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Glycosylation of a CNS-specific extracellular matrix glycoprotein, tenascin-R, is dominated by *O*-linked sialylated glycans and "brain-type" neutral *N*-glycans

Susanne Zamze[3], David J.Harvey, Penka Pesheva[1,4], Taj S.Mattu, Melitta Schachner[1,5], Raymond A.Dwek and David R.Wing[2]

Glycobiology Institute, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, UK and [1]Department of Neurobiology, ETH–Honggerberg, CH-8093 Zürich, Switzerland

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[2]To whom correspondence should be addressed
[3]Present address: The Edward Jenner Institute for Vaccine Research, Compton, Newbury, Berkshire RG20 7NN, UK
[4]Present address: Department of Physiology, Neurophysiology, University of Bonn, Wilhelmstrasse 31, D-53111 Bonn, Germany
[5]Present address: Zentrum für Molekulare Neurobiologie, Institut für Biosynthese Neuraler Strukturen, Martinistrasse 52, D-20246 Hamburg, Germany

As a member of the tenascin family of extracellular matrix glycoproteins, tenascin-R is located exclusively in the CNS. It is believed to play a role in myelination and axonal stabilization and, through repulsive properties, may contribute to the lack of regeneration of CNS axons following damage. The contrary functions of the tenascins have been localized to the different structural domains of the protein. However, little is known concerning the influence of the carbohydrate conjugated to the many potential sites for *N*- and *O*-glycosylation (10–20% by weight). As a first analytical requirement, we show that >80% of the *N*-glycans in tenascin-R are neutral and dominated by complex biantennary structures. These display the "brain-type" characteristics of outer-arm- and core-fucosylation, a bisecting *N*-acetylglucosamine and, significantly, an abundance of antennae truncation. In some structures, truncation resulted in only a single mannose residue remaining on the 3-arm, a particularly unusual consequence of the *N*-glycan processing pathway. In contrast to brain tissue, hybrid and oligomannosidic *N*-glycans were either absent or in low abundance. A high relative abundance of *O*-linked sialylated glycans was found. This was associated with a significant potential for *O*-linked glycosylation sites and multivalent display of the sialic acid residues. These *O*-glycans were dominated by the disialylated structure, NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc. The possibility that these *O*-glycans enable tenascin-R to interact in the CNS either with the myelin associated glycoprotein or with sialoadhesin on activated microglia is discussed.

Key words: CNS/extracellular matrix/*N*- and *O*-glycans/sialylation/tenascin-R

Introduction

Tenascin-R (TN-R), originally named J1-160/180, janusin, in rodents (Kruse *et al*., 1985; Pesheva *et al*., 1989, 1993) and restrictin in chicken (Rathjen *et al*., 1991) is a member of the tenascin multigene family of structurally-related, modular glycoproteins of the extracellular matrix (ECM) (Chiquet-Ehrismann, 1995). Molecules of the ECM play a key role in the development, maintenance, and repair of the nervous system (Reichardt and Tomaselli, 1991; Ruoslahti, 1996). The tenascins are multifunctional molecules that can either promote or inhibit cell adhesion and neurite outgrowth in specific assays (Faissner and Kruse, 1990; Morganti *et al*., 1990; Lochter *et al*., 1991; Erickson, 1993; Taylor *et al*., 1993; Lochter and Schachner, 1997). They are prominent in the development of the nervous system, where both adhesive and anti-adhesive interactions between neurons and the ECM are essential in neuronal pattern formation.

TN-R is detected exclusively in the CNS, where it is expressed by oligodendrocytes and subpopulations of neuronal cells (Pesheva *et al*., 1989; Bartsch *et al*., 1993; Wintergerst *et al*., 1993). The protein appears mostly associated with the surface of oligodendrocytes and myelinated axons in the white matter and with perineuronal nets of interneurons and motoneurons in different parts of the mammalian CNS (Pesheva *et al*., 1989; Bartsch *et al*., 1993; Angelov *et al*., 1998). This pattern of TN-R expression suggests a role in the process of CNS myelination, stabilization, and maintenance of nerve fiber tracts and axosomatic synaptic contacts, the latter required for normal connectivity and neuronal survival. TN-R has been shown to be adhesive for oligodendrocytes (sulfatide-mediated; Pesheva *et al*., 1997) and astrocytes (Pesheva *et al*., 1989; Morganti *et al*., 1990) and anti-adhesive for activated microglia (Angelov *et al*., 1998) and CNS neurons (Pesheva *et al*., 1989, 1993; Taylor *et al*., 1993). The latter action is mediated by the interaction of TN-R with the neuronal protein F3/F11/contactin (Pesheva *et al*., 1993; Xiao *et al*., 1996, 1997b, 1998), which also acts as a receptor for the 190 kDa isoform of tenascin-C (TN-C) (Zisch *et al*., 1992). The F3/F11-mediated inhibition of neurite outgrowth by TN-R (and probably TN-C) may also contribute to the marked lack of regeneration of CNS axons following injury. The contrary functions of tenascins localize to different structural domains of the molecule (Erickson, 1993; Schachner *et al*., 1994; Faissner and Schachner, 1995; Faissner *et al*., 1996; Norenberg *et al*., 1996; Fischer *et al*., 1997).

TN-R is composed of four structural motifs. The N-terminus, unique to the tenascin family, contains a cysteine-rich segment followed by EGF-like repeats (4.5 for TN-R) and eight motifs related to fibronectin type III-like domains. At the C-terminus, TN-R contains a 220 amino acid segment similar to the β and γ chains of fibrinogen, including a Ca^{2+} binding segment (Norenberg *et al*., 1996; Carnemolla *et al*., 1996). Rat, chicken, and human TN-R (Norenberg *et al*., 1992; Fuss *et al*., 1993; Carnemolla *et al*., 1996) show a high degree of sequence homology (typically at least 75%), including conservation of potential sites for *N*-glycosylation (14 in the rat). There are also numerous potential sites for *O*-glycosylation, the proportions of serine and threonine in several of the fibronectin type III domains indicating, moreover,

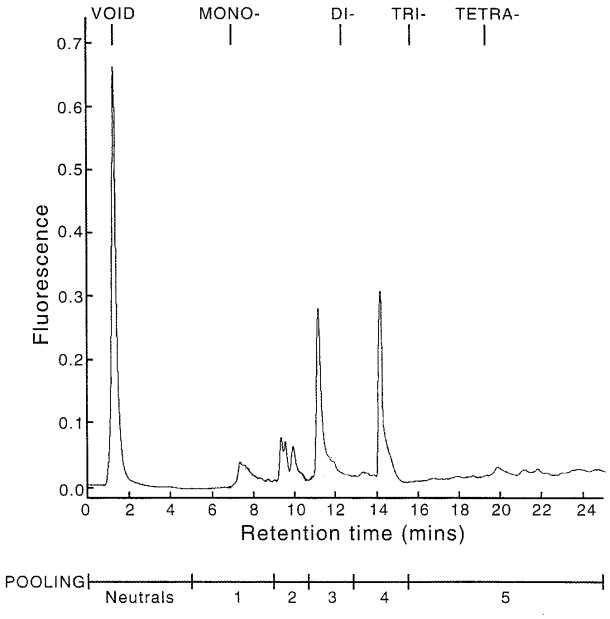

Fig. 1. WAX-HPLC of the total glycans released from TN-R. Fractions were collected, as shown at the bottom of the figure, into neutrals and pools 1–5. The column void and the elution positions of mono-, di-, tri-, and tetra-sialylated N-linked glycans obtained from fetuin are indicated at the top.

regions for potential *O*-linked domains (Wilson *et al*., 1991). Although tenascins contain in the region of 10–20% by weight of carbohydrate, nearly all structural and functional analyses to date have concentrated exclusively on the polypeptide. The possibility that binding between lecticans (e.g., the chondroitin sulfate proteoglycans versican, brevican) and TN-R may be mediated through a C-type lectin–carbohydrate interaction has been studied. However, the potential role of the glycoconjugate ligands of TN-R (e.g., the HNK-1 carbohydrate, containing a 3-sulfated glucuronic acid; see Schachner and Martini, 1995, for review) remained unclear (Aspberg *et al*., 1995, 1997).

For an understanding of the details of molecular interactions involving the glycans of TN-R, it is first necessary to describe the nature of the glycosylation of the glycoprotein. The results are presented in this paper for TN-R prepared from adult mouse brain and their potential biological significance is discussed.

Results

The total *N*- and *O*- linked glycans, released by hydrazinolysis, were subjected to weak anion-exchange (WAX) HPLC (Figure 1). A major, voided peak of neutral glycans was observed, followed by peaks eluting in the mono- to tri- sialyl region of the chromatogram (fractions 1–4), as determined by reference to standard sialylated glycans obtained from fetuin. Subsequently, a broad spectrum of components was seen, in low abundance, eluting from the tri-sialyl to the more highly charged regions of the chromatogram (fraction 5). The WAX HPLC partially resolves components according to size as well as charge, with smaller structures being retained to a greater extent than larger structures with the equivalent number of charges (Guile *et al*., 1994).

Neutral N-glycans

The Bio-Gel P4 profile of neutral *N*-linked glycans from TN-R, obtained from the WAX HPLC void fraction, is shown in Figure 2. Most (>80%) of the *N*-linked glycans released from TN-R were neutral (data not shown; retarded, acidic fractions of WAX HPLC, Figure 1, were mainly *O*-linked glycans; see below). Fractions a–f were collected preparatively as shown (Figure 2) and were analyzed by normal phase (NP-) and reverse phase (RP-) HPLC, matrix-assisted laser desorption/ionization (MALDI-) MS and exoglycosidase sequencing as described previously (Chen *et al*., 1998). The NP-HPLC profiles of P4 fractions a, c, d, and e/f are shown in Figure 3 and MALDI-MS data obtained from most of the NP-HPLC peaks are in Table I. P4 fraction b, as a shoulder of c (Figure 2), gave a similar NP-HPLC profile (not shown) to c (Figure 3). Overall the NPGU values (Figure 3), isobaric monosaccharide compositions (Table I), and sequential effects of the enzymatic digestions indicated that "truncated" complex biantennary glycans (Figure 4), similar or identical to those identified as major components of rat brain tissue (Chen *et al*., 1998) were particularly abundant. Truncation of the 3-arm as far as the mannose was observed in some structures, based on the use of *Diplococcus pneumoniae* β-hexosaminidase at arm-specific concentrations in the presence of a bisecting GlcNAc (see *Materials and methods*). The "boxed" structures shown in Figure 4 were major components of the neutral *N*-linked glycan pool from TN-R.

Table I. Masses and compositions of the neutral N-linked glycans found in TN-R arranged in order of complexity

		Mass[1]		Comp.[2]		
No.[3]	Fract.[4]	Found	Calc.	H	N	F
1	ef	*1377.6*	1377.5	5	2	0
2	ef	*1540.0*	1539.6	6	2	0
3	ef	—	1256.5	3	3	0
4	ef	*1402.5*	1402.6	3	3	1
5	ef	*1460.2*	1459.6	3	4	0
6	d,c	1605.4	1605.4	3	4	1
7	c	1605.7	1605.4	3	4	1
8	c	1768.2	1767.7	4	4	1
9	c	*1913.9*	1913.7	4	4	2
10	b	1784.1	1783.7	5	4	0
11	b	1930.3	1929.7	5	4	1
12	c	*1808.7*	1808.7	3	5	1
13	b	1971.4	1970.8	4	5	1
14	b	—	2132.8	5	5	1
15	b	—	2116.8	5	5	2
16	a	*2149.0*	2148.8	6	5	0

[1]Monoisotopic mass of the MNa^+ ion. Masses in italics were recorded with the AutoSpec instrument, other masses were recorded with the Voyager instrument. -, Minor compound (Figure 4) analyzed by sequential exoglycosidase digestion only.
[2]Monosaccharide composition: H, hexose (mannose, galactose); N, HexNAc (GlcNAc); F, deoxyhexose (fucose).
[3]Compound number for structure in Figure 4.
[4]P4 Fraction (Figure 2). Due to poor resolution on Bio-Gel P4, many of the compounds found in Fraction c (nos. 7, 8, 9, and 12) also contributed significantly to Fraction b.

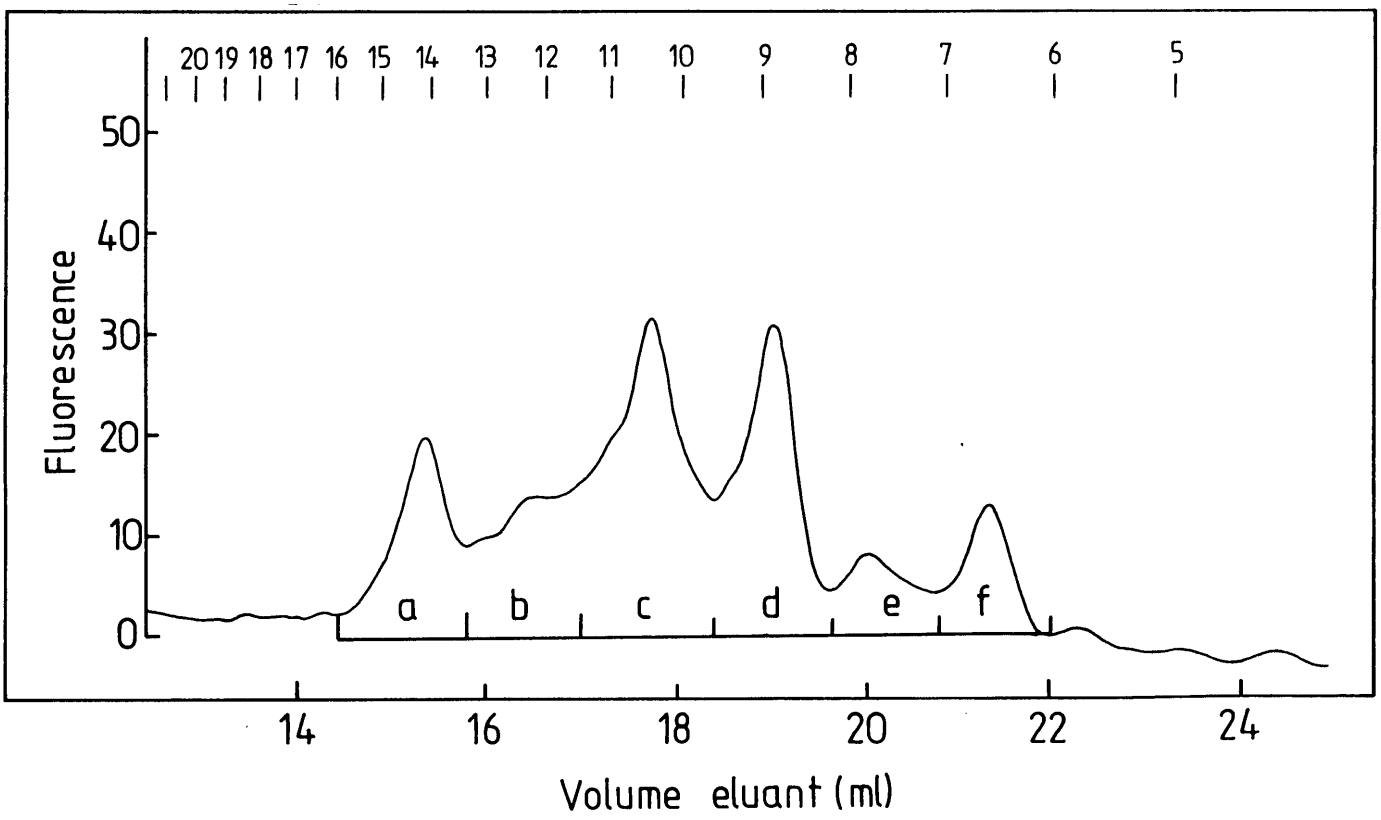

Fig. 2. BioGel P4 chromatogram of the neutral N-linked oligosaccharides from TN-R. Fractions a-f were pooled as shown for further analysis. The numbers at the top indicate the elution positions of glucose oligomers (P4 glucose units, P4GU) from a partial dextran hydrolysate, 2AB-labeled. Due to a slight charge interaction with the matrix, the presence of the 2AB-fluorescent label decreases the glucose unit value of a given oligosaccharide by 2.65 compared with the corresponding nonlabeled alditol. The void volume containing acidic glycans is not shown.

Oligomannosidic glycans $Man_{(6–5)}GlcNAc_2$ were the main components of P4 fractions e and f, respectively, and were the only constituents detected from this glycan family. Thus, in contrast to brain tissue, larger oligomannosidic glycans $Man_{(9–7)}GlcNAc_2$ were completely absent from TN-R, as were hybrid structures.

P4 fraction b contained a complex mixture of the more highly processed outer-arm- and core-fucosylated biantennary structures ± bisecting GlcNAc (Figure 4), similar to those in whole brain (Chen *et al*., 1998) and in fraction c (Figure 3). Fraction a contained a single component identified as a (2,4)-branched galactosylated triantennary glycan without either outer-arm or core fucosylation. Digestion with *Diplococcus pneumoniae* β-galactosidase removed three Gal residues. The product coeluted by HPLC with a (2,4)-branched agalactosylated triantennary structure and was sensitive to digestion with *Diplococcus pneumoniae* β-hexosaminidase at 0.01 U/ml with the removal of two β(1,2)-linked GlcNAc residues. This structure (though present as a minor constituent) had not been observed as a major component in the whole brain (Chen *et al*., 1998). It is possible that partially galactosylated, outer-arm fucosylated triantennary glycans such as found in rat brain (Chen *et al*., 1998) were contained in pool b, but these would only have been present as trace components.

Acidic glycans

Acidic *N*- and *O*- linked glycans were voided on the BioGel P4 column and were separated analytically by NP-HPLC (Figure 5a). Essentially all components eluting after the void and by 65 min (depicted by the horizontal bar) were *O*-linked glycans. The later-eluting region contained the much less abundant *N*-linked oligosaccharides. Separation of the acidic glycans, primarily by charge, was performed by WAX-HPLC and was illustrated in Figure 1. Each acidic pool was collected separately and was analyzed by NP-HPLC. This was coupled, in specific cases, with exoglycosidase sequencing and the following results were obtained.

WAX pools 1 and 2. These relatively minor pools of the WAX HPLC profile (Figure 1) contained essentially *N*-linked and *O*-linked glycans, respectively, as was indicated by the corresponding late and early retention times on NP-HPLC (not shown). Resistance of these profiles to change after incubation of the glycan fractions with *Arthrobacter ureafaciens* neuraminidase indicated that, in both cases, the carbohydrates were not sialylated but carried a different monoanionic charge (see *Discussion*).

WAX pool 3. This pool revealed homogeneity on NP-HPLC (Figure 5b) and showed complete neutralization after digestion with NDV neuraminidase. The 2-aminobenzamide (2-AB)-labeled product was identified by RP-HPLC (by reference to the standard profile in Figure 6a) as the monosaccharide galactose (not shown; see *Discussion*) and the original glycan as NeuAc α2–3 Gal.

WAX pool 4. This pool also showed homogeneity on NP-HPLC (Figure 5c). Incubation with Newcastle disease virus (NDV)

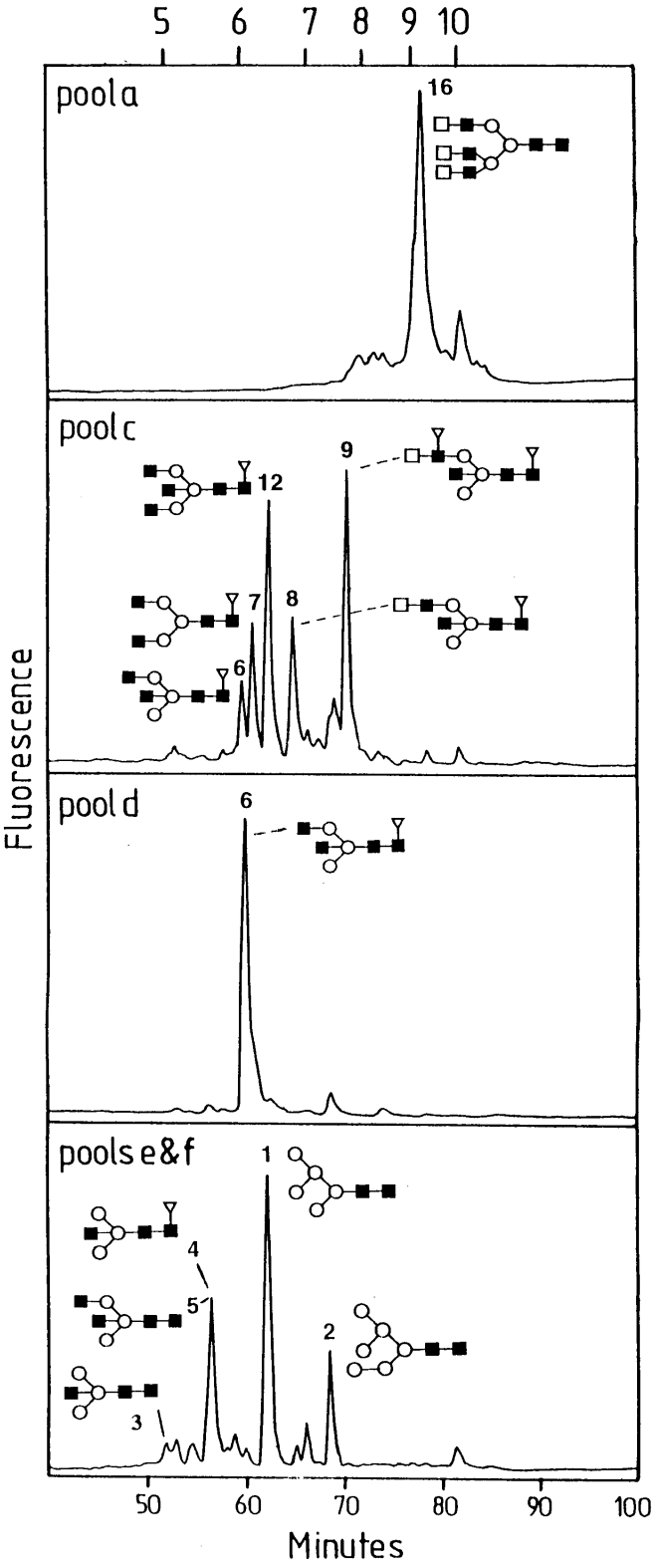

Fig. 3. NP-HPLC profiles of neutral N-glycan fractions from TN-R. Fractions shown are pools a, c, d, and e & f from BioGel P4 chromatography (see Figure 2). Numbers adjacent to peaks indicate compound number in Figure 4 (and Table I). Symbols: triangles, fucose; solid squares, GlcNAc; open circles, mannose; open squares, galactose. The numbers at the top of the NP-HPLC profiles indicate the elution positions of glucose oligomers (normal phase glucose units, NPGU) from a partial dextran hydrolysate, 2AB-labeled.

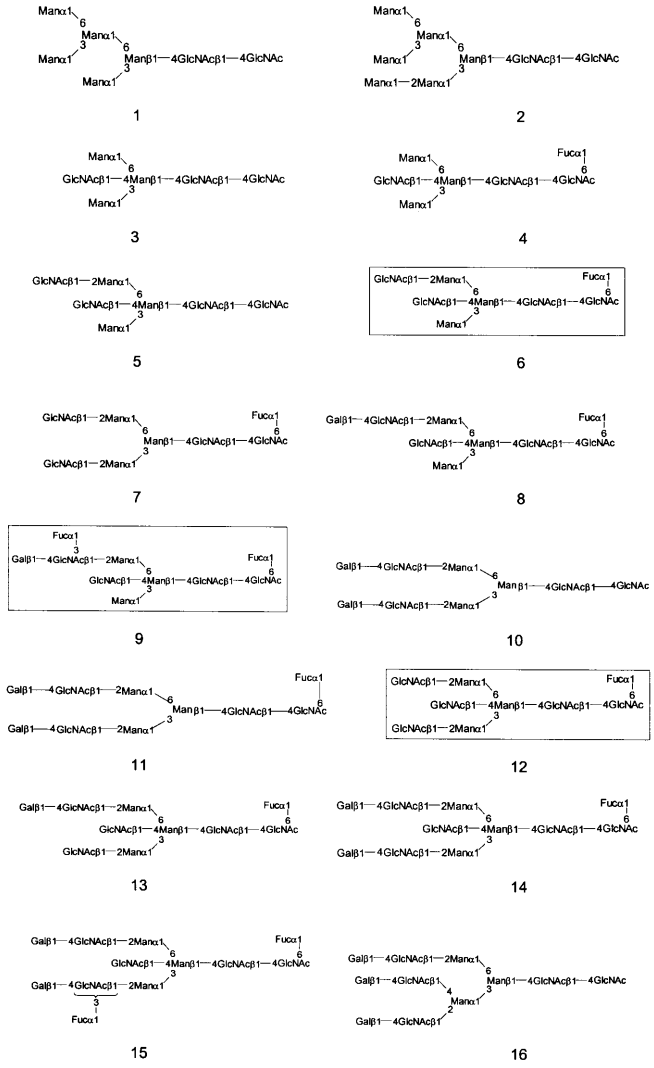

Fig. 4. Structures of the neutral N-glycans of TN-R. The compound numbers correspond to those given in Table I and to those peak numbers given in Figure 3. Components in boxes represent the most abundant glycans.

neuraminidase caused a loss of one NeuAc residue (not shown), and subsequent digestion with *Arthrobacter ureafaciens* neuraminidase produced complete neutralization. The neutral product coeluted with standard 2AB-labeled Galβ1–3GalNAc on both NP- and RP-HPLC. It was resistant to incubation with jack bean β-galactosidase but was digested by bovine testes β-galactosidase. Confirmation of the 2AB-labeled carbohydrate residue as GalNAc was provided by RP-HPLC (Figure 6b). The structure NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc was therefore proposed. This was confirmed when the glycan coeluted with the authentic disialylated oligosaccharide (see *Materials and methods*) on NP- and RP- HPLC. Further confirmation was obtained in parallel studies using the glycan labeled with tritium in its reduced state. In this case, the reducing terminal monosaccharide was detected as its alditol acetate by gas chromatographic analysis (data not shown).

WAX pool 5. This broad pool of minor components contained a mixture of *N*- and *O*-linked glycans. All appeared to be sialylated as there was a substantial change in the NP-HPLC profile after *A.ureafaciens* neuraminidase digestion (not shown). However, no

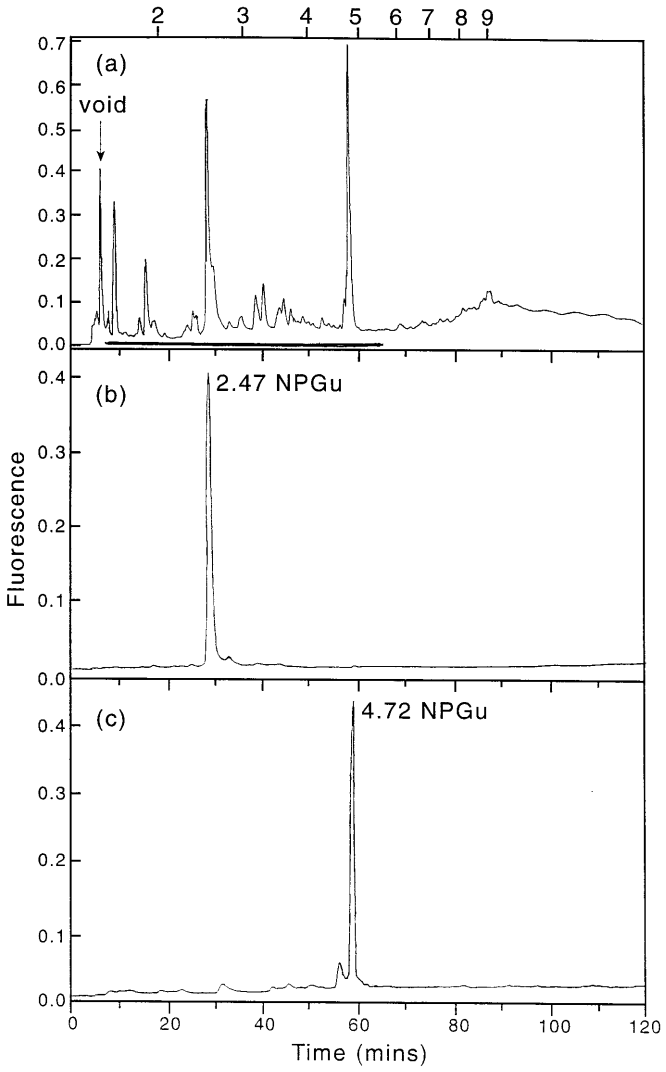

Fig. 5. NP-HPLC profiles of 2AB-labeled acidic glycans of TN-R. (**a**) Total acidic glycans. The horizontal bar indicates the elution period for *O*-linked glycans. (**b**) WAX pool 3. (**c**) WAX pool 4. The numbers at the top indicate the elution positions of glucose oligomers (NPGU) from a partial hydrolysate of dextran, 2AB-labeled.

glycans were neutralized by this treatment, indicating that all contained at least one other type of anionic charge.

The acidic *N*-glycans, detected in WAX pools 1 and 5 only, were present in a relatively low overall abundance and showed a high degree of structural heterogeneity. More than 90% contained charged groups other than or in addition to NeuAc. On account of the low abundance and complexity of these structures, further analysis was not performed.

Discussion

This study addresses the characterization of the most abundant glycans expressed on TN-R from adult mouse brain. The glycans comprised neutral *N*-linked oligosaccharides and *O*-linked sialylated structures.

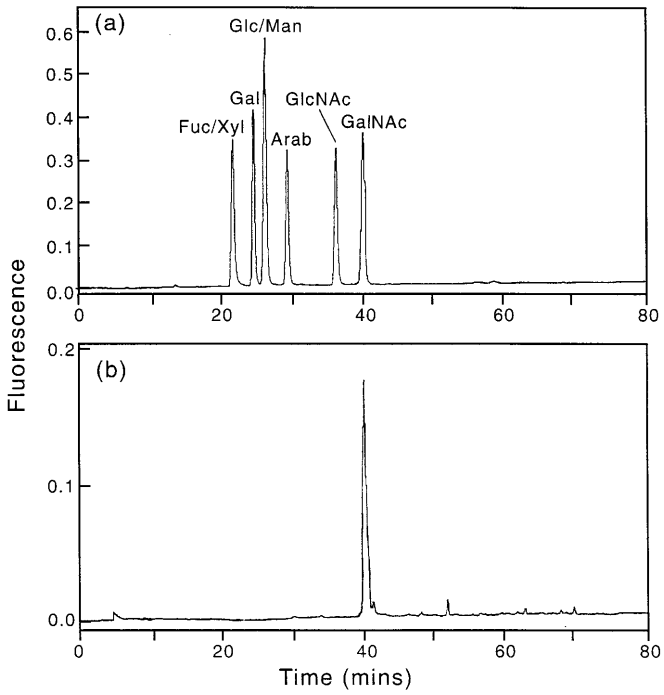

Fig. 6. RP-HPLC of 2AB-labeled monosaccharides. (**a**) Profile for a mixture of common standard monosaccharides. Abbreviations: Fuc, fucose; Xyl, xylose; Gal, galactose; Glc, glucose; Man, mannose; Arab, arabinose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine. (**b**) Profile for the reducing terminal monosaccharide of the component in WAX pool 4. The peak has a retention time corresponding to that of 2AB-labeled GalNAc.

N-Linked glycans

The neutral *N*-linked glycans comprised >80% of the *N*-linked oligosaccharide pool and consisted mainly of complex biantennary structures with outer-arm and core fucosylation, bisecting GlcNAc residues and arm truncation. The truncated complex biantennary glycans were major components of TN-R and are highlighted as boxed structures in Figure 4. These glycans were detected earlier in whole brain tissue (Shimizu *et al*., 1993; Chen *et al*., 1998). The involvement of N-acetylglucosaminyltransferase I in substituting the 3-arm mannose with GlcNAc is a prerequisite for subsequent N-glycan processing, and it was proposed that the absence of this monosaccharide may indicate the presence of a novel, specific β-hexosaminidase in brain tissue (Shimizu *et al*., 1993; Chen *et al*., 1998). The present results indicate that such a specific activity is likely to be prevalent in oligodendrocytes from which cell type TN-R is mainly expressed.

In contrast to brain tissue (Chen *et al*., 1998), hybrid structures were not expressed on TN-R. Moreover, oligomannosidic glycans, known to be expressed on several neural glycoproteins (Fahrig *et al*., 1990; Horstkorte *et al*., 1993), including synaptic glutamate receptors (Clark *et al*., 1998), were relatively minor components, consisting only of $Man_{(6-5)}GlcNAc_2$. Thus, the *N*-glycan processing pathway was directed almost entirely towards the production of complex oligosaccharides only. This suggested that there was an extensive array of processing enzymes in the oligodendrocyte cell and good accessibility to the glycosylation sites on TN-R for the completion of the processing.

TN-R contained one complex glycan, in addition to those described above, which was not identified as a major structure in rat brain tissue (Chen *et al*., 1998). This component was a

(2,4)-branched nonfucosylated triantennary oligosaccharide. The sialylated counterpart of this structure was seen, however, in rat brain (Zamze *et al*., 1998).

Most of the minor fraction of acidic *N*-glycans from TN-R possessed anionic charges in addition to NeuAc, with <10% containing NeuAc, either α2,3- or α2,6-linked, as their sole charged group. In accordance with this observation, TN-R is known to express the L2/HNK-1 epitope (Kruse *et al*., 1985; Pesheva *et al*., 1989), a characteristic carbohydrate structure of many neural glycoproteins based on a terminal 3-sulfated glucuronic acid moiety. It is concluded that those glycans carrying this epitope on TN-R were probably present in minor and heterogeneous fractions.

O-Linked acidic glycans

The most striking observation of the glycosylation of TN-R was the relatively large abundance of *O*-linked acidic glycans, and particularly the disialylated structure NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc (WAX pool 4, Figure 1). Potential *O*-linked domains (Wilson *et al*., 1991) could be seen within the region of the TN-R molecule containing the fibronectin type-III domains (Fuss *et al*., 1993). This type of glycosylation could be important in giving TN-R an extended three-dimensional structure (Jentoft, 1990) with a display of complex carbohydrates—in this case bearing sialic acid residues—presented in multivalent form. These would provide much higher avidities in carbohydrate–protein interactions than in monovalent form.

WAX pool 3 (Figure 1) also contained an abundant *O*-linked acidic glycan identified as NeuAcα2–3Gal. This probably resulted from base peeling of parent structures (such as that described above) during the hydrazinolysis procedure (Mattu *et al*., 1998). The other *O*-linked acidic structures described were relatively minor. Those present in WAX pool 2 (Figure 1) resisted digestion with *A.ureafaciens* neuraminidase and, from the retention time on WAX HPLC, most probably carried a single sulfate. The minor populations in WAX pool 5 (Figure 1), with a late retention time, were heterogeneous in their anionic charge, being sensitive to the neuraminidase but without being neutralized. It is possible that such *O*-linked structures could also carry the L2/HNK-1 carbohydrate epitope (Yuen *et al*., 1997).

The myelin-associated glycoprotein (MAG) is the major CNS-located member of the family of lectins recognizing sialic acids (Kelm *et al*., 1994; Probstmeier and Pesheva, 1999). Its specificity of recognition has been demonstrated by Yang *et al*. (1996) using a variety of gangliosides and related glycosphingolipids. An α2–3-N-acetylneuraminic acid residue on the terminal galactose was necessary for binding and additional sialic acid residues on the other core saccharides contributed significantly. (Additional supporting evidence has been provided recently by Strenge *et al*., 1998.) The physiological sugar ligands for MAG recognition are believed to be conjugated to protein (Kelm *et al*., 1994) since the interaction was trypsin-sensitive. Clearly the multivalent display of the disialylated *O*-linked glycan described above on TN-R would strongly point to this carbohydrate as a potential ligand for MAG. This possibility is accentuated by the fact that MAG is present in the noncompacted myelin loops at the node of Ranvier, where tenascin is accumulating (Bartsch *et al*., 1992, 1993). The elasticity of the tenascins (Oberhauser *et al*., 1998) additionally would make this type of molecule ideally suited for an intermediary role in such a process as myelination, substantiating results using oligodendrocytic cells (Jung *et al*., 1993; Pesheva *et al*., 1997).

In addition, the present results suggest that sialoadhesin may represent another cellular receptor for TN-R on activated microglia. This I-type lectin, known to favor recognition of sialic acids in α 2–3 linkage (Crocker *et al*., 1991; Kelm *et al*., 1994), is normally absent in the adult CNS. It becomes expressed, however, by a subpopulation of brain microglia/macrophages upon CNS injury (Perry *et al*., 1992) and is likely to mediate the repellent action of TN-R on activated microglia reported recently, i.e., such interaction may play a role in the proposed function of TN-R in neuronal protection against these cells (Angelov *et al*., 1998). During neurodegeneration following peripheral nerve axotomy, the downregulation of TN-R in the perineuronal net of motoneurons would (1) result in an impaired structural integrity of this specialized ECM, due to a disruption of the assembly of TN-R with ECM proteins and chondroitin sulfate proteoglycans, such as versican and phosphacan (Celio and Blumcke, 1994; Aspberg *et al*., 1997; Xiao *et al*., 1997a; Milev *et al*., 1998), and, as a consequence, (2) affect neuronal function and/or survival.

Materials and methods

Materials

All exoglycosidases were purchased from Oxford GlycoSciences (Abingdon, Oxon, UK) except for *Charonia lampas* α-fucosidase which was prepared in the Oxford Glycobiology Institute. Oligosaccharide standards were obtained from Oxford GlycoSciences and were fluorescently labeled with 2-AB as described below. Additional standards were prepared by exoglycosidase digestion of A2G0FB with *Diplococcus pneumoniae* β-hexosaminidase, 0.01 U/ml, to remove selectively the GlcNAc residue linked to the Man α(1–3)-arm, and digestion of A2G0FB with *C.lampas* α-fucosidase to remove core fucose. A disialylated tetrasaccharide standard, NeuAcα2–3Galβ1–3(NeuAcα2–6)-GalNAc, was obtained from IgA1 (Mattu *et al*., 1998).

Tenascin-R

TN-R was immunoaffinity purified from adult mouse brains as described previously (Pesheva *et al*., 1989). After dialysis against 0.1 M ammonium bicarbonate pH 7.0, the sample was lyophilized in preparation for anhydrous hydrazinolysis.

Release and labeling of oligosaccharides

The release of oligosaccharides from TN-R (1–2 mg) by small scale hydrazinolysis was carried out essentially as described previously (Ashford *et al*., 1987; Parekh *et al*., 1987) for the optimal release of *N*- and *O*-linked glycans (Patel *et al*., 1993). Oligosaccharides were fluorescently labeled with 2-AB by reductive amination according to the method of Bigge *et al*. (1995), using an Oxford GlycoSciences Signal labeling kit and following the manufacturer's instructions. Fluorescence was measured at λ_{em}420 nm and λ_{ex}330 nm.

Bio-Gel P4 gel filtration chromatography

The size fractionation of neutral glycans by Bio-Gel P4 gel filtration chromatography in water was performed as described previously (Ashford *et al*., 1987; Parekh *et al*., 1987). Neutral glycans are resolved by this method and are separated from acidic glycans which elute in the void volume of the column. Fluorescently labeled neutral oligosaccharides were analyzed on an Oxford GlycoSciences GlycoMap 1000 equipped with an on-line fluorescence monitor.

HPLC

Anion-exchange HPLC was carried out using a Vydac 301VHP575 7.5 × 50 mm WAX column (Hichrom Ltd., Reading, UK) with ammonium formate at pH 9.0 at a flow rate of 1 ml/min (Guile *et al*., 1994). The following linear stepwise gradient was used: an increase from 0 to 25 mM formate over 12 min, followed by increases to 105 mM over 13 min, 400 mM over 25 min, 500 mM over 5 min, and then held for 5min.

NP-HPLC of neutral 2-AB-labeled N-linked glycans was performed using a polyhydroxyethyl aspartamide column as described previously (Chen *et al*., 1998). Structures were assigned normal phase glucose unit (NPGU) values in relation to external calibration with a 2-AB-labeled partial hydrolysate of dextran (Guile *et al*., 1996). NP-HPLC of acidic 2-AB-labeled *N*- and *O*- linked glycans was also performed using a polyhydroxyethyl aspartamide column using conditions described in Guile *et al*. (1996).

RP-HPLC was performed on a Hypersil BDS C_{18} column as described previously (Chen *et al*., 1998) using 50 mM ammonium formate as solvent A and acetonitrile as solvent B. The following gradient conditions were used for the separation of 2-AB-labeled *N*- and *O*-linked glycans: an increase of 2–4% B over 25 min, followed by an increase of 4–8% B over 65 min and a further increase of 8–12% B over 5 min. The flow rate was 0.5 ml/min. This was then held at the final conditions for 5 min at 1 ml/min. For the separation of 2-AB-labeled monosaccharide residues: a linear gradient was used of 0–4% B over 50 min, followed by an increase of 4–9% B over 42 min and a further increase of 9–12 % B over 3 min. The flow rate was 0.5 ml/min. The final conditions were held for 5 min at 1 ml/min.

Mass spectrometry

Positive ion matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed on selected peaks from the NP-HPLC separations on either a Micromass AutoSpec-QFPD magnetic sector mass spectrometer or a PerSeptive Biosystems Voyager Elite mass spectrometer operated in the reflectron mode with delayed extraction. Samples were prepared by mixing 0.5 µl of the glycan solution, in water, with 3.0 µl (AutoSpec) or 0.5 µl (Voyager) of a saturated solution of 2,5-DHB in acetonitrile, allowing the mixture to crystallize and then recrystallizing it from ethanol. Spectra were acquired on the AutoSpec instrument with the array detector in the high resolution position to provide monoisotopic masses. The mass range was chosen to be appropriate to the compounds to be detected and spectra were accumulated until a satisfactory signal:noise ratio was obtained. During spectral acquisition, the laser spot was moved manually over the target surface to compensate for sample depletion. Spectra on the Voyager instrument were acquired with an accelerating voltage of 20 kV, a pulse decay of 75 ns, and a grid voltage of 70%; 256 laser shots were fired to produce each spectrum.

Exoglycosidase digestions

The digestion of 2-AB-labeled oligosaccharides was carried out in a volume of 20 µl for 18 h at 37 °C using the following enzymes: *D.pneumoniae* β-galactosidase, 0.4 mU/ml in 100 mM sodium acetate pH 5.5; bovine testes β-galactosidase 10 mU/ml in 100 mM citrate-phosphate, pH 4.0; jack bean β-hexosaminidase 10 U/ml in 100 mM citrate-phosphate, pH5.0; *Aspergillus saitoi* α(1,2)-mannosidase 1 mU/ml in 100 mM sodium acetate, pH5.0; almond meal α-fucosidase 1 mU/ml in 100 mM sodium acetate, pH 5.0 and *Charonia lampas* α-fucosidase 10 mU/ml in 50 mM sodium acetate, pH 4.5 containing 0.15 M sodium chloride. *D.pneumoniae* β-hexosaminidase was used at either 10 U/ml or, for conditions providing linkage specificity, at 0.01 U/ml in 100 mM citrate-phosphate, pH 6.0 (Yamashita *et al*., 1992). At the lower concentration, only GlcNAc residues linked β (1–2) to Man are cleaved, with two provisos: (1) that β (1–2) GlcNAc is not cleaved if the Man to which it is attached is also substituted at the C-6 position and (2) that β(1–2) GlcNAc linked to the Man α(1–6)-arm of the tri-mannosyl core is not cleaved in the presence of a bisecting GlcNAc.

Jack bean α-mannosidase was used at either 25 U/ml or 10 U/ml in 100 mM sodium acetate pH 5.0 containing 2 mM Zn^{2+}. At the lower concentration R-Manα1–6(Manα1–3)-Manβ1–4GlcNAcβ1–4GlcNAc but not R-Manα1–3(Manα1–6)-Manβ1–4GlcNAcβ1–4GlcNAc is susceptible where R is not H or Man (Yamashita *et al*., 1980). Mixed digests of *D.pneumoniae* β-galactosidase (0.4 U/ml) and almond meal α-fucosidase (1 mU/ml) were carried out in 100 mM sodium acetate, pH 5.5.

Digestion of acidic glycans with NDV neuraminidase (specificity for α2–3(8)NeuAc) was performed at 0.2 U/ml in 50 mM sodium acetate, pH 5.5; and *Arthrobacter ureafaciens* neuraminidase (specificity for α2–6(3,8)NeuAc) was used at 2 U/ml in either 100 mM ammonium acetate or 50 mM sodium acetate, pH 5.0.

Reactions were terminated and samples deproteinated using 0.45 µm cellulose nitrate Pro-Spin Micro centrifugal filters. After application to the filter, samples were left at room temperature for 60 min. Glycans were then recovered by centrifugation three times with 30 µl 5% acetonitrile.

Monosaccharide analysis

The identity of the terminal reducing monosaccharide of the *O*-linked glycans was determined (1) by RP-HPLC of the 2-AB-labeled monosaccharide following complete exoglycosidase digestion of the glycan to its reducing monosaccharide unit and (2) by GC of the radiolabeled alditol acetate following reduction of the glycans with ^{3}H-labeled sodium borohydride and acid hydrolysis (Ashford *et al*., 1987, and references therein).

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Abbreviations

A2G0FB, agalactosylated biantennary N-glycan with core fucose and bisecting N-acetylglucosamine; 2-AB, 2-aminobenzamide; CNS, central nervous system; 2,5-DHB, 2,5-dihydroxy benzoic acid; ECM, extracellular matrix; EGF, epidermal growth factor; GC, gas chromatography; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; GU, glucose unit; IgA, immunoglobulin A; MAG, myelin-associated glycoprotein; MALDI-MS, matrix-assisted laser desorption/ionization-mass spectrometry; Man, mannose; NDV, Newcastle disease virus; NeuAc, N-acetyl neuraminic acid; NP, normal phase; RP, reverse phase; TN-C, tenascin-C; TN-R, tenascin-R; WAX, weak anion exchange.

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