B, arrow) and anti-HNK-1 (Fig. 9C, arrow) indicating that terminal β1,4-linked GalNAc-4-SO₄ and GlcUA-3-SO₄, respectively, are present on the TN-R produced by these cultured neuroblastoma cells. An additional glycoprotein with an Mₙ >250,000 (Fig. 9, B and C, arrowhead) co-immunoprecipitated with the TN-R but did not react with the anti-TN-R (Fig. 9A). The co-immunoprecipitated protein did, however, react with Cys-Fc and anti-HNK-1 (Fig. 9, B and C).

Digestion of immunoprecipitated TN-R with PNGase-F resulted in a loss of Cys-Fc reactivity (Fig. 10A). Following the digestion with PNGase-F the TN-R migrated with an Mₙ of 150,000 (Fig. 10B) indicating that the N-linked oligosaccharides had indeed been released. [³⁵S]SO₄-labeled TN-R was immunoprecipitated from both the medium and Triton X-100 extract of metabolically labeled SH-SY5Y cells using pTN-R (Fig. 10, C and D). The protein with an Mₙ >250,000 was co-precipitated from both the medium and the Triton X-100 extract (Fig. 10C, arrowhead). Digestion with PNGase-F released the [³⁵S]SO₄ label from both TN-R and the co-precipitated protein (Fig. 10D). Thus, SH-SY5Y neuroblastoma cells express a single form of TN-R that, like TN-R present in the cerebellum and hippocampus, bears N-linked oligosaccharides that are modified with either β1,4-linked GalNAc-4-SO₄ or β1,4-linked GlcUA-3-SO₄.

**DISCUSSION**

The studies we have presented indicate that TN-R displays a remarkably complex pattern of glycosylation with distinct sulfated carbohydrate structures. TN-R was initially identified as a glycoprotein bearing the HNK-1 epitope, SO₄-3-GlcUAβ1,3GlcNAcβ1,4GlcNAc (12). It was subsequently reported that TN-R also contains O-linked structures (27) and low levels of chondroitin sulfate glycosaminoglycans (15). In addition to these sulfated glycans, we have determined that TN-R synthesized in the cerebellum, hippocampus, cerebral cortex, and by a neuroblastoma cell line, SH-SY5Y, bears N-linked oligosaccharides terminating with SO₄-4-GalNAcβ1,4GlcNAc. Thus, TN-R...
cane bear three distinct and unique sulfated oligosaccharide structures. Synthesis of these sulfated structures is protein-specific because one or more of the transferases responsible for the synthesis of each of these sulfated oligosaccharides are themselves protein-specific. For example, the β1,4-GalNAc transferase that accounts for the addition of terminal SO₄⁻⁴-GalNAcβ1,4GlcNAc to N-linked structures on the TN-R and the glycoprotein hormones recognizes a peptide determinant that increases the catalytic efficiency for GalNAc transfer to the oligosaccharide by 500-fold (28). Sulfate is subsequently added to the β1,4-linked GalNAc by a GalNAc-4-sulfotransferase (29, 30). As a result only glycoproteins with the appropriate peptide determinant are efficiently modified with terminal GalNAcβ1,4SO₄ in vivo.

Important consequences of the protein-specific addition of GalNAcβ1,4SO₄ and GlcUA-3-SO₄ are that: 1) only a limited number of glycoproteins bear these modifications, and that 2) the synthesis of these sulfated structures can be regulated with respect to when and where they are synthesized, and the extent to which oligosaccharides on target glycoproteins are modified. Terminal β1,4-linked GalNAcβ1,4SO₄ is present in multiple regions of the brain and in each region TN-R is one of the major glycoproteins that bears these modifications. The granular layer of the cerebellum contains large amounts of TN-R but does not stain with Cys-Fc, whereas the Purkinje cell and molecular layers of the cerebellum contain TN-R and are stained intensely by Cys-Fc. This pattern of expression, along with the inability to retain all TN-R in cerebellar extracts on immobilized Cys-Fc (14), indicates that there are forms of TN-R that are not modified with terminal GalNAcβ1,4SO₄. The presence of forms of TN-R that are and are not modified with terminal GalNAcβ1,4SO₄ in specific regions of the brain supports the view that the presence of terminal GalNAcβ1,4SO₄ contributes to the function of TN-R in some regions of the brain but not in others.

The pattern of TN-R oligosaccharide modification with terminal GlcUA-3-SO₄ (HNK-1) and GalNAcβ1,4SO₄ changes over the course of cerebellar postnatal development in the rat and mouse. Levels of both TN-R and GlcUA-3-SO₄ increase throughout cerebellar development, reaching a maximum between postnatal days 20 and 25. Levels of TN-R and GlcUA-3-SO₄ are significantly lower in the adult cerebellum. In contrast,
GalNAc-4-SO₄ only appears on cerebellar TN-R late in postnatal development beginning at postnatal day 15 and does not decrease in the adult. Thus, modification with GalNAc-4-SO₄ becomes predominant in the adult, whereas modification with GlcUA-3-SO₄ declines. The amount of the 160-kDa isoform of GalNAc-4-SO₄-modified TN-R also becomes more predominant in the adult. Because the 160-kDa form likely arises by proteolysis of the 180-kDa form of TN-R and loss of the amino-terminal region, TN-R in the ECM that bears terminal GalNAc-4-SO₄ may be subject to further modification by proteolysis. The changing patterns of TN-R modification with GalNAc-4-SO₄ and GlcUA-3-SO₄ during postnatal development and in the adult indicate that GalNAc-4-SO₄ and GlcUA-3-SO₄ addition are regulated independently and likely have different roles in vivo.

Cys-Fc staining is especially intense in the molecular layers of the cerebellum and the hippocampal formation that are rich in dendrites and ECM components. The time course for the complex changes in the pattern of TN-R glycosylation described above correspond to a time when there is rapid growth of dendrites in the molecular layer and active synapse formation (31, 32). Whereas the precise role played by TN-R in these processes is not well defined, TN-R is known to bind to a number of ECM components as well as to cellular receptors and to exhibit both adhesive and anti-adhesive properties when examined using in vitro culture systems (3). Changes in the extent of sulfate addition and in which sulfated structures are present on the N-linked oligosaccharides of TN-R may determine which ECM components and receptors are bound and modulate its adhesive and growth regulatory properties.

TN-R is synthesized by oligodendrocytes and neurons in the developing CNS, however, expression by oligodendrocytes is down-regulated, whereas expression by neurons is not down-regulated (10). The TN-R produced by oligodendrocytes is associated with myelinated axons in the white matter of the CNS that does not stain with Cys-Fc, suggesting that oligodendrocytes do not express the protein-specific GalNAc transferase and GalNAc-4-sulfotransferase required for GalNAc-4-SO₄ addition to TN-R. In contrast, TN-R expressed by neurons is associated with perineuronal nets around interneurons and motorneurons in various regions of the brain including the cortex, hippocampus, and cerebellum. The time course for the postnatal appearance and location of Cys-Fc staining in the CNS including perineuronal nets and motorneurons in various regions of the brain including the cortex, hippocampus, and cerebellum. The time course for the postnatal appearance and location of Cys-Fc staining in the CNS including perineuronal nets and motorneurons in various regions of the brain including the cortex, hippocampus, and cerebellum. The time course for the postnatal appearance and location of Cys-Fc staining in the CNS including perineuronal nets and motorneurons in various regions of the brain including the cortex, hippocampus, and cerebellum.

Further support for the neuronal origin of TN-R bearing GalNAc-4-SO₄ is provided by the neuronal cell line SH-SY5Y. We previously reported that SH-SY5Y cells express both the protein-specific GalNAc transferase and the GalNAc-4-sulfotransferase (21). We identified 4–5 glycopolypeptides synthesized by SH-SY5Y cells as bearing N-linked oligosaccharides terminating with GalNAc-4-SO₄ based on their binding to immobilized Cys-Fc including TN-R. Remarkably, TN-R synthesized by SH-SY5Y cells is recognized by both Cys-Fc and anti-HNK-1, indicating that both terminal GalNAc-4-SO₄ and GlcUA-3-SO₄ are present. SH-SY5Y cells were derived from a neuroblastoma that originated in the lung and have characteristics of sympathetic neurons from the peripheral sympathetic nervous system (33–35). They nonetheless synthesize TN-R with the same two unique sulfated structures that are produced in the cerebellum and other regions of the central nervous system. It has been reported that TN-R is expressed by other neural cell lines such as PC12 cells that also originated from the peripheral nervous system (36). SH-SY5Y cells only produced a single form of TN-R that was assembled into disulfide-linked oligomers because TN-R monomers were only observed following reduction. If SH-SY5Y cells are representative of the cells producing TN-R in the central nervous system, individual molecules of TN-R may be modified with both GalNAc-4-SO₄ and GlcUA-3-SO₄ on their N-linked oligosaccharides. In this instance, the relative levels of the β1,3-glucuronotransferase and the β1,4-GalNAc-transferase would determine the extent to which TN-R is modified with one or both sulfated structures.

Our current studies demonstrate that the addition of GalNAc-4-SO₄ and GlcUA-3-SO₄ to TN-R is differentially regulated during development. HNK-1 expression was previously shown to decrease during postnatal cerebellar development (20, 37), whereas the addition of terminal GalNAc-4-SO₄ was shown to increase during late postnatal development (14). It was uncertain, however, whether the decrease in HNK-1 and increase in GalNAc-4-SO₄ in the cerebellum was because of a change in the amount of synthesis of the underlying proteins or of the carbohydrate epitopes. Our findings indicate that the levels of both TN-R and the associated HNK-1 decrease in the adult, whereas TN-R-associated GalNAc-4-SO₄ increases. Throughout postnatal development modification of TN-R with HNK-1 parallels the level of protein expression, whereas modification with terminal GalNAc-4-SO₄ is independently regulated from TN-R expression.

The relative amounts of the 180- and 160-kDa TN-R isoforms also change during cerebellar development. Whereas early in development TN-R 180 is the dominant form, later in development and in adulthood TN-R 160 is more prominent. A previous report postulated that the 160-kDa form reflects alternative splicing of the TN-R message resulting in a loss of one of the FN-III repeats (10). However, our studies indicate that TN-R 160 arises by proteolytic cleavage near the NH₂ terminus of TN-R 180 (see Fig. 3) and that TN-R 160 is no longer covalently associated with the TN-R 180 subunits of the disulfide-bonded trimer. Early in postnatal development terminal GalNAc-4-SO₄ is found predominantly on the 180-kDa form of TN-R, whereas in the adult GalNAc-4-SO₄ is predominantly on the 160-kDa form of TN-R. The increase in Cys-Fc staining intensity observed for the 160-kDa form in the adult suggests that a greater fraction of TN-R bearing terminal GalNAc-4-SO₄ undergoes proteolytic cleavage in the adult.

In summary, we have shown that TN-R undergoes a series of post-translational modifications including the addition of sulfated carbohydrates and proteolytic cleavage. Studies are now ongoing to define the role of these modifications in the modulation of adhesive and regulatory functions that have been attributed to TN-R in the CNS.

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