

## Structure of the HNK-1 Carbohydrate Epitope on Bovine Peripheral Myelin Glycoprotein P0\*

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Hans Voshol†, Carol W. E. M. van Zuylen‡, Georg Orberger, Johannes F. G. Vliegthart‡, and Melitta Schachner¶

From the Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, CH-8093 Zürich, Switzerland and the ‡Department of Bio-Organic Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, P. O. Box 80.075, NL-3508 TB Utrecht, The Netherlands

The HNK-1 carbohydrate epitope, expressed by many neural recognition molecules, is involved in cell interactions that control cell type-specific neurite outgrowth and regeneration. It is also the target for autoimmune IgM antibodies in demyelinating neuropathies of the peripheral nervous system in humans. Despite its acknowledged importance in cell interactions, the HNK-1 carbohydrate structure, when expressed on glycoproteins, is still unknown. Here, we describe the structure of one of the predominant HNK-1-bearing glycans of bovine P0. The epitope consists of the sulfated trisaccharide  $\text{SO}_4\text{-3GlcA}\beta\text{1-3Gal}\beta\text{1-4GlcNAc}$ , attached to the  $\alpha\text{1-6}$  arm of a diantennary core with a bisecting *N*-acetylglucosamine. It is the first example of a terminal 3-sulfated glucuronic acid on an asparagine-linked carbohydrate. Because the similarity between the glycoprotein-derived structure and the glycosphingolipids carrying HNK-1 is restricted to the terminal sulfated trisaccharide, we conclude that this element is sufficient for HNK-1 immunoreactivity. Knowledge of the HNK-1 structure on proteins is an important prerequisite for the elucidation of its functional role in development and disease.

Peripheral neuropathies in humans can be accompanied by high levels of IgM autoantibodies in the circulation (1). Initially, these autoantibodies were described to be directed against the myelin-associated glycoprotein, but in many cases the actual antigen turned out to be a carbohydrate, related to the HNK-1 epitope (1). This carbohydrate, which was first described as an antigen of human natural killer cells (2), is also present on other glycoconjugates in myelin, such as P0 and the HNK-1 glycolipids (3). The correlation between the HNK-1

autoantibodies and the damage to nervous tissue was recently confirmed by the observation that antibodies from human patients cause demyelination in chicken (4). Besides its role in neuropathies, the HNK-1 carbohydrate has been recognized as an important mediator of molecular recognition in normal development of the nervous system. It is expressed by recognition molecules of the immunoglobulin superfamily, members of the tenascin family, integrins, and proteoglycans (5), and it binds to certain isoforms of laminin (6) and to P- and L-selectins (7). There are also indications that the HNK-1 carbohydrate is involved in the maintenance of the blood-brain barrier (8). Differential expression of the epitope in motor and sensory axons of the femoral nerve in adult mice appears to be responsible for guidance of regenerating axons after a lesion.<sup>1</sup> Furthermore, the HNK-1 carbohydrate represents a molecular memory of degenerating myelinating Schwann cells associated with the motor axons before the lesion (9). The HNK-1 epitope is found on the single *N*-glycan of P0, the glycoprotein which is responsible for formation and maintenance of peripheral myelin (10). Carbohydrates containing the HNK-1 determinant appear to be of functional importance for P0, since this glycoprotein loses its adhesive properties, when the biosynthesis of complex-type carbohydrates is blocked (11, 12). Moreover, there is evidence that homophilic binding between P0 molecules is partly mediated by a lectin-like interaction between P0 and the HNK-1 carbohydrate (13). So far, detailed information on the carbohydrate structure of the epitope has been restricted to the glycolipid-bound form (14). Attempts to identify the HNK-1 carbohydrate on glycoproteins, using mass spectrometry (15) or various chromatographic techniques (16–18), have not provided the conclusive data that are necessary for a rigorous elucidation of its role in development and disease. In this study, we describe the structure of the major HNK-1 reactive oligosaccharide of bovine P0.

### EXPERIMENTAL PROCEDURES

**Isolation of P0**—Bovine sciatic nerves (40 g) were ground to a fine powder under liquid nitrogen and suspended in 250 ml of a 0.29 M sucrose solution. The suspension was layered in 25-ml aliquots over 15 ml of 0.85 M sucrose and centrifuged for 1 h at  $100,000 \times g$  at 4 °C. Myelin was collected from the interface between the two sucrose layers and washed twice with  $\text{H}_2\text{O}$  to remove sucrose. The myelin was delipidated by two extractions with chloroform/methanol. The methanol/water phase with the precipitated protein was collected and dried by evaporation under reduced pressure. The delipidated myelin was quickly frozen in and homogenized under liquid nitrogen. After suspension in 100 ml of phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) with 1% SDS, the mixture was heated for 10 min at 100 °C and incubated at room temperature under continuous mixing. After 16–40 h, the myelin suspension was centrifuged for 1 h at  $100,000 \times g$  to remove undissolved material and concentrated by ultrafiltration (YM10 membrane, Amicon). P0 was purified from the solubilized myelin proteins by sequential gel filtration on a  $50 \times 1.6\text{-cm}$  Superose 12 prep grade column and on a  $60 \times 1.6\text{-cm}$  Superdex 75 prep grade column (Pharmacia Biotech Inc.), run in phosphate-buffered saline with 0.1% SDS.

**Release and Isolation of the Carbohydrate Chains**—After adding SDS and 2-mercaptoethanol (0.5% each), the purified P0 preparation (1–2.5 mg/ml) was boiled for 5 min, diluted 1:1 with enzyme buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.4) containing 2% Nonidet P-40, and

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‡ Present address: Ciba-Geigy AG, K681–5.03, Postfach, CH-4002, Basel, Switzerland.

¶ To whom correspondence should be addressed. Tel.: 41-1-633-3396; Fax: 41-1-633-1046.

<sup>1</sup> T. M. Brushart, R. Martini, and M. Schachner, unpublished observations.

treated with 1 unit/ml PNGase F<sup>2</sup> (EC 3.5.1.52, Boehringer Mannheim) for 20–40 h at room temperature. To remove proteins and non-ionic detergents, the PNGase F digest was extracted twice with chloroform/methanol. The combined water/methanol phases were dried and desalted on Bio-Gel P2 (Bio-Rad). Further fractionation, by high pH anion-exchange chromatography (HPAEC) (19), was performed using a CarboPac PA 100 column (Dionex DX-500 system, Dionex, Sunnyvale, CA) with a 30-ml gradient from 100 mM to 600 mM sodium acetate in 0.1 M NaOH. Corresponding fractions from different runs were pooled, neutralized, and desalted on P2.

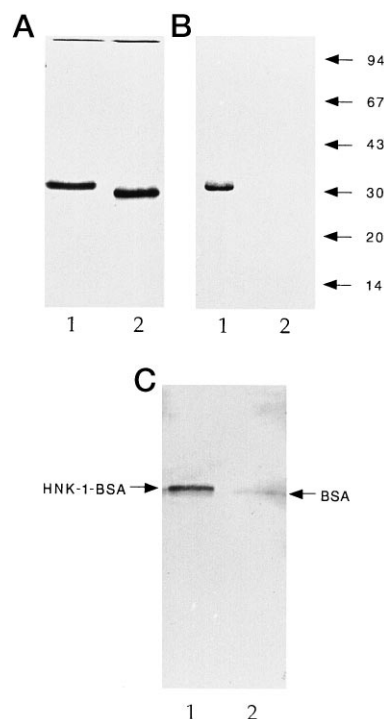
**Immunostaining of HNK-1**—For immunostaining, proteins were separated by SDS-PAGE and transferred to nitrocellulose. Optimal transfer of P0 was observed in a transfer buffer containing 27 mM sodium phosphate and 10% methanol, pH 6.5. To test their HNK-1 reactivity, glycans isolated from P0 were coupled to BSA by reductive amination (20). The HNK-1 epitope was detected with the 412 (L2) (21) HNK-1 monoclonal antibody.

**NMR Spectroscopy**—Prior to NMR-spectroscopic analysis in <sup>2</sup>H<sub>2</sub>O, samples were exchanged twice in 99.9% <sup>2</sup>H<sub>2</sub>O with intermediate lyophilization and finally dissolved in 450  $\mu$ l of 99.96% <sup>2</sup>H<sub>2</sub>O (Isotec). <sup>1</sup>H NMR measurements were carried out on a Bruker AMX-500 or AMX-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University). The <sup>1</sup>HO<sup>2</sup>H signal was suppressed by presaturating for 0.8–1 s. Experiments were performed at a probe temperature of 285 K unless indicated otherwise. Chemical shifts ( $\delta$ ) are expressed by reference to internal acetone ( $\delta$  2.225 ppm). To achieve phase sensitive detection in the  $t_1$  direction, the TPPI method (22) was applied. Two-dimensional spectra were recorded with 400–600  $t_1$  experiments, and 96–152 (8–24 for trisaccharide) free induction decays of 2048 data points were collected per  $t_1$  experiment. Data sets were processed using Bruker UXNMR software. In short, time-domain data were multiplied with a shifted squared sine bell function. After zero-filling and Fourier transformation, the resulting data sets of 1024  $\times$  1024 (or 2048  $\times$  2048) data points were base-line corrected in both frequency domains by fifth order polynomial fits. Two-dimensional TOCSY spectra were recorded using a “clean” MLEV-17 spin-lock pulse sequence of 100 ms preceded by a 2.5-ms trim-pulse (23–25). Two-dimensional ROE spectra (26) were recorded at a field strength of 2.3 kHz with the offset at 5.5 ppm and a spin lock time of 150 ms (or 300 ms for the trisaccharide).

**Hexosaminidase Digestion**—Fraction D8 of the HPAEC separation was treated with  $\beta$ -N-acetylhexosaminidase (EC 3.2.1.30; from *Streptococcus pneumoniae*, Oxford Glycosystems) for 16 h at 37 °C, according to the manufacturer's instructions. The ratio of enzyme to substrate was lowered to approximately 15 milliunits of enzyme for 70 nmol of glycan (amount estimated from NMR spectra). Glycans were separated from BSA (from the enzyme preparation) and salts by gel filtration.

## RESULTS

P0 was extracted from bovine sciatic nerve and purified by repeated gel filtration to a final purity of more than 90% (Fig. 1A). Enzymic deglycosylation of P0 with PNGase F was quantitative, judging from the 2-kDa shift in mobility on SDS-PAGE and the virtual disappearance of reactivity with HNK-1 monoclonal antibodies (Fig. 1, A and B). After separation from the protein, the carbohydrate pool was fractionated by high pH anion-exchange chromatography (19), resulting in five major (10–25% each) and three minor (<5% each) fractions (data not shown). One-dimensional 500 MHz <sup>1</sup>H-NMR spectroscopy<sup>3</sup> showed that three of the major carbohydrate fractions contained structural reporter group resonances, similar to the SO<sub>4</sub>-3GlcA element of the synthetic HNK-1 reactive trisaccharide YM677 (SO<sub>4</sub>-3GlcA $\beta$ 1-3Gal $\beta$ 1-4GlcNAc), prepared by Ogawa and co-workers (27). At this stage of the purification, one of the three fractions, designated D8, was homogeneous, as judged from the NMR spectrum. To confirm its HNK-1 immunoreactivity, D8 was coupled to bovine serum albumin and reacted with two independent monoclonal antibodies to HNK-1



**FIG. 1. Purification of P0 and isolation of HNK-1 immunoreactive glycans.** PNGase F treatment of P0 results in quantitative release of glycans, as illustrated by a higher electrophoretic mobility of the enzyme-treated P0 (lane 2) compared to the untreated protein (lane 1) (panel A), and the presence of HNK-1 staining before (lane 1) and the disappearance after (lane 2) PNGase F treatment (panel B). C, coupling of the major HNK-carrying glycan D8 renders BSA HNK-1 positive (lane 1), whereas untreated BSA is HNK-1 negative (lane 2). Staining for the HNK-1 epitope was performed with mAb 412 (shown in B and C) and with mAb HNK-1 (Becton Dickinson; data not shown). Staining patterns of the two antibodies were identical (data not shown), albeit that the IgM monoclonal HNK-1 showed much weaker staining both with P0 and HNK-1-BSA, possibly because of the monovalent presentation of the epitope.

(Fig. 1C). Further structural analysis using combined one- and two-dimensional NMR spectroscopy was carried out with fraction D8 (Fig. 2 and Table I).

The structural reporter group (28) signals of D8 show that this oligosaccharide contains two modifications of the common Asn-linked pentasaccharide core (Fig. 3): a fucose  $\alpha$ 1-6 linked to the first N-acetylglucosamine (GlcNAc) and a so-called bisecting GlcNAc, linked to the first core mannose (Man) as a third extension (29). The two outermost Mans are both substituted with an additional GlcNAc. This basic structure can be deduced from the chemical shifts of H-1 and H-2 of Man-3/Man-4, H-1 of Man-4' and H-1, H-4, H-5 and NAc, of GlcNAc-9 (Table I), which are comparable to those of a diantennary structure with a bisecting GlcNAc (29).

The trisaccharide YM677 provided the necessary reference data for the assignment of terminal SO<sub>4</sub>-3GlcA $\beta$ 1-3Gal linked to GlcNAc. Analysis of two-dimensional NMR spectra of YM677 and D8 reveals identical spin patterns in both compounds, confirming that the HNK-1 trisaccharide element is present in D8 (Table I and Fig. 2). In order to determine the branch location of SO<sub>4</sub>-3GlcA $\beta$ 1-3Gal, D8 was treated with hexosaminidase, which removes unsubstituted GlcNAcs (Fig. 3). A two-dimensional TOCSY spectrum of this sample (Fig. 2) showed the disappearance of the signals corresponding to GlcNAc-5 and GlcNAc-9 (Fig. 3) and changes in the chemical shifts of the core mannoses (3, 4, and 4'). The chemical shifts are now in agreement with a structure, in which only Man 4' is extended (30). This points to the location of the HNK-1 epitope in

<sup>2</sup> The abbreviations used are: PNGase F, peptide-N<sub>4</sub>-(N-acetyl- $\beta$ -glucosaminyl)asparagine amidase; HPAEC, high pH anion-exchange chromatography; ROE, rotating frame nuclear Overhauser enhancement; TOCSY, total correlated spectroscopy.

<sup>3</sup> C. W. E. M. van Zuylen, H. Voshol, M. Schachner, and J. F. G. Vliegthart, manuscript in preparation.

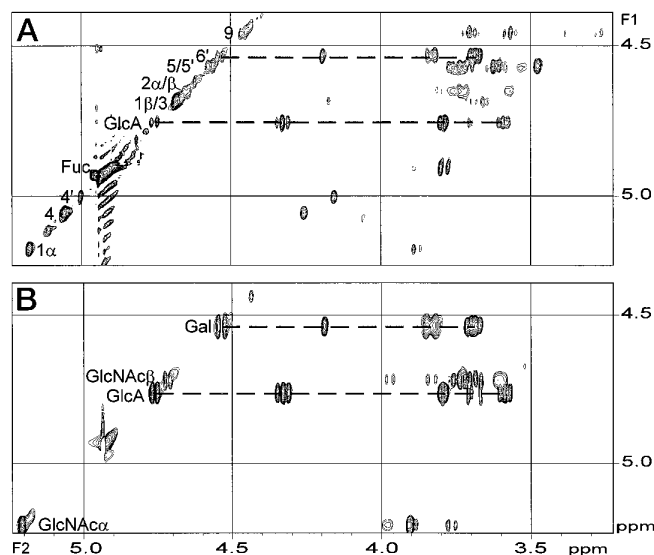


FIG. 2. Analysis of the HNK-1 carbohydrate structure by TOCSY. Parts of TOCSY spectra, recorded at 500 MHz, are shown of D8 (A) and of the synthetic trisaccharide YM677 (B). Specific spin patterns observed for GlcA and Gal are indicated by dashed lines, showing that they are identical in both spectra. For comparison the structures are given in Fig. 3.

the  $\alpha$ 1-6-linked branch. This is confirmed by ROE contacts that prove the sequential linkages from GlcA to Gal-6' to GlcNAc-5' to Man-4' (Table I). For further confirmation of the structure deduced from the NMR data, an aliquot of D8 was permethylated and analyzed using positive-mode fast atom bombardment-mass spectrometry. The most intense pseudomolecular ion was observed at  $m/z$  2552.49 corresponding to  $M + Na - 1$  Me of the permethylated deduced structure. Some less intense pseudomolecular ions, stemming from multiply undermethylated structures, were also detectable.

#### DISCUSSION

In this study we present the complete structure of an N-glycan carrying the HNK-1 epitope, isolated from the immunoglobulin superfamily recognition molecule P0 from bovine peripheral nervous system. This is the first structure of an integral glycoprotein-derived HNK-1 carbohydrate chain that has been firmly established by NMR parameters. Comparison of this P0-derived structure with the carbohydrate structure of the HNK-1-containing glycolipids (14) shows that they have a sulfated trisaccharide, corresponding to the reference trisaccharide YM677 (Fig. 3), in common. Apparently, since it is the only common element, this trisaccharide is sufficient for reactivity with the HNK-1 antibodies. Schmitz *et al.* (27) describe this part of the glycolipid-derived structure to be responsible for biological activity, whereas a glycolipid with only a sulfated glucuronic acid is inactive. Whether the "backbone" part of the oligosaccharide is of functional significance remains to be clarified.

The HNK-1 epitope is prominently expressed on P0, since approximately 80% of all P0 molecules from bovine sciatic nerve have HNK-1-reactive oligosaccharides.<sup>3</sup> The single N-glycosylation site of P0 carries the HNK-1 carbohydrate on three or four different glycans,<sup>3</sup> of which the most predominant one was analyzed in the present study. The degree of HNK-1 substitution of the P0 glycans is highly relevant, since its putative function, maintaining the structural integrity of myelin by homophilic adhesion, depends on its glycosylation status (see Introduction). Previous data (31) indicate that the carbohydrate composition of rat P0 varies with age, suggesting that the expression of the HNK-1 epitope could be varied to

TABLE I  
<sup>1</sup>H NMR parameters of the HNK-1 oligosaccharides

NMR parameters are given of the synthetic trisaccharide YM677 and of the P0-derived fraction D8, before and after digestion with  $\beta$ -N-acetylhexosaminidase. Values are chemical shifts (in ppm) of the important structural reporter groups, derived from one-dimensional NMR measurements at 300 K, unless indicated otherwise. For reference, the structures and the numbering of the residues are given in Fig. 3. The HNK-1 determinant is characterized by the signals of the three residues of YM677, which are also present in D8. After hexosaminidase digestion, the signals belonging to GlcNAcs 5 and 9 have disappeared (see also text). Rotating frame NOE (ROE) contacts are observed between GlcA-H1 and Gal6'-H3/H4, between Gal6'-H1 and GlcNAc5'-H4 and between GlcNAc5'-H1 and Man4'-H1/H2, proving the linkages between these residues.

Reporter group	Residue	Chemical shift (ppm) in		
		YM677	D8 <sup>a</sup>	D8-digested
H-1	GlcNAc1 $\alpha$		5.180	5.180
	GlcNAc2		4.651	4.659
	Man3		4.685	4.77 <sup>b</sup>
	Man4		5.056	5.101
	Man4'		4.999	4.925
	GlcNAc5/5'	$\alpha$ 5.207, $\beta$ 4.717 <sup>b</sup>	4.557/4.571	—/4.574
	Gal6'	$\alpha$ 4.535, $\beta$ 4.530	4.533	4.528
	GlcA-SO <sub>4</sub>	4.760 <sup>b</sup>	4.760	4.77 <sup>b</sup>
	GlcNAc9		4.459	
	Fuca/β		4.899/4.907	4.893/4.90
H-2	Man3		4.174	4.253
	Man4		4.249	4.068
	Man4'		4.149	4.12 <sup>c</sup>
H-3	Gal6'	4.185	4.188	4.187
H-4	GlcA-SO <sub>4</sub>	4.329	4.328	4.330
H-5	GlcNAc9		3.265	
NAc	GlcNAc9		3.403	
	Fuca/β		4.11 <sup>c</sup> /4.14 <sup>c</sup>	4.10 <sup>c</sup> /4.141
	GlcNAc1		2.037	2.042
	GlcNAc2		2.097	2.096
	GlcNAc5/5'	2.041	2.064/2.037	—/2.042
CH <sub>3</sub>	GlcNAc9		2.052	
	Fuca/β		1.206/1.218	1.209/1.221

<sup>a</sup> Measured at 295 K.

<sup>b</sup> Measured at 285 K.

<sup>c</sup> Value determined from two-dimensional ROESY or TOCSY spectrum at 285 K.

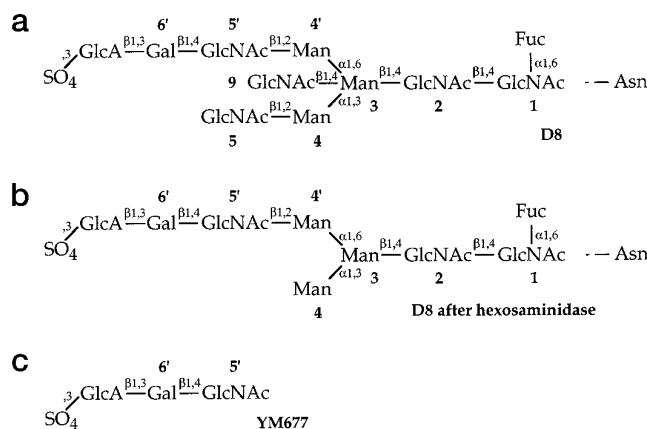


FIG. 3. Structures of the HNK-1 oligosaccharides. Structures are given of D8 before (a) and after (b) hexosaminidase digestion and of the synthetic trisaccharide YM677 (c).

accommodate for alterations in external requirements. In contrast to its highly homologous counterparts from human, rat, and bovine, P0 from the mouse is not HNK-1 reactive, even though the carbohydrate is expressed in peripheral myelin (32). It is tempting to speculate that, when an HNK-1 epitope is functionally important for a particular glycoprotein in several animal species, structurally related carbohydrates, which are not recognized by HNK-1 antibodies, serve as structural and functional substitutes in cases where no HNK-1 reactivity is

observed. These structures will await further elucidation.

Knowledge of the carbohydrate structure of the HNK-1 epitope now opens the way for further steps in the elucidation of the involvement of this carbohydrate in recognition processes and for analysis of the glycosyltransferases involved in its biosynthesis. With respect to the latter aspect, it should be noted that different glucuronyltransferases appear to be involved in the biosynthesis of the glycolipid- and glycoprotein-bound forms of the epitope (33). Since the linkage of glucuronic acid to lactosamine is the same in both forms, this suggests that other structural or environmental factors play a role in acceptor recognition by the glucuronyltransferase.

Furthermore, by modifying the carbohydrate structure, one can now study the structural requirements for the function of the HNK-1 carbohydrate in terms of molecular details. Since autoimmunoreactivity against the HNK-1 epitope is responsible for damage to nervous tissue in demyelinating neuropathies (see Introduction), compounds that block the binding site of the autoantibodies to HNK-1 have potential therapeutic applications. Obviously, the HNK-1 trisaccharide YM677 could be used for this purpose, but the disadvantage of this compound, and of oligosaccharides in general, is that their chemical synthesis is a laborious enterprise. This problem could be solved by trying to find a "minimal" structure, which is less complex and easier to prepare, but which retains high affinity for the autoantibodies. Further structural studies of HNK-1 oligosaccharides and the functionally relevant HNK-1 related carbohydrates might provide clues for the development of such functional "mimics." On the other hand, as soon as glycosyltransferases to produce the HNK-1 structure enzymically are available, application of HNK-1 carbohydrates as therapeutic compounds in autoimmune demyelinating neuropathies represents a feasible option.

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