Characterization of N-glycans from mouse brain neural cell adhesion molecule


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The N-glycosylation pattern of the neural cell adhesion molecule (NCAM), isolated from brains of newborn mice, has been analyzed. Following digestion with trypsin, generated glycopeptides were fractionated by serial immunoaffinity chromatography using immobilized monoclonal antibodies specifically recognizing polysialic acid (PSA) units or the HNK1-carbohydrate epitope. Subsequent analyses of the resulting (glyco)peptides by Edman degradation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) revealed polysialylated glycans to be exclusively linked to glycosylation sites 5 (Asn431) and 6 (Asn460), whereas glycans carrying the HNK1-epitope could be assigned to sites 2 (Asn497), 5, 6, and, to a lesser extent, site 3 (Asn329). PSA-, HNK1-, and non-PSA/HNK1-glycan fractions were characterized by carbohydrate constituent and methylation analyses as well as MALDI-TOF-MS in conjunction with chromatographic fractionation techniques. The results revealed that the core structures of PSA-glycans represented predominantly fucosylated, partially sulfated 2,6-branched isomers of triantennary as well as tetraantennary complex-type glycans, whereas carbohydrate chains bearing the HNK1-epitope were dominated by diantennary species carrying in part bisecting GlcNAc residues. Non-PSA/HNK1-glycans exhibited a highly heterogeneous pattern of partially truncated, mostly diantennary structures being characterized by the presence of additional fucose, bisecting GlcNAc and/or sulfate residues. In conclusion, our results revealed that the glycosylation pattern of murine NCAM displays high structural and regional selectivity, which might play an important role in controlling the biological activities of this molecule.

Key words: HNK1-epitope/NCAM/N-glycans/polysialic acid

Introduction

The fine-tuning of cell interactions is an important requirement for the specification of neural-cell contacts, which ultimately underlie the orderly development of neural networks. Recognition molecules of the immunoglobulin superfamily are particularly interesting with regard to their cell specification in the context of their carbohydrate composition (Schachner and Martini, 1995). Among them, the neural cell adhesion molecule (NCAM) was the first to be recognized to carry an interesting carbohydrate, the ε2,8-linked polysialic acid (PSA), which is characteristic of NCAM (Finne et al., 1983) and some sodium channels (James and Agnew, 1987; Zuber et al., 1992). All the major isoforms of NCAM carry this carbohydrate, which has been estimated to consist of up to 200 sialic acid residues (Livingston et al., 1988). PSA-substituted NCAM had been originally considered as the embryonic form of NCAM, because it is more highly expressed during development than in the adult (Hekmat et al., 1990). However, some brain regions, particularly those involved in synaptic plasticity, retain expression of this carbohydrate structure in the adult (Seki and Arai, 1993; Ronn et al., 2000). Under conditions of regeneration and plasticity, up-regulation of the expression of PSA has been described (Müller et al., 1994, 1996; Becker et al., 1996). Its expression is also highly correlated with enhanced neurite outgrowth in cell culture and migration of precursor cells in the olfactory system of mammals (Doherty et al., 1990; Eckhardt et al., 2000). The bulky and highly negatively charged PSA chains modulate the functional properties of NCAM by rendering it less adhesive to itself (homophilic binding; Rao et al., 1993, 1994) or to other molecules (heterophilic binding; Kadmon et al., 1990, Reyes et al., 1990; Kiselyov et al., 1997). The question is still open as to whether PSA may be an essential entity in signal transduction pathways. It has been hypothesized to function as a receptor or ligand that is recognized by partner molecules (Joliot et al., 1991; Storms and Rutishauser, 1998) either within the surface membrane of one and the same cell, the extracellular matrix, or the cell surface of a neighboring cell.

In addition to polysialylated glycans, NCAM is known to carry the L2/HNK1-carbohydrate epitope (Kruse et al., 1984) consisting of a 3′-sulfated glucuronic acid attached to C3 of a N-acetyllactosamine unit (Chou et al., 1986; Ariga et al., 1987; Voshol et al., 1996). The HNK1-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 (Ig) superfamily, members of the tenascin family, integrins, proteoglycans (Schachner and Martini, 1995), as well as acidic glycolipids of the peripheral nervous system (Chou et al., 1986; Ariga et al., 1987). It binds to certain isoforms of laminin and to P and L
selectins (Hall et al., 1993; Needham and Schiina, 1993). The HNK1-epitope displays a high degree of phylogenetic conservation because it occurs not only in vertebrates but also in glycoproteins and glycolipids of insects (Dennis et al., 1988). Its expression appears to be associated with developmental events involving the movement of cells and extensions of their processes. For example, there is a correlation of the presence of the HNK1-carbohydrate epitope with the migration of chick embryo cells, such as epiblasts (Canning and Stern, 1988) and neural crest cells (Vincent et al., 1983; Rickmann et al., 1985; Bonner-Fraser, 1987; Loring and Erickson, 1987). Expression of HNK1 is also spatially and temporally regulated during other aspects of neural development (Schwarting et al., 1987; Yoshiihara et al., 1991) as well as during outgrowth of astrocytic and neuronal processes and glial cell migration (Künemund et al., 1988; Martini et al., 1992). Furthermore, there is evidence that this carbohydrate is involved in neuron to glial cell adhesion (Keilhauer et al., 1985).

Because of the functional importance of NCAM carbohydrates, a precise knowledge of the structures, location on the protein backbone, and the regulation of their biosynthesis is clearly mandatory. Studies performed by Finne (1982) provided early evidence for the presence of fucosylated tri- and tetraantennary core structures of the PSA glycan chains from NCAM of fetal rat brain. Later, additional sulfate groups, type 1 (Galβ3GlcNAc) and type 2 (Galβ4GlcNAc) antennae and lactosamine repeating units were demonstrated to occur in the polysialylated oligosaccharides from NCAM of embryonic chick brains (Kudo et al., 1996). In the case of chicken NCAM, it could be further verified that polysialylation occurs exclusively on N-glycan chains in the fifth Ig-like domain, where two of the three potential N-glycosylation sites have been shown to be polysialylated (Nelson et al., 1995). In the present study, we have initiated the characterization of the entire pattern of N-glycans obtained from newborn mouse NCAM. In this context, particular emphasis is laid on the specific location and the characteristic structural features of the different classes of carbohydrate chains present to achieve more detailed information on the carbohydrate structure of this glycoprotein.

**Results**

**Isolation and characterization of NCAM**

NCAM was extracted from brains of 2- to 3-day-old mice and purified by immunoaffinity chromatography using the anti-NCAM monoclonal antibody (mAb) H28, which recognizes all isoforms of the protein (Hirn et al., 1981). An aliquot of the NCAM preparation obtained was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis. Silver staining of the gel and immunostaining with anti-NCAM mAb H28 displayed the characteristic diffuse, polydisperse “smear” (Hoffman et al., 1982) of polysialylated NCAM in a range of about 190 kDa (Figure 1A,B). Treatment with endo-β-N-acetylglucosaminidase N (endo N) resulted in a clear shift in the electrophoretic mobility of NCAM (Barschels et al., 1987), it can be deduced that each of the six potential N-glycosylation sites has been located in an individual trypptic peptide. Therefore, trypsin was used for proteolytic digestion. The subsequent strategy adopted for the analysis of glycans and peptide moieties is shown in Figure 2. In brief, resulting glycopeptides were fractionated by sequential immunoaffinity chromatography using an anti-PSA mAb 735 column and an anti-HNK1 mAb 412 column, thus yielding three individual fractions termed “PSA-glycopeptides,” “HNK1-glycopeptides,” and “non-PSA/HNK1-glycopeptides.”

![Fig. 1. Characterization of NCAM from mouse brain by SDS–PAGE and Western blotting. (A) Silver stained SDS-gel; (B–D) Western blot analyses employing anti-NCAM mAb H28 (B), anti-PSA mAb 735 (C), and anti-HNK1 mAb 412 (D). Lane 1: starting material; lane 2: NCAM after treatment with endo N; lane 3: NCAM after treatment with PNGase F; lane 4, buffer control. Apparent molecular masses of standard proteins are indicated in kDa.](image-url)
Edman degradation of the PSA–glycopeptide fraction revealed predominantly two peptide sequences comprising the fifth and the sixth potential N-glycosylation site, both of which are located in the fifth Ig-like domain of the molecule (Figure 3). The signals of Asn residues 431 and 460, being both part of the typical consensus sequence Asn-X-Ser/Thr and presumed to be modified by N-glycans, displayed a significantly reduced intensity, indicating that they were indeed N-glycosylated. The identified sequences D423GQLLPSS(N)YSNIK436 and I437YNTPSASYLEVTPDS-ENDFGNY(N)CTAVNR466 are consistent with the primary structure of murine NCAM reported in the database (Swiss-Prot P13595). To corroborate this finding, deglycosylated PSA-peptides (see Figure 2) were fractionated by reversed-phase high-performance liquid chromatography (RP-HPLC) (Figure 4), and individual peptide fractions were identified by Edman degradation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Table I). The results obtained clearly confirmed the assignment of the PSA-modified glycans to N-glycosylation sites 5 and 6 (Figure 3). Tryptic peptides including other glycosylation sites were neither detected by MALDI-TOF-MS nor identified by Edman sequencing.

Likewise, HNK1-glycopeptides could be demonstrated to comprise preponderantly the N-glycosylation sites 2, 5, and 6. Edman degradation of major glycopeptide fractions revealed the following sequences: I292TYVE(N)QTAME302 (site 2), D423GQLLPSS(N)YSN434 (site 5), and I437YNTPS_SYLE447 (N-terminal sequence of the glycopeptide making up site 6). Again, glycosylated Asn residues (given in parentheses) were not registered due to their carbohydrate substitution. In addition, minor peptide species with the N-terminal sequence of (N329)ISSEE334 indicated the presence of compounds including also glycosylation site 3.

Characterization of PSA glycans

The different glycan fractions from murine NCAM were characterized by carbohydrate constituent and linkage analyses as well as MALDI-TOF mass measurement. For all glycan fractions obtained, neutral monosaccharide constituent analysis revealed the exclusive presence of Fuc, Man, Gal, and GlcNAc, whereas GalNAc could not be detected in any of these oligosaccharide pools. Hence, the presence of N-glycans with GalNAcβ4GlcNAc (LacdiNAc) units could be excluded. For the characterization of the PSA chains, aliquots of the PSA glycopeptides were subjected to methylation analysis in combination with esterification and reduction of the carboxyl group of Neu5Ac, resulting in the corresponding partially methylated d-erythro-1-manno/gluc-o-nitol acetates. Due to incomplete methylation—probably as a consequence of the bulk of neuraminic acid residues present—as well as the small amounts of material available, resulting partially methylated...
monosaccharide derivatives were only qualitatively characterized (Table II). The presence or absence of the respective alditol acetates, however, was clearly significant in all cases. The analysis of the PSA glycopeptides revealed the presence of terminal and 8-substituted Neu5Ac, terminal fucose, 3-substituted and small amounts of terminal galactose, 2-substituted as well as 2,4-, 2,6- and 3,6-disubstituted mannose residues in addition to 4-substituted N-acetylglucosamine. From the relative amounts of 2-substituted, 2,4-disubstituted, and 2,6-disubstituted mannosyl residues found, it is proposed that the respective oligosaccharides comprise fucosylated diantennary, triantennary, and tetraantennary complex-type core structures in a
were recorded as \([M+Na]^+\) or \([M-H]^-\) pseudomolecular ions, respectively. Glycans were analysed in the positive-ion (A) or negative-ion (B) reflectron mode and Fig. 5. MALDI-TOF mass spectra of native desialylated PSA glycans. Glycans were fractionated according to charge by anion-exchange HPLC (Fig. 6). The results revealed a preponderance of compounds carrying two negative charges in agreement with the chemical structure of the HNK1 epitope (see below). Compounds with one, three, and four negatively charged groups, occurring only in minor proportions, might reflect glycans carrying either a single sulfate group or a GlcA residue, the latter of which are also bound by mAb 412 (Schmitz et al., 1994), as well as sugar chains bearing one or two sulfate and/or GlcA substituents. Due to the limited amounts of material available, only the doubly charged 2-AB-glycans, pooled as indicated in Fig. 6, were further characterized by methylation analysis (Table II) and mass spectrometry (Fig. 7).

Linkage data and MALDI-TOF-MS analyses in the negative-ion mode provided evidence for the presence of mostly core-fucosylated, partially incomplete triantennary complex-type glycans in addition to small amounts of triantennary chains and hybrid-type species (cf. Figure 7 and Table III). As a striking feature, the majority of triantennary and triantennary glycans carried an additional bisecting GlcNAc residue. From the simultaneous presence of 2,4- and 2,6-disubstituted mannosyl residues (Table II), it may be further deduced that triantennary species comprised both 2,4- and 2,6-branched isomers. All registered pseudomolecular ions included the mass increment of both hexuronic acid and sulfate residues. Because this glycan fraction had been isolated by immuno-affinity chromatography using the HNK1-specific mAb 412, it may be concluded that GlcA and sulfate groups are both constituents of a HNK1 epitope \((SO_4^--3GlcA(\beta1-4)GlcNAc)\), which is in agreement with methylation data. Its precise linkage position (i.e., \([\alpha1-3]-\) or \([\alpha1-6]-\)linked antenna), however, has not been assigned. Negative-ion MALDI-TOF-MS of HNK1-2-AB-glycans in the reflectron mode further revealed the formation of characteristic fragment ions, possibly due to metastable decay. Respective fragment ions differed from the corresponding pseudomolecular ions by a mass increment of about 86 mass units and appeared to be of high diagnostic value, because they have been also observed in the case of oligosaccharide standards carrying the HNK1 epitope but not in the case of sulfated glycans lacking glucuronic acid residues (H. Geyer, unpublished observation). The precise mechanism of fragmentation, however, has not been investigated in this study.

Characterization of non-PSA/HNK1-glycans

Non-PSA/HNK1-(glyco)peptides obtained with the flow-through of both immunoaffinity columns (see Fig. 2) were preparatively desialylated by mild acid treatment. Oligosaccharides were released by incubation with PNGase F, freed from residual peptides by RP-HPLC, and characterized by MALDI-TOF-MS

ratio approaching 10:45:45, respectively, the majority of which (about 90%) is substituted at C3 of the terminal Gal. The molar ratio of 2,4-disubstituted versus 2,6-disubstituted mannosyl residues was found to be 1:2. Therefore, it can be assumed that the core structures of triantennary PSA glycans represent predominantly the 2,6-branched type of isomers.

After desialylation of the PSA glycopeptides, methylation analysis revealed a significant increase in terminal Gal resi-...
In the positive-ion reflectron mode (Figure 8). To increase sensitivity (Geyer et al., 1999), remaining glycans were converted into 2-aminopyridine (PA) derivatives by reductive amination and, subsequently, subjected to MALDI-TOF-MS in the negative-ion reflectron mode. In parallel, resulting PA-glycans were examined by linkage analysis (Table II). The results demonstrated that non-PSA/HNK1-oligosaccharides represented mainly core-fucosylated diantennary species, the majority of which comprised truncated N-acetyllactosamine chains as well as additional bisecting GlcNAc and/or one to two sulfate residues (Table III). In addition, oligosaccharides with the compositions Hex$_2$HexNAc$_2$Hex$_2$, Hex$_2$HexNAc$_2$dHex$_2$, Hex$_2$HexNAc$_2$dHex$_2$SO$_4$, Hex$_2$HexNAc$_2$Hex$_2$SO$_4$, Hex$_2$HexNAc$_2$dHex$_2$SO$_4$, Hex$_2$HexNAc$_2$Hex$_2$SO$_4$, and Hex$_2$HexNAc$_2$dHex$_2$ were detected, reflecting partially sulfated structural variants with more than one fucosyl residue and/or minor amounts of triantennary carbohydrate chains (see Table III). Due to the presence of trace amounts of 2,4- and 2,6-disubstituted mannose residues (cf. Table II), the latter species are again assumed to represent both 2,4- and 2,6-branched isoforms. Because all glycans were released by PNGase F treatment, difucosylation as additional bisecting GlcNAc and/or one to two sulfate groups is suggested that some residues may be linked to the C3 of terminal galactose units. In contrast to the aforementioned study, however, the assignment of polyasialylated structures in this study was not based on site-directed mutagenesis, which might also have indirect effects on protein glycosylation. Instead, respective glycosylated peptides were directly identified by N-terminal amino acid sequencing and MALDI-TOF-MS. Edman degradation.

### Discussion

Our results demonstrate that the overall glycosylation pattern of NCAM from newborn mouse brain is characterized by a remarkable structural diversity. In agreement with earlier studies on chicken NCAM, polysialylated N-glycans were found to be restricted to the fifth and sixth N-glycosylation site located in the fifth Ig-like domain (Nelson et al., 1995). In contrast to the aforementioned study, however, the assignment of polyasialylated structures in this study was not based on site-directed mutagenesis, which might also have indirect effects on protein glycosylation. Instead, respective glycosylated peptides were directly identified by N-terminal amino acid sequencing and MALDI-TOF-MS. Edman degradation.

Table II. GC-MS data obtained after methylation analysis of NCAM oligosaccharides

<table>
<thead>
<tr>
<th>Alditol acetate</th>
<th>PSA glycopeptides</th>
<th>Desialylated PSA glycopeptides</th>
<th>Desialylated HNK1 oligosaccharides$^a$</th>
<th>Desialylated non-PSA/HNK1-oligosaccharides$^b$</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5(Me)AcMe$_{4,7,9}$ol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Neu5Ac(2→)</td>
</tr>
<tr>
<td>Neu5(Me)AcMe$_{4,7,8}$ol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Neu5Ac(2→)</td>
</tr>
<tr>
<td>Fuc-Me$_{2,3}$ol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Fuc(1→)</td>
</tr>
<tr>
<td>Man-Me$_{3,4}$ol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Man(1→)</td>
</tr>
<tr>
<td>Man-Me$_{3,6}$ol</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>Man(1→)</td>
</tr>
<tr>
<td>Man-Me$_{3,4,6}$ol</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>Man(1→)</td>
</tr>
<tr>
<td>Man-Me$_{2,4}$ol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Man(1→)</td>
</tr>
<tr>
<td>Gal-Me$_{3,4,6}$ol</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Gal(1→)</td>
</tr>
<tr>
<td>Gal-Me$_{2,4,6}$ol</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>Gal(1→)</td>
</tr>
<tr>
<td>GlcN(Me)AcMe$_{3,4,6}$ol</td>
<td>–</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>GlcNAc(1→)</td>
</tr>
<tr>
<td>GlcN(Me)AcMe$_{3,4}$ol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>GlcNAc(1→)</td>
</tr>
<tr>
<td>GlcN(Me)AcMe$_{3}$ol</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND, not determined</td>
</tr>
<tr>
<td>GlcN(Me)AcMe$_{2}$ol</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND, not determined</td>
</tr>
<tr>
<td>GlcN(Me)AcMe$_{1}$ol</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND, not determined</td>
</tr>
</tbody>
</table>

PSA-glycopeptides, desialylated PSA-glycopeptides, desialylated HNK1-2-AB-oligosaccharides and desialylated non-PSA/HNK1-PA-oligosaccharides were subjected to methylation analysis (for details see Materials and methods). Owing to the small amounts of material and, in part, incomplete methylation, fractions were only qualitatively characterized. The absence or presence of respective alditol derivatives is indicated by – or +, respectively. Neu5(Me)AcMe$_{4,7,8,9}$ol, 1,2,6,8-tetra-O-acetyl-3,5-dideoxy-5-(N-methyl)acetamido-4,7,9-tetra-O-methyl-b-erythro-L-manno/glucono-nitrol, Neu5(Me)AcMe$_{4,7}$ol, 1,2,6,8-tetra-O-acetyl-3,5-dideoxy-5-(N-methyl)acetamido-4,7,9-tri-O-methyl-d-erythro-L-manno/glucono-nitrol; Fuc-Me$_{2,3}$ol, 1,5-di-O-acetyl-2,3,4-tri-O-methylfucitol; GlcN(Me)AcMe$_{3,4,6}$ol, 1,5-di-O-acetyl-2-deoxy-2-(N-methyl)acetamido-3,4,6-tri-O-methylfucitol, etc; (+), trace; ND, not determined.

*2-AB derivatives.

*3-AB derivatives.

+4PA derivatives.

In the positive-ion reflectron mode (Figure 8). To increase sensitivity (Geyer et al., 1999), remaining glycans were converted into 2-aminopyridine (PA) derivatives by reductive amination and, subsequently, subjected to MALDI-TOF-MS in the negative-ion reflectron mode. In parallel, resulting PA-glycans were examined by linkage analysis (Table II). The results demonstrated that non-PSA/HNK1-oligosaccharides represented mainly core-fucosylated diantennary species, the majority of which comprised truncated N-acetyllactosamine chains as well as additional bisecting GlcNAc and/or one to two sulfate residues (Table III). In addition, oligosaccharides with the compositions Hex$_2$HexNAc$_2$Hex$_2$, Hex$_2$HexNAc$_2$dHex$_2$, Hex$_2$HexNAc$_2$dHex$_2$SO$_4$, Hex$_2$HexNAc$_2$Hex$_2$SO$_4$, Hex$_2$HexNAc$_2$dHex$_2$SO$_4$, Hex$_2$HexNAc$_2$Hex$_2$SO$_4$, and Hex$_2$HexNAc$_2$dHex$_2$ were detected, reflecting partially sulfated structural variants with more than one fucosyl residue and/or minor amounts of triantennary carbohydrate chains (see Table III). Due to the presence of trace amounts of 2,4- and 2,6-disubstituted mannose residues (cf. Table II), the latter species are again assumed to represent both 2,4- and 2,6-branched isoforms. Because all glycans were released by PNGase F treatment, difucosylation of the innermost GlcNAc residue can be excluded (Tretter et al., 1991). Instead, the additional fucose residue(s) may be assumed to reside on either of the lactosamine antennae. From the presence of 3,4-disubstituted GlcNAc residues (see Table II), it is possible that this fucose is linked to C3 of GlcNAc, thus forming a Lewis X determinant. Similar to desialylated PSA-glycans, MALDI-TOF-MS of non-PSA/HNK1-PA-glycans in the negative-ion mode provided evidence for the presence of sulfated compounds (Table III). The composition of these prevalent structures suggests the presence of incomplete, mostly diantennary species, as detected in the positive-ion mode, which carry an additional sulfate group in an as yet undefined position. Trace amounts of 3-substituted Gal, however, suggests that some residues may be linked to the C3 of terminal galactose units.
Murine NCAM glycosylation

Table III. Molecular masses of major mouse NCAM core glycans as determined by MALDI-TOF-MS

<table>
<thead>
<tr>
<th>Assigned structure</th>
<th>Composition</th>
<th>PNA-glycans&lt;sup&gt;[M+Na]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>HNK1-glycans&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NeuFCA/HNK1-glycans&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid type:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HexA + SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>HexHexNAcHexSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1804.0</td>
<td>1804.2</td>
<td></td>
</tr>
<tr>
<td>Basic structure:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GaLacNAcMan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GalLacNAcMan&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2014.1</td>
<td>2012.8</td>
<td></td>
</tr>
<tr>
<td>Structural modifications:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Hex</td>
<td>HexHexNAcHexSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2201.7</td>
<td>2201.6</td>
<td></td>
</tr>
<tr>
<td>+ Hex + HexNAc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HexHexNAcHexSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>2259.0/2246.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2259.0/2246.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
| Glycans were analyzed in the positive-ion and/or negative-ion reflectron mode. Given mass values indicate average masses. Bold numbers mark main species; masses in parentheses represent minor compounds.  
<sup>a</sup>Based on monosaccharide composition and linkage analyses as well as MALDI-TOF-MS.  
<sup>b</sup>2-aminobenzamide(2-AB)-derivatives.  
<sup>c</sup>2-aminopyridine(PA)-derivatives.  
<sup>d</sup>According to methylation data, additional HexNAc residues represent mostly bisecting GlcNAc residues.  
<sup>e</sup>Specific fragment.  
<sup>f</sup>Sodium salt.  

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revealed, in part, unusual tryptic cleavage, which might be due to the autolysis of trypsin, resulting in a different substrate specificity of the enzyme (Konigsberg and Steinman, 1977).

Although the ratio of terminal and 8-substituted sialic acid derivatives observed after linkage analysis did not allow any conclusions on the length of the PSA chains, the results obtained clearly verified the polymeric nature of these substituents. Respective oligosaccharide core structures comprised predominantly 2,6-branched isomers of triantennary as well as tetraantennary complex-type N-glycans. Similar to NCAM from embryonic chick brains (Kudo et al., 1996), the carbohydrate chains were found to carry a 6-linked fucosyl residue attached to the innermost GlcNAc residue and, in part, an additional sulfate group in as yet undefined position(s). Due to the experimental strategy used, the presence of small amounts of glycans with N-acetyllactosamine repeats could not be ruled out. In contrast to the above study, however, roughly 10% of the formerly polysialylated glycans were found to be of the diantennary type, indicating that, at least in murine NCAM, 2,6-branching of the (α1-6)-linked mannosyl residues is obviously not an absolute requirement for polysialylation, which is in agreement with data reported by Angata and coworkers (Angata et al., 1998). In addition, there is evidence that a minor proportion of these glycans carried bisecting GlcNAc residues. Carbohydrate substituents carrying an HNK1 epitope could be assigned to the N-glycosylation sites 5 and 6, similar to polysialylated glycans, as well as to site 2 and, in minor proportion, also to site 3 of the molecule. This result is in partial agreement with an earlier study on chicken NCAM in which the HNK1 epitope has been assigned to glycosylation site 1 or 2 (Cole and Schachner, 1987). The present study on murine NCAM extends this information insofar as glycans bearing this epitope could be also allocated to sites 5 and 6. HNK1 glycopeptides with amino acid sequences comprising the first and the fourth potential N-glycosylation of NCAM could not be detected in the present study. Hence, although showing a higher degree of spreading, the presence of glycans with an HNK1 epitope is again restricted to distinct sites of the molecule. From these results, it might be concluded that the enzyme activities involved in polysialylation and HNK1 biosynthesis depend on different structural parameters of the glycoprotein.
set of variants of triantennary species as well as hybrid-type glycans were only found as minor components. In contrast to PSA glycans, triantennary oligosaccharides were found to comprise both 2,4- and 2,6-branched isomers. Furthermore, tetrantennary species representing major compounds in the case of polysialylated sugar chains could not be detected in the HNK1–glycan fraction, thus displaying a separate, highly characteristic structural profile.

Due to limited amounts of material, residual non-PSA/ HNK1-glycans could not be assigned to distinct N-glycosylation sites. Therefore, the question remains open as to whether the first or the fourth potential N-glycosylation sites of murine NCAM are actually used for carbohydrate substitution. Structurally, these glycans represented a highly heterogeneous population of mostly diantennary species that were individually modified by partial truncation and, in particular, by the presence of additional bisecting GlcNAc as well as deoxyhexose residues and/or one to two sulfate groups. Similar to oligosaccharides comprising the HNK1 epitope, triantennary or tetrantennary species were only present in small amounts or not detectable, respectively, underlining again the different structural properties of the three classes of N-glycans present in NCAM from newborn mouse brain.

The present study was designed to characterize and to assign the entire panel of N-glycans to individual glycosylation sites of NCAM. Carbohydrate constituent analyses (data not shown) of the peptides obtained from the non-PSA/HNK1-glycopeptide fraction after PNGase F treatment also revealed the presence of GalNAc, which might be indicative for the simultaneous occurrence of O-linked glycans in murine NCAM. The question as to the precise structure(s) and localization of the respective O-glycan(s) was, however, not addressed in this study.

NCAM plays a fundamental role during the development of the nervous system by mediating intercellular recognition and adhesion (see Introduction). The modulation of its biological activities is usually discussed in conjunction with its degree of polysialylation. In agreement with studies on the polysialylated N-glycans of NCAM from chicken embryos (Kudo et al., 1996) our study demonstrates that NCAM glycans contribute negative charges not only due to their polysialic acid substituents but also by sulfate groups, which have been demonstrated to occur in all classes of N-glycans characterized. Hence, the degree in sulfation may further subtly modulate NCAM bioactivity and may also be subject to developmental regulation. To our knowledge, however, systematic investigations concerning the degree of expression of the HNK1 epitope and/or the sulfation of NCAM N-glycans during development have not yet been performed.

Materials and methods

Materials

The monoclonal antibodies used (mAb H28, mAb 735, and mAb 412, interacting specifically with murine NCAM, PSA chains, and the HNK1 epitope, respectively) have been described previously (Hirm et al., 1981; Kruse et al., 1984; Frosch et al., 1985; Schmitz et al., 1994). For immunoaffinity chromatography, monoclonal antibodies were coupled to preswollen CNBr-activated Sepharose 4B gel (Pharmacia, Freiburg, Germany) according to the manufacturer’s instructions (40 mg mAb H28, 16 mg mAb 735, and 16 mg mAb 412 coupled to 15 ml gel, 2 ml gel, and 8 ml gel, respectively). Endo-N was purified from the Escherichia coli bacteriophage PK1E as described elsewhere (Gerardy-Schahn et al., 1995). All reagents used were of analytical grade.

Isolation of NCAM

Brains of 2- to 3-day-old mice (60 g; stored at −80°C) were thawed and immediately homogenized (20 min; Ultra-Turrax, Janke & Kunkel, Staufen, Germany) on ice in 60 ml buffer containing 30 mM sodium phosphate (pH 7.4), 0.5 mM PMSF, 0.1 mM DTE, and 1 mM EDTA. Soluble material was removed by centrifugation at 17,000 × g and 4°C for 90 min. The pellet was resuspended for solubilization in 300 ml buffer containing 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 0.5 mM PMSF, 0.1 mM DTE, 1 mM EDTA, and 1% (v/v) Triton X-100. The mixture was shaken overnight at 4°C and finally centrifugated at 100,000 × g and 4°C for 1 h. The supernatant was diluted six times with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) and added in 300-ml aliquots to a column (1 × 20 cm) containing immobilized mAb H28. The column was washed with 30 column volumes of PBS with 1% Triton X-100, 10 column volumes of PBS containing 300 mM NaCl, and again with 10 column volumes of PBS. Bound NCAM was eluted with buffer containing 100 mM diethylamine, pH 11.5, 150 mM NaCl, 2 mM EDTA, and 0.1% (v/v) sodium deoxycholate. The eluted material was quickly neutralized by addition of 500 mM Tris–HCl buffer, pH 6.8, concentrated by ultrafiltration (YM100 membrane, Millipore, Eschborn, Germany) and desalted on a PD-10 column (1 × 20 cm; BioRad, Munich, Germany).

SDS–PAGE

Analytical SDS–PAGE of NCAM was carried out according to Laemmli (1970) in slab gels containing 7.5% polyacrylamide. Proteins were detected by silver staining. For immunostaining, proteins were blotted onto nitrocellulose. NCAM, PSA, and the HNK1 epitope were detected with mAb H28, mAb 735, and mAb 412, respectively.

Treatment of NCAM with PNGase F and endo N

Deglycosylation of NCAM using PNGase F from Flavobacterium meningosepticum (Oxford GlycoSciences, Abingdon Oxfordshire, UK) was performed in 20 mM sodium phosphate buffer, pH 7.2 at 37°C for 24 h (2 µU PNGase F per 10 µg of NCAM). The same conditions were employed for treatment of NCAM with endo-N (0.2 µg endo-N per 10 µg of NCAM) as well as in control experiments in which protein was similarly incubated without enzyme.

Isolation of tryptic glycopeptides

NCAM was carboxymethylated and digested with trypsin (25 µg of trypsin per mg of NCAM; sequencing grade; Sigma, Deisenhofen, Germany) at 37°C for 24 h. For fractionation, the sample was first applied to an immunoaffinity column (40 mg AbH28, 16 mg Ab735, and 16 mg Ab412) and, subsequently, the flow-through to a column (1 × 10 cm) containing mAb 412. Both columns were washed with five column volumes of PBS containing 0.5% (v/v) Triton X-100 and five column volumes
of PBS. Bound material was eluted with buffer containing 100 mM diethylylamine, pH 11.5, 150 mM NaCl, and 2 mM EDTA. Eluted glycopeptides were neutralized by addition of 500 mM Tris–HCl buffer, pH 6.8, and desalted on a Bio-Gel P30-column (1.5 × 20 cm; BioRad) with 50 mM NH₄HCO₃. Fractions were monitored by absorption at 206 nm and tested for carbohydrates by orcinol/H₂SO₄ staining. Positive fractions were pooled and lyophilized.

Isolation of oligosaccharides and peptide moieties
Glycans were released from the different glycopeptide fractions by treatment with PNGase F (20 mU of enzyme at 37°C for 24 h; Roche, Mannheim, Germany) and separated from residual peptides by RP-HPLC using a column filled with ODS-Hypersil C₁₈ (2.1 × 250 mm; 3 μm; Shandon, UK). Elution was carried out at 120 μl/min with a linear gradient from 0.1% trifluoroacetic acid to 60% acetonitrile containing 0.1% trifluoroacetic acid in 45 min at 30°C. Peptides were monitored by absorption at 220 nm; oligosaccharides were detected by orcinol/H₂SO₄ staining. Fractions of 60–120 μl were collected, pooled, and immediately evaporated to dryness. Pooled glycan fractions were desalted using Bio-Gel P2 (BioRad) as described earlier (Geyer and Geyer, 1993).

Desialylation of glycopeptides and oligosaccharides
Sialic acid residues were removed by mild acid hydrolysis in 500 μl of 1 M trifluoroacetic acid for 30 min at 80°C. After drying the sample in a SpeedVac concentrator, residual acid was removed by repeated addition of methanol and evaporation under vacuum (Geyer et al., 1992).

Derivatization of oligosaccharides
Oligosaccharides were pyridylaminated according to Kondo et al. (1990). Excess 2-aminopyridine and reaction byproducts were removed by gel filtration using a column of Fractogel HW-40F (Merck, Darmstadt, Germany) at a flow rate of 1 ml/min. A linear gradient of 0–300 mM potassium phosphate buffer, pH 4.4, was applied within 60 min for elution (Lochnit and Geyer, 1995). Reductive amination with 2-aminobenzamide was performed as described by Bigge et al. (1995) using the Signal™ Labelling Kit (Oxford GlycoSciences).

Anion-exchange HPLC
Anion-exchange HPLC was performed at 25°C at a flow rate of 1 ml/min using a Mikropak AX-10 column (0.46 × 25 cm; Varian, Walnut Creek, CA). For elution, a linear gradient of 0–300 mM potassium phosphate buffer, pH 4.4, was applied within 60 min (Liedtke et al., 1994).

Size-fractionation HPLC
Non-PSA/HNK1-PA-oligosaccharides were subjected to size-fractionation HPLC (Ohara et al., 1991) using a MN-Carbohydrate column (0.46 × 25 cm; Macherey & Nagel, Düren, Germany) at a flow rate of 1 ml/min. A linear gradient of 25–50% (v/v) 200 mM acetic acid-triethylamline buffer pH 7.3 in acetonitrile in 60 min was used. PA-oligosaccharide samples were dissolved in 75% aqueous acetonitrile prior to injection and monitored using a fluorescence spectrophotometer (Lochnit and Geyer, 1995).

Peptide sequencing
(Glyco)peptides were amino-terminally sequenced by automated Edman degradation using a Modular Sequencer (Knauer, Berlin, Germany) that had been modified to allow isotopic identification of the phenylhydantoin amino acid derivatives as described earlier (Frank, 1989; Strobl et al., 1997).

Carbohydrate constituent and methylation analysis
Carbohydrate constituents were identified as alditol acetates as detailed elsewhere (Geyer et al., 1982). For linkage analysis, glycopeptides or oligosaccharides were permethylated (PazParente et al., 1985) and hydrolyzed. Partially methylated alditol acetates obtained after reduction and acetylation were analyzed by capillary combined gas liquid chromatography/mass spectrometry (GLC-MS) using the instrumentation and microtechniques described earlier (Geyer and Geyer, 1994). For determination of sialic acid linkages, PSA glycopeptides were first treated with 250 μl of 0.05 M HCl in methanol for 1 min at room temperature. Then 500 μl of diazomethane were added. After 12 h at ambient temperature, the sample was dried in a stream of nitrogen, permethylated (Kvernheim, 1987), hydrolyzed, and reduced as above. Thereafter, the sample was again treated with 1 M HCl in methanol (5 min) and dried, and the carboxyester was reduced with sodium borohydride (see above). Following peracetylation, the sample was analysed by GLC-MS using a Hewlett-Packard Model 5989 instrument equipped with a HP-5MS capillary column (Hewlett-Packard, Waldbronn, Germany) using a temperature gradient of 150–320°C at 5°C/min. Electron impact spectra were recorded at 70 keV, and chemical ionization spectra were obtained with ammonia as reactant gas.

MALDI-TOF-MS
Data were obtained using a Vision 2000 mass spectrometer (Finnigan MAT, Bremen, Germany), equipped with a UV-nitrogen laser (λ = 337 nm). Mass spectra were recorded at an accelerating voltage of 5 kV in the positive- or negative-ion reflectron mode. Typically, obtained spectra result from the accumulation of 5–25 laser shots. Either 2,5-dihydroxybenzoic acid (10 mg/ml in 0.1% [v/v] aqueous trifluoroacetic acid, 30% [v/v] acetonitrile) or 6-aza-2-thiothymine (5 mg/ml in twice distilled water; Geyer et al., 1999) were used as matrix. One microliter of analyze solution was mixed on the stainless steel target with 1 μl of matrix solution and allowed to air-dry. In the positive-ion mode, the instrument was calibrated with an external mixture of isomaltosyl oligosaccharides containing 5–15 glucose units (measurement of oligosaccharides) or, in the case of peptides, with human angiotensin and bovine insulin (both from Sigma). For calibration in the negative-ion mode, isomaltosyl oligosaccharide derivatives with anthranilic acid tags were prepared (Anumula, 1994). Given mass values represent average masses. Overall mass accuracy was about 0.025%.

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**Abbreviations**

2-AB, 2-aminoazobenzamide; dHex, deoxyhexose; endo N, endo neuraminidase N; GLC-MS, combined gas liquid chromatography/mass spectrometry; Hex, hexose; HexNAc, N-acetylhexosamine; HNK1 epitope, SO₃Gal(β1-3)Gal(β1-4)GlcNAc; Ig, immunoglobulin; LaciNIac, GalNαC(β1-4)GlcNAc; mAb, monoclonal antibody; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NCAM, neural cell adhesion molecule; PA, pyridylamine; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide-N-deglycosylation; RP-HPLC, reversed-phase high-performance liquid chromatography.

**References**


