Expression Cloning of a Human Sulfotransferase That Directs the Synthesis of the HNK-1 Glycan on the Neural Cell Adhesion Molecule and Glycolipids*

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The HNK-1 carbohydrate is expressed on various adhesion molecules in the nervous system and is suggested to play a role in cell-cell and cell-substratum interactions. Here we describe the isolation and functional expression of a cDNA encoding a human sulfotransferase that synthesizes the HNK-1 carbohydrate epitope. A mutant Chinese hamster ovary cell line, Lec2, which stably expresses human neural cell adhesion molecule (N-CAM) (Lec2-NCAM), was first established. Lec2-NCAM was co-transfected with a human fetal brain cDNA library, a cDNA encoding the rat glucuronyltransferase that forms a precursor of the HNK-1 carbohydrate, and a vector encoding the polyoma large T antigen. The transfected Lec2-NCAM cells expressing the HNK-1 glycan were enriched by fluorescence-activated cell sorting. Sibling selection of recovered plasmids resulted in a cDNA encoding a sulfotransferase, HNK-1ST, that directs the expression of the HNK-1 carbohydrate epitope on the cell surface. The deduced amino acid sequence indicates that the enzyme is a type II membrane protein. Sequence analysis revealed that there is a short amino acid sequence in the presumed catalytic domain, which is highly homologous to the corresponding sequence in other Golgi-associated sulfotransferases so far cloned. The amount of HNK-1ST transcript is high in fetal brain compared with fetal lung, kidney, and liver. Expression of HNK-1ST resulted in the formation of the HNK-1 epitope on N-CAM and a soluble chimeric form of HNK-1ST was shown to add a sulfate group to a precursor, GlcAβ1→3Galβ1→4GlcNAcβ1→R, forming sulfogluco-3GlcAβ1→3Galβ1→4GlcNAcβ1→R. The results combined together indicate that the cloned HNK-1ST directs the synthesis of the HNK-1 carbohydrate epitope on both glycoproteins and glycolipids in the nervous tissues.

Neural cells express unique carbohydrates that are often shared by immune cells (1, 2). One of them is the HNK-1 carbohydrate epitope, originally discovered by a monoclonal antibody raised against human natural killer cells (3), although its role in immune cells is not known. The functional significance of the HNK-1 carbohydrate was first recognized as an auto-antigen involved in peripheral demyelinating neuropathy. The structural analysis of glycolipids reacting with these auto-antibodies led to the discovery that the HNK-1 epitope is sulfogluco-3GlcAβ1→3Galβ1→4GlcNAcβ1→R (4, 5).

By using HNK-1-specific antibodies and carbohydrate structural studies, the HNK-1 glycan has been found in a number of neural cell adhesion molecules including N-CAM, myelin-associated glycoprotein, L1, contactin, and P0 (2, 6–9). The studies, using either monoclonal antibodies or isolated carbohydrates, demonstrated that the HNK-1 glycan is involved in cell-cell and cell-substratum interactions (10, 11). In one study, the inhibition by HNK-1 oligosaccharide was abolished by desulfation of the HNK-1 glycan indicating the critical role of the sulfate group (11). The expression of the HNK-1 epitope is spatially and developmentally regulated, and is found on migrating neural crest cells, cerebellum, and myelinating Schwann cells in motor neurons but not on those in the sensory neurons (12–14). In addition, the HNK-1 carbohydrate was shown to bind to P- and L-selectins (15), suggesting that the interactions between immune cells and the nervous system may be mediated through the binding of the HNK-1 carbohydrate in neural cells.

The HNK-1 carbohydrate is synthesized in a stepwise manner by the addition of a β,1,3-linked glucuronic acid to a precursor N-acetyllactosamine followed by the addition of a sulfate group to GlcAβ1→3Galβ1→4GlcNAcβ1→R (16, 17). Recently, Terayama et al. (18) reported the cloning of a β,1,3-glucuronyltransferase, GlcAT-P, that forms an HNK-1 precursor carbohydrate, GlcAβ1→3Galβ1→4GlcNAcβ1→R, in glycoproteins (18). As a part of our systematic studies on neural cell glycoconjugates (19–22, 45), we describe herein the expression cloning of HNK-1 sulfotransferase, HNK-1ST. Using the cDNA isolated, the expression profile of the HNK-1ST transcripts was compared with that of GlcAT-P transcripts for various fetal and adult tissues. We also demonstrate the HNK-1ST activity in vivo and in vitro using N-CAM, synthetic oligosaccharides and glycolipids as acceptors.

EXPERIMENTAL PROCEDURES

Preparation of Recipient Cells and Plasmids—A mutant cell line of Chinese hamster ovary cells, Lec2, was used as recipient cells. β-Glucuronolysis of N-acetyllactosamines is extremely efficient in Lec2 cells (18) because sialylation is absent in this cell line (23). Lec2 cells were first transfected with pH3Ap-1-neo-NCAM 140 (24) and a stable cell line expressing human N-CAM, Lec2-NCAM, was selected as described before (19). For cloning of GlcAT-P, the cDNA was synthesized from poly(A)⁺ RNA of rat brain (CLONTECH) using a reverse transcription-PCR kit (Stratagene). Using the cDNAs synthesized as templates, PCR

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF033827.

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1 The abbreviations used are: N-CAM, the neural cell adhesion molecule; GlcAT-P, glycoprotein-specific glucuronyltransferase; HNK-1ST, HNK-1 sulfotransferase; PCR, polymerase chain reaction; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.
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was performed to amplify the GlcAT-P sequence under the conditions described previously (21). The 5′- and 3′-primers correspond to nucleotides −32 to −10 and nucleotides 1047–1027, respectively, of the reported rat GlcAT-P sequence (18). The 5′- and 3′-primers also contain HindIII and XhoI sites, respectively. The PCR product was digested with HindIII and XhoI, then cloned into pcDNAI, resulting in pcDNA3-GlcAT-P.

Isolation of a Human HNK-1ST cDNA Clone—Lec2-NCAM cells were found to be negative for the HNK-1 antigen after pcDNA3-GlcAT-P was transiently expressed. Lec2-NCAM cells were thus co-transfected with 15 μg of a human fetal brain cDNA library in pcDNAI (19), 6 μg of pcDNA3-GlcAT-P, and 6 μg of pPSVE1-PyE harboring the polyoma large T DNA (25), using LipofectAMINE™ (Life Technologies, Inc.) as described previously (19). After 62 h, the transfected cells were dissociated into monodisperse cells using the enzyme-free cell dissociation solution (Hanks’ based, purchased from Cell and Molecular Technologies, Lavellette, NJ), followed by fluorescence-activated cell sorting of the HNK-1-positive cells using anti-HNK-1 monoclonal antibody (Becton Dickinson). Plasmid DNA from the sorted cells was isolated by the Hirt (26) procedure and amplified in the host bacteria Escherichia coli MC1061/P3 in the presence of ampicillin and tetracycline. The pcDNAI vector contains the supF suppressor tRNA, so that MC1061/P3 cells containing pcDNAI are resistant to both ampicillin and tetracycline. In contrast, MC1061/P3 cells harboring pcDNA3-GlcAT-P, pcPSVE1-PyE are resistant to ampicillin but not to tetracycline. Because of this difference, only plasmids derived from pcDNAI were rescued and amplified by this procedure (19), allowing the isolation of plasmids responsible for the HNK-1 glycan expression.

Bacteria harboring plasmids, which were isolated by the Hirt procedure, were divided into 20 plates. Plasmid DNA was prepared from each plate and separately transfected into Lec2-NCAM cells together with pcDNA3-GlcAT-P. The transfectants were screened by immunofluorescence microscopy using anti-HNK-1 antibody to identify a plasmid pool that directed the expression of the HNK-1 glycan. By narrowing down the plasmid pools using the same procedure, it was possible to isolate a single clone containing the plasmid DNA (pcDNA-HNK-1ST) encoding a human sulfotransferase that directed the expression of the HNK-1 carbohydrate epitope.

Construction of VectorsHarboring Short 5′- and 3′-Untranslated Sequences—To shorten the long 3′-untranslated sequence of pcDNA-HNK-1ST, pcDNA-HNK-1ST cDNA was digested utilizing an internal EcoRI site 15 nucleotides downstream of the stop codon and cloned into pcDNA3, resulting in pcDNA3-HNK-1ST (short). A truncated cDNA containing only 9 and 6 nucleotides of 5′- and 3′-untranslated sequences in addition to the coding sequence was prepared by PCR. The 5′- and 3′-primers for the PCR were 5′-GTCAGCTTTGTGACAAA-3′ and 5′-GCCCTGCGATGTAATGTTGACAAAATGCACCACCCAGTGGCT-3′. HindIII and XhoI sites are singly underlined, while HNK-1ST-coding sequences are doubly underlined. After restriction enzyme digestion, the PCR product was cloned into pcDNA3, yielding pcDNA3-HNK-1ST (ORF). Nucleotide sequences were determined in both strands by an automated sequencer (Applied Biosystems 373XL).

Northern Blot Analysis of Various Human Tissues—Human multiple tissue Northern blots of poly(A)+ RNA (CLONTECH) were hybridized sequentially with gel-purified cDNA inserts of pcDNA-HNK-1ST (ORF) and pcDNA3-GlcAT-P, after labeling with 32P(dCTP) by random oligonucleotide primers (Prime-IT II labeling kit, Stratagene).

Western Blot Analysis of N-CAM Expressing the HNK-1 Carbohydrate—Lec2-NCAM cells were transiently transfected with pcDNAI-HNK-1ST and pcDNA3-GlcAT-P, pcDNA3-HNK-1ST alone or pcDNA3-GlcAT-P alone. Forty-eight h after transfection, cell lysates were made from the transfected cells and incubated with a mouse anti-human N-CAM monoclonal antibody (ERIC-1, Santa Cruz Biotechnology) (27), followed by protein G-agarose (Pierce). After solubilization, the immunoprecipitates were separated by SDS-polyacrylamide (5%) gel electrophoresis and transferred onto nitrocellulose membrane. The blot was then incubated with the anti-N-CAM antibody, anti-HNK-1 antibody, or M6749 antibody (28) followed by horseradish peroxidase-conjugated sheep anti-mouse immunoglobulins and visualized with ECL (Amersham Corp.).

Expression of the Protein A-HNK-1ST Fusion Protein—The cDNA fragment encoding the stem region plus catalytic domain of HNK-1ST was prepared by PCR using pcDNA3-HNK-1ST (short) as a template and fused with the cDNA encoding a signal peptide sequence and the IgG binding domain of Staphylococcus aureus protein A (20, 29). The 5′-primer for this PCR is 5′-TATGATCTAGAGTTGCTAAGTGGC-3′, where Bgl II site is underlined, and the coding sequence of HNK-1ST is doubly underlined. The 3′-primer is SP6 promoter sequence. The PCR product was digested by BglII and XhoI and then cloned into BamHI and XhoI sites of pcDNAI-A (20), yielding pcDNAI-A-HNK-1ST. pcDNAI-A-HNK-1ST and pcDNAI-A were separately transfected to COS-1 cells and the enzyme was adsorbed to IgG-Sepharose 6FF (Pharmacia Biotech Inc.) as described previously (20).

Assay of In Vitro Activity of HNK-1ST—Galβ1→4GlcNAcβ1→octyl, GlcAβ1→3Galβ1→4GlcNAcβ1→octyl, and sufo-3GlcAβ1→3Galβ1→4GlcNAcβ1→octyl were synthesized according to the reported procedures (30, 31) with a slight modification. The detailed procedure for the synthesis of these oligosaccharides will be published elsewhere.2 Key 1H NMR (600 MHz, D2O) for GlcAβ1→3Galβ1→4GlcNAcβ1→octyl is included; δ 4.680 (d, J = 8.0 Hz, H-1 GlcA), 4.520 (secondary order, J1,2 = 8.1 Hz) and 4.502 (d, J = 7.9 Hz) (H-1 Gal and H-1 Glc), 4.190 (d, J = 3.1, H-4 Gal), 2.040 (s, NOCH3), GlcAβ1→3Galβ1→4GlcNAcβ1→octyl was prepared by acid hydrolysis from its sulfated form (4) and kindly provided by Dr. Firoze Jungalwala.

The reaction mixtures (100 μl total) contained 0.02 mM [35S]PAPS, 25 μl of IgG bead-bound enzyme suspension, 0.1 mM acceptor oligosaccharides or 0.025 mM acceptor glycolipids in 100 mM Tris-HCl, pH 7.2, 0.1% Triton X-100, 10 mM MnCl2, and 2.5 mM ATP (17). After incubation for 2 h at 37 °C, the reaction products were adjusted to 0.25 mM ammonium formate, pH 4.0, and applied to C18 reverse phase column (Alltech). After washing the column with the same solution, the product was eluted with 70% methanol. The radioactivity was measured by scintillation counting.

The products were separated by thin layer chromatography using silica gel. After separation in chloroform, methanol, 0.25% KCl (5:4:1, v/v/v), the thin layer plate was exposed to X-ray film (Kodak BioMax) for 16 h at room temperature. Standard oligosaccharides were then detected by spraying with 0.2% orcinol in 2 M H2SO4.

RESULTS

Isolation of a cDNA Clone That Directs the Expression of the HNK-1 Carbohydrate Epitope—Lec2-NCAM cells were co-transfected with a human fetal brain cDNA library in pcDNAI, pcDNA3-GlcAT-P, and pPSVE1-PyE (25). The transfected cells were incubated with anti-HNK-1 antibody followed by fluorescein isothiocyanate-conjugated secondary antibody, then subjected to cell sorting. Plasmid DNA, recovered from anti-HNK-1 antibody-positive Lec2-NCAM cells, was immediately subjected to sibling selection with sequentially smaller, active pools, identifying a single clone containing the plasmid, pcDNAI-HNK-1ST, that directed the expression of the HNK-1 glycan (Fig. 1C). Weak staining was observed when only HNK-1ST was present (Fig. 1B) probably due to endogenous expres-
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A truncated cDNA starting from 9 base pairs upstream of the initiation codon was constructed in pcDNA3, yielding pcDNA3-HNK-1ST (ORF). This plasmid directed the expression of the HNK-1 carbohydrate (Fig. 1D), confirming that nucleotides 1–1068 encode the coding region of HNK-1ST (Fig. 2).

There are three potential N-glycosylation sites (see asterisks in Fig. 2) in the human HNK-1ST sequence. A consensus sequence for polyadenylation signal is present at nucleotides 2432–2437 followed by a poly(A) tail. Judging from the size of
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Expression of HNK-1ST mRNA in Human Tissues—Northern blots of poly(A)+ RNA derived from various human tissues were examined (Fig. 4). An HNK-1ST transcript of ~3 kilobases was prominently detected in fetal brain, moderately in lung, and kidney but barely in liver. The same transcript was strongly detected in adult brain, testis, ovary, and moderately in heart, skeletal muscle, pancreas, spleen, and thymus, but weakly in other tissues. Among various parts of the brain, HNK-1ST transcript is expressed more in the frontal lobe than the other parts.

The transcript of GlcAT-P is almost exclusively expressed in both fetal and adult brains, as shown previously for rat tissues (18). Moreover, the GlcAT-P transcript is ubiquitously expressed in various parts of the brain and it is most abundant in the corpus callosum and hippocampus (Fig. 4). Since GlcAT-P is necessary to form the HNK-1 glycan in glycoproteins, expression of the HNK-1 carbohydrate in glycoproteins of nervous tissue may be determined by the regulation of GlcAT-P expression. In other tissues, it is possible that HNK-1ST may utilize acceptors other than those synthesized by GlcAT-P.

Expression of HNK-1 Epitope on N-CAM—To determine if HNK-1ST is capable of adding the HNK-1 epitope on N-CAM, Lec2-NCAM cells were transiently transfected with pcDNAI-HNK-1ST and pcDNA3-GlcAT-P, pcDNAI-HNK-1ST alone or pcDNA3-GlcAT-P alone. Western blot analysis of N-CAM derived from those transfected cells demonstrated that the HNK-1 glycan was formed on N-CAM when both HNK-1ST and GlcAT-P were expressed while the HNK-1 glycan was not expressed in the absence of either enzyme (Fig. 5, lanes 1–3).

The expression of GlcAT-P alone resulted in the binding of GlcA to ceramide, Galβ1-4GlcNAcβ1-octyl, Galβ1-4GlcNAcβ1-octyl (lanes 1 and 2), or Galβ1-4GlcNAcβ1-octyl (lanes 3 and 4) or with GlcAβ1-3Galβ1-4GlcNAcβ1-octyl (lanes 5 and 6). The activity is expressed as picomoles of sulfate transferred from PAPS/μl of the culture medium.

Demonstration of In Vitro Activity of HNK-1ST—To formally prove that the cloned cDNA encodes HNK-1ST, a soluble chimeric HNK-1ST was expressed in COS-1 cells. The enzyme adsorbed to IgG-Sepharose was then incubated with acceptor oligosaccharides or glycolipid and [35S]PAPS. As shown in Fig. 6, a substantial amount of [35S]-sulfate was incorporated to GlcAβ1-3Galβ1-4GlcNAcβ1-octyl (lane 4), while no incorporation was detected using the medium from mock-transfected COS-1 cells (lane 3), or using Galβ1-4GlcNAcβ1-octyl as an acceptor (lane 2). Similarly, [35S]-sulfate was incorporated into the glycolipid acceptor (lane 6). Considering that the con-
centration of the oligosaccharide acceptors is 3.8 times higher than that of the glycolipid acceptor, HNK-1ST added sulfate to the oligosaccharide as efficiently as to the glycolipid. The reaction products shown in lanes 2–6 were subjected to thin layer chromatography followed by autoradiography. The sulfated product migrated at the same position of a standard synthetic ceramide, respectively. Lanes 1–3 represent standard oligosaccharides detected by the orcinol-H$_2$SO$_4$ reagent. Lanes 1–6 and lanes 7 and 8 were run separately.

**DISCUSSION**

In this study, we describe the isolation of a cDNA clone encoding a human sulfotransferase, HNK-1ST, the enzyme responsible for the formation of a sulfate group attached to the C-3 of GlcA attached to N-acetyllactosamine, forming sulfo-GlcAβ1→3Galβ1→4GlcNAcβ1→octyl. Our studies also demonstrate that the transcript of HNK-1ST is more widely distributed in different tissues compared with that of GlcAT-P. In the brain, both the HNK-1ST and GlcAT-P transcripts are highly expressed, suggesting that the cloned HNK-1ST and GlcAT-P are most likely responsible for the formation of the HNK-1 glycan in the nervous tissues. It has been shown that there are two glucuronyltransferases specific for forming the HNK-1 precursor structure in glycoproteins and glycolipids, respectively. In contrast, HNK-1ST cloned in the present study was shown to add a sulfate group to both glycoproteins and glycolipids. The HNK-1ST present in tissues other than the brain may act on glycolipid acceptors. Alternatively, there is another GlcAT-P acting on glycoprotein acceptors, which may differ in tissue distribution than the one that has been cloned.

As described in the Introduction, the HNK-1 carbohydrate is associated with a number of cell adhesion molecules in the nervous tissues. The addition of the HNK-1 glycan to these various glycolipids and glycoproteins is thought to modulate cell-cell and cell-substratum interactions. The HNK-1 cDNA obtained in the present study will be a powerful molecular tool to manipulate the expression level of the HNK-1 glycan in specific cell types, allowing us to dissect the intricate and complex cell processes of cell-cell interactions during development.

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