Biosynthesis of HNK-1 Glycans on O-Linked Oligosaccharides Attached to the Neural Cell Adhesion Molecule (NCAM)

THE REQUIREMENT FOR CORE 2 β1,6-N-ACETYLGLUCOSAMINYLTRANSFERASE AND THE MUSCLE-SPECIFIC DOMAIN IN NCAM

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Edgar Ong‡, Misa Suzuki, Frederic Belot§, Jiunn-Chern Yeh¶, Isabelle Franceschini†, Kiyohiko Angata, Ole Hindsaul, and Minoru Fukuda**

From the Glyobiology Program, Cancer Research Center, The Burnham Institute, La Jolla, California 92037

The HNK-1 glycan, sulfo-3GlcAβ1→3Galβ1→4-GlcNAcb1→R, is highly expressed in neuronal cells and apparently plays critical roles in neuronal cell migration and axonal extension. The HNK-1 glycan synthesis is initiated by the addition of β1,3-linked GlcA to N-acetyllactosamine followed by sulfation of the C-3 position of GlcA. The cDNAs encoding β1,3-glucuronyltransferase (GlcAT-P) and HNK-1 sulfotransferase (HNK-1ST) have been recently cloned. Among various adhesion molecules, the neural cell adhesion molecule (NCAM) was shown to contain HNK-1 glycan on N-glycans. In the present study, we first demonstrated that NCAM also bears HNK-1 glycan attached to O-glycans when NCAM contains the O-glycan attachment scaffold, muscle-specific domain, and is synthesized in the presence of core 2 β1,6-N-acetylglucosaminyltransferase, GlcAT-P, and HNK-1ST. Structural analysis of the HNK-1 glycan revealed that the HNK-1 glycan is attached on core 2 branched O-glycans, sulfo-3GlcAβ1→3Galβ1→4-GlcNAcb1→6Galβ1→3GalNAc. Using synthetic oligosaccharides as acceptors, we found that GlcAT-P and HNK-1ST almost equally act on oligosaccharides, mimicking N- and O-glycans. By contrast, HNK-1 glycan was much more efficiently added to N-glycans than O-glycans when NCAM was used as an acceptor. These results are consistent with our results showing that HNK-1 glycan is minimally attached to O-glycans of NCAM in fetal brain, heart, and the myoblast cell line, C2C12. These results combined together indicate that HNK-1 glycan can be synthesized on core 2 branched O-glycans but that the HNK-1 glycan is preferentially added on N-glycans over O-glycans of NCAM, probably because N-glycans are extended further than O-glycans attached to NCAM containing the muscle-specific domain.

Neural cell adhesion molecules undergo critical post-translational modifications that modulate their affinity and in some cases alter their specificity for their cognate ligands (1, 2). In particular, many neural cell adhesion molecules are heavily glycosylated, and the extent and composition of attached carbohydrates modify their adhesive properties. Among those, polysialic acid and HNK-1 are particularly notable. Polysialic acid is mostly attached to NCAM,1 and polysialylated NCAM is abundantly present in embryonic brain (1, 2). In the adult brain, polysialylated NCAM is restricted to certain tissues, such as the hippocampus and olfactory bulb, where neuronal regeneration persists. Polysialic acid is a linear homopolymer of α2,8-linked sialic acid residues formed on α2,3- or α2,6-linked sialic acid in N-acetyllactosamine of N-glycans (3–6). Polysialic acid is attached to two N-glycosylation sites in the fifth immunoglobulin-like domain in NCAM and thought to attenuate the adhesive property of NCAM (7–9).

The HNK-1 carbohydrate epitope was originally defined by a monoclonal antibody raised against human natural killer (HNK) cells (10). In nervous tissues, the HNK-1 carbohydrate was first recognized as an autoantigen involved in peripheral demyelinating neuropathy (11, 12). The structural analysis of glycolipids reacting with these autoantibodies demonstrated that the HNK-1 epitope is sulfo-3GlcAβ1→3Galβ1→4GlcNAcβ1→R (11, 12). HNK-1 glycan is widely distributed in glycoproteins, glycolipids, and proteoglycans (2, 11–13). The expression of HNK-1 glycan is spatially and developmentally regulated and found on migrating neuronal crest cells, cerebellum, and myelinating Schwann cells in motor neurons but not on those in the sensory neurons (14–16). Although it has been reported that HNK-1 glycan is attached to the N-glycosylation site in the third immunoglobulin-like domain of NCAM, the recent studies showed that HNK-1 glycan is attached to the second, third, fifth, and sixth N-glycosylation sites (17).

HNK-1 apparently plays critical roles in neural cell migration and axonal extension. HNK-1 glycolipids coated on plates facilitated neurite outgrowth, whereas no facilitation of neurite outgrowth was observed on sulfatide, 3′-sulfogalactosyl ceramide. The effect of HNK-1 glycan was abolished once the sulfate group was removed (18, 19). The HNK-1 glycan was identified in NCAM and N-glycans isolated from P0 glycoprotein (20, 21). It was also found as rather unusual structures of O-linked oligosaccharides, sulfo-3GlcAβ1→3Galβ1→4GlcNAcβ1→2Man, which then are attached to serine or threonine (22). This glycan can be

1 The abbreviations used are: NCAM, neural cell adhesion molecule; GlcA, glucuronic acid; MSD, muscle-specific domain; HNK-1ST, HNK-1 sulfotransferase; GlcAT-P, β1,3-glucuronyltransferase; VASE, variable alternatively spliced exon; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; GnT-I, β1,2-N-acetylglucosaminyltransferase I; Core2GlcNAcT, core 2 β1,6-N-acetylglucosaminyltransferase; ConA, concanavalin A.

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†Present address: Genset Corp., San Diego, CA 92121.
‡Present address: Department of Neuroscience, Institute Pasteur, 75724 Paris cedex 15, France.
§Present address: Centre de Chimie Organique, Institute Pasteur, 10901 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-646-3144; Fax: 858-646-3193; E-mail: minoru@burnham.org.
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attached to dystroglycan, and its defect may cause muscular dystrophy (23).

NCAM has numerous isoforms due to alternative splicing of precursor mRNAs. Among them, NCAM(MSD) contains an additional domain, the so-called muscle-specific domain (MSD), consisting of 37 amino acids between two fibronectin type III-like domains (24). This NCAM(MSD) is mostly present in skeletal myotubes and often anchored to the plasma membrane through glycoprophatidylinositol (24). The MSD is highly enriched with serine, threonine and proline and O-linked oligosaccharides were shown to attach to this domain (25). It has been shown that NCAM(MSD) from C2C12 myoblast cell line contains mucin-type O-linked oligosaccharides \( \pm NeuAc \) \( \rightarrow 3Gal\beta1 \rightarrow 3(\pm NeuAc2 \rightarrow 6Gal\)NaAc \( \rightarrow Thr/Ser \) (25). However, no studies have addressed whether O-glycans in NCAM contain an HNK-1-capping structure.

The HNK-1 glycan is synthesized in a stepwise manner by the addition of a \( \beta,\)\( 1,3 \)-linked glucuronic acid to precursor N-acetyllactosamine by \( \beta,\)\( 1,3 \)-glucosaminyltransferase (GlcAT-P) followed by the addition of a sulfate group by HNK-1ST to GlcA followed by the addition of a sulfate group by HNK-1 sulfo-I site is underlined, and the rest of the sequence is nucleotides (26).

HNK-1 glycans on NCAM, although GlcAT-P and HNK-1ST can be formed on core 1 O-glycans but not to core 1 O-glycans present in NCAM, although GlcAT-P and HNK-1ST utilize O-glycan and N-glycan oligosaccharides almost equally as an acceptor.

**EXPERIMENTAL PROCEDURES**

**Plasmids Encoding NCAM, NCAM(MSD), GlcAT-P, and HNK-1ST**—pig-NCAM-IgG and pig-NCAM(MSD)-IgG encoding NCAM-IgG and NCAM(MSD)-IgG chimeric proteins, respectively, were constructed from pig-NCAM/VASE, MSD,IgG, originally provided by Dr. David Simmons at Oxford University (31), as described previously (32). All products derived from these different vectors were found to react with anti-NCAM antibody (Erie-1) by Western blotting, confirming the sequences and sizes of the products (32).

The cDNA encoding rat \( \beta,\)\( 1,3 \)-glucuronyltransferase, GlcAT-P (27), was cloned as described previously using reverse transcription-PCR and subcloned into pcDNA3, resulting in pcDNA3(neo)-GlcAT-P (29). The cDNA encoding a catalytic domain of GlcAT-P was obtained by PCR (27) using pcDNA3(neo)-GlcAT-P as a template. 5′- and 3′-primers for the PCR are 5′-CGQAATCCAGCTCGACACTTGGCTTCGCT-3′ (the BamHI site is underlined, and the rest is nucleotides 120–141 of GlcAT-P) and 5′-ACTCGAGTCAGATCTCCACCGAGGGGTGTC-3′ (the XhoI site is underlined, and the rest of the sequence is nucleotides 1044–1024 of GlcAT-P). After BamHI and XhoI digestion, the cDNA fragment was ligated into the same sites of pcDNA3(neo)-GlcAT-P (29).

**Preparation of Lec2 Cells Expressing HNK-1 Precursors and HNK-1 Glycans**—To avoid the formation of HNK-1 in N-glycans, the majority of the experiments were carried out using a mutant Chinese hamster ovary cell line, Lec1. Since Lec1 cells are deficient in GnT-I, all of the NCAM glycosylation in Lec1 cells is synthesized in the presence of zeocin (Invitrogen) and then by immunofluorescent staining using anti-NCAM antibody (Eric-1) by Western blotting, confirming the sequence and sizes of the products (32).

**Metabolic Labeling of Carbohydrate Moiety of NCAM-IgG**—Lec1 cells expressing GlcAT-P and HNK-1ST, Lec2-HNK-1, were established as described previously (29). Lec2-HNK-1 cells were selected using anti-HNK-1 monoclonal antibody. Lec2 cells expressing GlcAT-P were similarly established and selected by immunofluorescent staining using M6749 antibody, with a secondary antibody as described previously (29). Lec2-HNK-1 cells were selected using anti-HNK-1 monoclonal antibody. Lec2 cells expressing GlcAT-P were similarly established and selected by immunofluorescent staining using M6749 monoclonal antibody. Lec2 cells do not synthesize sialylated oligosaccharides due to the defect in CMP-sialic acid transporter in the Golgi (40).

**Isolation of O-Linked Oligosaccharides Containing HNK-1 Glycans**—Metabolically labeled NCAM/MSD-IgG was digested with Pronase, and labeled glycopeptides were isolated by Sephadex G-25 gel filtration as described previously (41). O-Glycan-containing glycopeptides were separated from high mannose glycopeptides and other glycosylating ConA-Sepharose affinity chromatography. The purified glycopeptides were subjected to alkaline reductive treatment to release O-linked oligosaccharides from NCAM-IgG (36). The digested NCAM-IgG samples were applied to a Poly-Prep column (Bio-Rad) containing 2 ml of anion-exchange QAE-Sephadex A-25. The resin was equilibrated with 2 mM pyridine-acetate buffer, pH 5.5, and eluted in a stepwise manner with 60 mM, 250 mM, and 1 M NaCl in 2 mM pyridine-acetate buffer, pH 5.5. Fractions (1 ml) were collected, and aliquots were taken for determination of radioactivity.
The oligosaccharides eluted with 1 mM NaCl were digested with Arthrobacter siailandicus (Sigma) to remove sialic acid and subjected to QAE-Sephadex A-25 column chromatography. Those eluted with 1 mM NaCl after sodium treatment were subjected to structural characterization.

Structural Characterization of O-Glycans Containing HNK-1 Glycan—The O-linked glycans were subjected to solvolysis to remove a sulfate group as described previously (41, 42). The resultant oligosaccharides, purified by QAE-Sephadex chromatography, were digested with 125 milliunits of Escherichia coli β-glucuronidase (Sigma) in 50 μl of the reaction mixture under the same conditions as described previously (43). The obtained oligosaccharides were analyzed on an AX-5 amino-bonded column using a Gilson 306 HPLC apparatus at a flow rate of 0.8 ml/min. The column was equilibrated with 90% solvent A (80% acetonitrile, 20% 15 mM KH2PO4) and 10% of solvent B (40% acetonitrile, 60% KH2PO4). After 5 min, the elution was carried out by linear gradient from 10% of solvent B to 100% of solvent B in 60 min. The column was finally washed for 5 min with solvent B. Fractions were collected every minute, and aliquots were taken for determination of the radioactivity by scintillation counting.

Periodate Oxidation of Oligosaccharides—Oligosaccharides were oxidized with 50 mM NaOCl in 50 mM sodium acetate buffer, pH 4.5, at room temperature, and oxidized samples were reduced as described previously (41). The samples, after destroying NaBH4 by acetic acid, were applied to a small column of Dowex 50 × 8 and dried after the addition of methanol. The samples were then hydrolyzed in 0.1M HCl at 80 °C for 1 h, neutralized with 0.1 M Tris-HCl buffer, pH 8.5, and applied to a column (1 × 120 cm) of Bio-Gel P-4 (200–400-mesh) equilibrated with 0.1 mM pyridine-acetate buffer. pH 5.5. Oligosaccharides were also separated by QAE-Sephadex A-25 column chromatography as described above.

Assays for GlcAT-P and HNK-1ST Activity—To assay the activities of GlcAT-P and HNK-1ST, soluble protein A-GlcAT-P and soluble protein A-HNK-1ST were prepared and assayed to IgG-Sepharose as described previously (33). To assay for GlcAT-P activity, the reaction mixture (100 μl) consisted of 500 μl acceptor, 0.1 mM HEPES buffer, pH 6.5, 10 mM MnCl2, 10 mM of galactonic acid-β-lactone, 50 μl of the soluble chimeric enzyme attached to beads, and 50 μl UDP-14C GlcA (284 μCi/mmol, PerkinElmer Life Sciences). The reaction mixture was incubated for 3 h at 37 °C, and the reaction was stopped by adding 50 mM (final concentration) EDTA.

To assay for HNK-1ST activity, the donor, 3′-phosphate 5′-phosphosulfate, and various amounts of an acceptor were incubated with a solution of the 50% suspension of the chimeric protein A-HNK-1ST attached to the conditions described previously (29, 33). The reaction was stopped by 250 mM ammonium formate, pH 4, and labeled products were obtained by C18 reverse-phase chromatography as described previously (29). The Km for GlcAT-P and HNK-1ST was determined using a Lineweaver-Burk plot at various concentrations of the acceptor (2.5–2000 μM).

Synthesis of Standard Oligosaccharides—The acceptors GlcAβ1-3Galβ1-4GlcNAcβ1-6Manβ1-6Manβ1-2OH(CH2)7CH3 (octyl) (compound 1), GlcAβ1-3Galβ1-4GlcNAcβ1-2Manβ1-6Manβ1-2OH ( Compound 2) and glucosaminyltransferase I, which is the first key enzyme to form complex N-glycans (36). Because this enzyme is absent in Lecl cells, N-glycans remain as the high mannose type, and N-acetyllactosamine, which is a precursor for HNK-1 glycan, is not formed in Lecl cells.

Lecl cells also lack core 2 β1,6-N-acetylgalactosaminyltransferase (Core2GlcNAcT) as in wild-type Chinese hamster ovary cells. Since mucin-type oligosaccharides can acquire N-acetyllactosamine when core 2 branches are formed by Core2GlcNAcT, Lecl cells expressing HNK-1 glycan (Lecl-HNK-1) were further transfected with cDNA encoding Core2GlcNAcT-I (see Fig. 1). As shown in Fig. 2A, HNK-1 was detected by Western blotting in NCAM(MSD)-IgG synthesized in Lecl cells expressing HNK-1ST and GlcAT-P, together with Core2GlcNAcT-I (lanes 4). When HNK-1ST was absent, glucuronylated precursor glycan was detected by anti-gluconic acid antibody, M6749 (GlcA), but not by anti-HNK-1 antibody (HNK-1) (lanes 6 in Fig. 2A). Multiple HNK-1-positive bands may represent NCAM(MSD)-IgG containing different numbers of sulfate groups, since glucuronylated molecules correspond mainly to the lowest molecular weight band (Fig. 2A, lanes 6). Moreover, NCAM(MSD)-IgG did not react with either anti-HNK-1 antibody or M6749 antibody when Lecl cells were not transfected with Core2GlcNAcT cDNA (Fig. 2A, lanes 1–3). These results establish that Core2GlcNAcT, GlcAT-P, and HNK-1ST are all necessary to form the HNK-1 glycan in NCAM(MSD).

To determine whether HNK-1 glycans can be attached to O-glycans in sequences other than MSD, NCAM-IgG lacking the MSD sequence was also expressed in the same Lecl cells expressing Core2GlcNAcT-I and HNK-1ST glycan on the cell surface. As shown in Fig. 2B, NCAM synthesized in these Lecl cells did not bear the HNK-1 glycan or glucuronylated precursor regardless of whether Core2GlcNAcT-I was present or absent. Minor HNK-1-positive glycoproteins were detected when Lecl cells expressed Core2GlcNAcT-I, GlcAT-P, and HNK-1ST (Fig. 2B, lane 4). They were judged to be contaminating glycoproteins other than NCAM based on their mobility in SDS-polyacrylamide gel electrophoresis. These results indicate that the MSD sequence is essential for acquiring the HNK-1 glycan in O-glycans attached to NCAM.

Structural Characterization of the HNK-1 Glycan Attached to the MSD Sequence in NCAM—To determine the structure of the HNK-1 glycan attached to NCAM, NCAM(MSD)-IgG was transiently expressed in Lecl-core 2 HNK-1 cells expressing HNK-1 glycan and core 2 branched O-glycans. Twenty-four h
after the transfection, the transfected cells were cultured for an additional 48 h in sulfate-free medium in the presence of [3H]GlcN, [3H]Gal, and [35S]sulfate. Similarly, the transfected cells were cultured in glucose-free medium with [35S]sulfate. NCAM(MSD)IgG molecules purified from the cultured cells were then subjected to SDS-polyacrylamide gel electrophoresis and fluorography. The results showed that [35S]sulfate, [3H]galactose, and [3H]glucosamine were incorporated into NCAM(MSD)IgG (data not shown).

As a first series of experiments for structural characterization, NCAM(MSD)IgG labeled with [3H]galactose, [3H]glucosamine, and [35S]sulfate was used. Glycopeptides were prepared and applied to ConA-Sepharose chromatography. The results shown in Fig. 3 demonstrated that [35S]labeled material was not bound to ConA-Sepharose. The glycopeptides eluted with 10 mM methyl-α-glucoside and 250 mM methyl-α-mannoside represent bi-antennary and high mannose type N-glycans, respectively, and they did not contain sulfate. The glycopeptides unbound to ConA-Sepharose (shown in the horizontal bar in Fig. 3), representing O-glycans and multiantennary N-glycans, were then subjected to alkaline borohydride treatment to release O-linked oligosaccharides.

The treated sample was separated into two peaks after Sephadex G-25 gel filtration. The first peak of higher molecular weight contained both [35S] and [3H] radioactivity, whereas the second peak contained only [3H] radioactivity (data not shown). Upon QAE-Sephadex column chromatography, all of the [35S]-labeled material was recovered in the fraction eluted with 1 M NaCl in the pyridine-acetate buffer (Fig. 4A, Q3). This [35S]- and [3H]-labeled material eluted again in this highly anionic fraction after sialidase treatment (Fig. 4B). Since no [3H] radioactivity was released, peak Q3 did not contain sialic acid, which can be labeled from [3H]glucosamine precursor. In contrast, the glycopeptides eluted with 2–250 mM NaCl (Q0, Q1, and Q2 in Fig. 4A) were mostly eluted at the void volume after sialidase treatment (data not shown), consistent with the fact that they were devoid of [35S]sulfate.

Since no sulfatase isolated to date is known to remove a sulfate group from HNK-1 glycan, we opted to remove sulfate by solvolysis. The sample after solvolysis eluted with 70 mM pyridine-acetate buffer (Fig. 4C, D), while a small amount of remaining HNK-1 glycan existed (peak d in Fig. 4D). Peak b in Fig. 4C, desulfated HNK-1 glycan, was subjected to Bio-Gel P-4 gel filtration before and after β-glucu-
results established that HNK-1 glycan, shown as peak a in Fig. 4B, has the structure sulfate\(\rightarrow\)GlcA\(\beta1\)\(\rightarrow\)Gal\(\beta1\)\(\rightarrow\)4GlcNAc\(\beta1\)\(\rightarrow\)6(Gal\(\beta1\)\(\rightarrow\)3)GalNAcOH.

**Periodate Oxidation of HNK-1 Glycan and Its Desulfated Form**—The above results establish that the HNK-1 glycan is attached to core 2 branched O-glycans. The above experiments, however, did not determine the linkage and attachment sites of sulfate and glucuronic acid. In order to obtain this information, the HNK-1 glycan and its nonsulfated form (peaks a and b in Fig. 5, A and B, respectively) were subjected to periodate oxidation followed by reduction and mild acid hydrolysis (Smith degradation). Periodate oxidation takes place in a cis-glycol group.

As a control experiment, core 2 O-glycan, Gal\(\beta1\)\(\rightarrow\)4GlcNAc\(\beta1\)\(\rightarrow\)6(Gal\(\beta1\)\(\rightarrow\)3)GalNAc (Fig. 5C, c) was subjected to Smith degradation. This treatment produced two peaks: f and g (Fig. 5F). Peaks f and g represent GlcNAc and glycerol derived from galactose, respectively. Peak c derived from HNK-1 glycan yielded the same results. When peak b in Fig. 5B, nonsulfated HNK-1 glycan, was subjected to the same treatment, peaks e and g were produced (Fig. 5E). Peak e corresponds to Gal\(\beta1\)\(\rightarrow\)4GlcNAc, which was confirmed by exoglycosidase digestion.

These results show that glucuronic acid prevented galactose from oxidation, indicating that glucuronic acid residue was attached to C-3 of galactose. Otherwise, peak b in Fig. 5B and peak c in Fig. 5C would produce the same product. When peak a in Fig. 5A was subjected to the same treatment, peak d was obtained (Fig. 5D). Peak d was larger than peak e and was judged to be produced from sulfated glycans. Peak e in Fig. 5D was probably produced from nonsulfated glycans that were formed during various preparation steps. These results thus indicate that sulfate protected the GlcA residue from oxidation, and peak d corresponds to sulfo\(\rightarrow\)3GlcA\(\beta1\)\(\rightarrow\)3Gal\(\beta1\)\(\rightarrow\)4GlcNAc. The presence of sulfate and glucuronic acid in peak d was confirmed by the fact that peak d still had two anionic charges as assessed by QAE-Sephadex column chromatography (data not shown).

These results, combined together, establish the structure of HNK-1 glycan as sulfo\(\rightarrow\)3GlcA\(\beta1\)\(\rightarrow\)3Gal\(\beta1\)\(\rightarrow\)4GlcAc\(\beta1\)\(\rightarrow\)6(Gal\(\beta1\)\(\rightarrow\)3)GalNAc.

**NCAM in Mouse Embryonic Brain and Heart and C2C12 Cells Contain Negligible Amounts of HNK-1 Glycans Attached to O-glycans**—We then examined whether NCAM in mouse embryonic brain and heart contain HNK-1 glycans attached to O-glycans. NCAM present in brain at embryonic day 12 exhibit broad high molecular weights that were converted to bands of 200 and 140 kDa after N-glycanase treatment (Fig. 6A, lanes 1 and 2). When the same blot was incubated with anti-HNK-1 antibody, no band corresponding to N-glycanase-treated NCAM reacted with the antibody (Fig. 6A, lanes 2 and 6). The same experiment showed that small amounts of HNK-1 glycan were attached to N-glycans in NCAM, displaying two bands that are slightly larger than 220 kDa (Fig. 6A, lane 5). The remaining HNK-1 antigen was mostly associated with molecules larger than 220 kDa, suggesting that those HNK-1 glycans are associated with proteoglycans. HNK-1 was barely detected in heart tissue, although NCAM was detected (Fig. 6A, lanes 3, 4, 7, and 8).

By using similar methods, the myoblast cell line, C2C12, was subjected to analysis. C2C12 cells were derived from mouse cells and can be induced to form myotubes (25). Our preliminary results showed that HNK-1 glycan was barely detected by Western analysis of cell lysates. We thus use \(^{35}\)S-sulfate incorporation to detect sulfated glycans. The results shown in Fig. 6B illustrate that after 4 days of induced differentiation, C2C12 cells expressed \(^{35}\)S-sulfate molecules that were heterogeneous.
Fig. 5. Bio-Gel P-4 gel filtration of HNK-1 glycans before and after various treatments. A–C, peak a in Fig. 4B (A) and peak b in Fig. 4C before (B) and after β-glucuronidase treatment (C) were subjected to Bio-Gel P-4 gel filtration. D–F, the samples shown in A–C were subjected to Smith degradation and subjected to the same Bio-Gel P-4 gel filtration. Peaks a–c correspond to sulfos-GlcAβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl (K_m = 358 μM). Core 2 branched O-glycan is an intermediate between these N-glycan acceptors (K_m = 283 μM). These results combined indicate that core 2 branched O-glycan is a good acceptor as N-glycans for both GlcAT-P and HNK-1ST.

Despite the fact that the enzymes responsible for HNK-1 synthesis can utilize N-glycan and O-glycan acceptors almost equally well, HNK-1 was barely detected in NCAM O-glycans in tissues examined. These results obtained as a whole thus suggest that HNK-1 may be more efficiently added to N-glycans than O-glycans when they are attached to NCAM. In order to test this hypothesis, Lec1 cells expressing GlcAT-P and HNK-1ST were transiently transfected with cDNA encoding GrT-I. This transfection allows Lec1 cells to gain complex N-glycans, which can then become substrates for both GlcAT-P and HNK-1ST. The amount of HNK-1 glycan was significantly increased in NCAM(MSD) derived from Lec1 cells expressing GrT-I, compared with NCAM(MSD) derived from the parent Lec1 cells (Fig. 9A, compare lanes 7 and 9). The large majority of HNK-1 glycans were removed after N-glycanase treatment (Fig. 9A, lane 10), indicating that the majority of HNK-1 glycan is associated with newly synthesized N-glycans.

It is possible that sialylation inhibits HNK-1 glycan formation in core 2 branched O-glycans, since sialylation and glucoronylation compete for the same N-acetyllactosamine. To test this possibility, Lec2 cells that are deficient in sialylation were used to express HNK-1 glycan. Transfected Lec2 cells bearing both N-glycans and O-glycans expressed significant amounts of HNK-1 glycan, but almost all HNK-1 glycans were removed after N-glycanase treatment (Fig. 9A, lanes 11 and 12). Almost identical results were obtained when NCAM(MSD) were labeled with [35S]sulfate (Fig. 9B). These results as a whole indicate that HNK-1 synthesis is shifted from O-glycans to N-glycans once O-glycan acceptor substrates are available.

**DISCUSSION**

The present study demonstrated that HNK-1 can be synthesized in O-glycans when three enzymes, GlcAT-P, HNK-1ST, and Core2GlcNAcT, are transfected into Lec1 cells, which lack the ability to form the HNK-1 glycan attached to complex and hybrid N-glycans. Since Lec1 cells lack Core2GlcNAcT, a simultaneous expression of Core2GlcNAcT is essential to form HNK-1 glycan on O-glycans. Our structural analysis of HNK-1 glycan indeed demonstrated that HNK-1 glycan is synthesized on N-acetyllactosamine in core 2 branched O-glycans (Fig. 1). This requirement of Core2GlcNAcT was also demonstrated for sialyl Lewis x synthesis in mucin-type O-glycans, since sialyl Lewis x, Sialo2→3Galβ1→4(Fuca1→3)GlcNAcβ1→R, is also...
synthesized on N-acetyllactosamine backbone, which is formed by Core2GlcNAcT (41, 48).

The present study also demonstrated that mucin-type O-glycans bearing HNK-1 can be synthesized only when NCAM contains an MSD. MSD is enriched with threonine, serine, and proline, which is characteristic for amino acid sequences that attach mucin-type O-glycans (23, 24). A short amino acid sequence that consists of these amino acids is also present in the other part of NCAM, but it apparently does not serve for O-glycosylation attachment sites. MSD is encoded by four different exons, and differential splicing of NCAM precursor mRNA leads to the formation or absence of MSD. The expression of MSD-containing NCAM is temporally and spatially restricted, and myotubes express NCAM containing MSD (24).

Since the amino acid sequence of MSD is enriched with proline, threonine, and serine, MSD most likely does not have a unique conformation, such as an α-helical or β-sheet structure (49). Since O-glycosylation takes place in the Golgi apparatus, O-glycosylation sites need to be exposed to the environment after a protein folds. Non-α-helical and non-β-sheet structure satisfies such a requirement. In addition, it is most likely that such an amino acid sequence does not take a particular conformation, and its structure is flexible (49). It is thus possible that MSD may function as a hinge region where O-glycans are attached. This situation is similar to the hinge region of IgA, where multiple O-glycans are attached (50, 51). Lysosomal membrane glycoproteins (LAMP-1 and LAMP-2) also contain a hingelike structure, and the hingelike structure of human LAMP-1 has homology to the IgA hinge region (52, 53). Future studies are of importance to determine whether MSD in NCAM also functions as a hingelike structure.
Despite the fact that HNK-1 glycan can be added to O-glycans to NCAM(MSD) upon transfection with GlcAT-A and HNK-1, NCAM isolated from embryonic brain, heart, and C2C12 cells did not bear detectable amounts of HNK-1 epitope in O-glycans. This is most likely not due to the absence of Core2GlcNAC-T, since Core2GlcNAC-T-I is expressed in the brain and heart (54). One possible explanation for this discrepancy is that the expression levels of GlcAT-P and HNK-1ST are lower in these tissues compared with Lec1 cells or HeLa cells (data not shown) transfected with GlcAT-P and HNK-1ST. On the other hand, the same experiment clearly detected HNK-1 glycan in molecules with high molecular weights. Since these HNK-1-positive molecules still showed a polydispersity after N-glycanase treatment, it is likely that these molecules represent proteoglycans. These results indicate that the method used was sensitive enough to detect HNK-1 glycan. As a whole, it can be concluded that HNK-1 glycan is only barely, if at all, present on O-glycans attached to NCAM in the tissues examined.

The above results could be obtained if GlcAT-P and HNK-1 utilize N-glycans as acceptors better than O-glycans. However, GlcAT-P and HNK-1ST did not utilize N-glycan acceptors better than O-glycans when synthetic oligosaccharides that mimic N-acetyllactosamines, presumably on the O-glycans (59). These results strongly suggest that synthesis of O-glycans acceptors is available.

HNK-1 glycan has been shown to bind to laminin (2), whereas sialylated core 1 O-glycan, NeuNacO–6GalNAcO, was shown to bind Siglec such as myelin-associated glycoprotein (57). These results suggest that the addition of a HNK-1-capping structure on NCAM plays a critical role in neural cell development.

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REFERENCES

1. Edelman, G. M., and Crossin, K. L. (1991) Annu. Rev. Biochem. 60, 155–190
2. Schachter, H., and Martini, R. (1995) Trends Neurosci. 18, 183–191
3. Finne, J. (1982) J. Biol. Chem. 257, 11966–11970
4. Kudo, M., Kitajima, K., Inoue, S., Shikishita, K., Morris, R. R., Dell, A., and Inoue, Y. (1996) J. Biol. Chem. 271, 22667–22677
5. Angata, K., Suzuki, M., McAlluliffe, J., Ding, Y., Hingdaeg, O., and Fukuda, M. (2000) J. Biol. Chem. 275, 18694–18691
6. Nakayama, J., Angata, K., Keng, E., Katayama, T., and Fukuda, M. (1996) Pathol. Int. 46, 665–677
7. Nelson, R. W., Bates, P. A., and Rutishauer, U. (1995) J. Biol. Chem. 270, 17171–17175
8. Angata, K., Suzuki, M., and Fukuda, M. (1998) J. Biol. Chem. 273, 28524–28532
9. von der Ohe, M., Wheeler, S. F., Wahrer, M., Harvey, D. J., Liedkte, S., Muhlenhoff, M., Gehr, B., Geyer, H., Dwek, R. A., Geyer, R., Wing, D. R., and Schachter, M. (2002) Glycobiology 12, 47–63
10. Abo, T., and Balle, C. M. (1991) J. Immunol. 127, 1024–1029
11. Chou, D. K., Byea, A. A., Evans, J. E., Costello, C., Quailes, R. H., and Jungalwala, B. F. (1986) J. Biol. Chem. 261, 11717–11725
12. Ariga, T., Kohriyama, K., Inoue, S., Shikishita, K., Kon, K., Ando, S., Saito, M., Latov, N., Freddo, L., and H.N.K.-1ST. On the other hand, the same experiment clearly detected HNK-1 glycan in molecules with high molecular weights. Since these HNK-1-positive molecules still showed a polydispersity after N-glycanase treatment, it is likely that these molecules represent proteoglycans. These results indicate that the method used was sensitive enough to detect HNK-1 glycan. As a whole, it can be concluded that HNK-1 glycan is only barely, if at all, added on O-glycans attached to NCAM in the tissues examined.

The above results could be obtained if GlcAT-P and HNK-1 utilize N-glycans as acceptors better than O-glycans. However, GlcAT-P and HNK-1ST did not utilize N-glycan acceptors better than O-glycans when synthetic oligosaccharides that mimic a portion of N-glycans or O-glycans were used as acceptors. Moreover, the synthesis of HNK-1 glycan on NCAM was substantially increased when Lec1 cells were converted to synthesize complex-type N-glycan acceptors for HNK-1 glycan by transfecting with GnT-I. The amount of HNK-1 glycan on O-glycans is much less than that on N-glycans (for Lec1 cells bearing complex type N-glycans). These results indicate that GlcAT-P and HNK-1ST are more accessible to N-glycan acceptors than to O-glycan acceptors in glycoproteins such as NCAM. This is probably because N-glycans are more extended from the polypeptide backbone than O-glycans. On the other hand, PSGL-1 glycoprotein containing numerous O-glycans has an extended riodlike structure and has been shown to have sialylated N-acetyllactosamines, presumably on the O-glycans (55, 56). These combined results strongly suggest that MSD of NCAM may not display as preferable a conformation for enzymes that synthesize O-glycans as the conformation of PSGL-1.

HNK-1 glycan has been shown to bind to laminin (2), whereas sialylated core 1 O-glycan, NeuNacO–6GalNAcO, was shown to bind Siglec such as myelin-associated glycoprotein (57). These results suggest that the addition of a HNK-1-capping structure on NCAM O-glycans may interact with the interaction of NCAM with myelin-associated glycoprotein to that with laminin. Such switching probably plays a critical role in neural cell development.

Our studies demonstrated that HNK-1 synthesis may be synthesized in a complex manner and depends on how much N-glycan acceptors versus O-glycan acceptors are available. Similarly, it has been demonstrated that GlcNac-6-O-sulfotransferase preferentially acts on N-glycans when glycoprotein acceptors such as CD34 bear both N- and O-glycans (41). By contrast, the same enzyme can act efficiently on O-glycans when the enzyme acts on GlyCAM-1, which almost exclusively contains O-glycans (58). In addition, it has been demonstrated that N-acetyllactosamine formation in core 2 branched O-glycans is much more efficient than in N-glycans (59). These results suggest that the synthesis of N-acetyllactosamine in core 2 glycans is a rate-limiting step for the addition of HNK-1 epitope structure on O-glycans. These results combined indicate that the synthesis of terminal, functional oligosaccharide groups in O-glycans is regulated in a complex manner and that the availability of acceptors in N- or O-glycans and their formation play a critical role in displaying such functional groups in different glycan classes.
43. Carlsson, S. R., Sasaki, H., and Fukuda, M. (1986) J. Biol. Chem. 261, 12787–12795
44. Belot, F., and Jacques J. C. (2000) Carbohydr. Res. 326, 88–97
45. Ujita, M., McAuliffe, J., Hindsaull, O., Sasaki, K., Fukuda, M. N., and Fukuda, M. (1999) J. Biol. Chem. 274, 16717–16726
46. McAuliffe J. C., Fukuda, M., and Hindsaull, O. (1999) Bioorg. Med. Chem. Lett. 9, 2855–2858
47. Misra, A. K., Fukuda, M., and Hindsaull, O. (2001) Bioorg. Med. Chem. Lett. 11, 2667–2669
48. Li, F., Wilkins, P. P., Crawley, S., Weinstein, J., Cummings, R. D., and McEver, R. P. (1996) J. Biol. Chem. 271, 3255–3264
49. Carlsson, S. R., Nakanishi, K., and Fukuda, M. (1993) Arch. Biochem. Biophys. 304, 65–73
50. Tsuzukiya, Y., Wang, C. C., and Putnam, F. W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1104–1108
51. Baenziger, J., and Kornfeld, S. (1984) J. Biol. Chem. 249, 7270–7281
52. Viitala, J., Carlsson, S. R., Siebert, P. D., and Fukuda, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3743–3747
53. Fukuda, M. (1991) J. Biol. Chem. 266, 21327–21330
54. Yeh, J. C., Ong, E., and Fukuda, M. (1999) J. Biol. Chem. 274, 3215–3221
55. Li, F., Erickson, H. P., James, J. A., Moore, K. L., Cummings, R. D., and McEver, R. P. (1996) J. Biol. Chem. 271, 6342–6348
56. Figarella-Branger, D., Daniel, L., Andrie, P., Guia, S., Renaud, W., Monti, G., Vivier, E., and Rougon, G. (1999) Am. J. Pathol. 155, 1261–1269
57. Kelm, S., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., De Biard, M. E., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994) Curr. Biol. 4, 965–972
58. Bistrup, A., Bhaktia, S., Lee, J. K., Belov, Y. Y., Gunn, M. D., Zan, F. R., Huang, C. C., Kannagi, R., Rosen, S. D., and Hemmerich, S. (1999) J. Cell Biol. 145, 899–910
59. Ujita, M., McAuliffe, J., Schwientek, T., Almeida, R., Hindsaull, O., Clausen, H., and Fukuda, M. (1998) J. Biol. Chem. 273, 34843–34849
60. Maemura, K., and Fukuda, M. (1992) J. Biol. Chem. 267, 24379–24386
61. Priatel, J. J., Chui, D., Hiraoka, N., Simmons, C. J. T., Richardson, K. B., Page, D. M., Fukuda, M., Varki, N. M., and Marth, J. D. (2000) Immunity 12, 273–283
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Edgar Ong, Misa Suzuki, Frederic Belot, Jiunn-Chern Yeh, Isabelle Franceschini,
Kiyohiko Angata, Ole Hindsgaul and Minoru Fukuda

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