# Molecular Cloning and Substrate Specificity of Human NeuAc α2,3Galβ 1,3GalNAc GalNAc α2,6-Sialyltransferase (hST6GalNAc IV)

Kyoung-Sook Kim, Sang-Wan Kim, Kwan-Sik Min*, Cheorl-Ho Kim**, Young-Kug Choo*** and Young-Choon Lee†

Division of Biotechnology, Faculty of Natural Resources and Life Science, Dong-A University, Busan 604-714
*Division of Breeding and Reproduction, National Livestock Research Institute, Suwon 441-350
**Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Kyung-Pook 780-350
***Divison of Biological Science, College of Natural Science, Wonkwang University, Iksan, Chonbuk 570-749, Korea

**Abstract** The cDNA encoding human NeuAc α2,3Gal β 1,3GalNAc GalNAc α2,6-Sialyltransferase (hST6GalNAc IV) was isolated by screening of human fetal liver cDNA library with a DNA probe generated from the cDNA sequence of mouse ST6GalNAc IV (mST6GalNAc IV). The cDNA sequence included an open reading frame coding for 302 amino acids, and comparative analysis of this cDNA with mST6GalNAc IV showed that each sequence of the predicted coding region contains 88% and 85% identities in nucleotide and amino acid levels, respectively. The primary structure of this enzyme suggested a putative domain structure, like that in other glycosyltransferases, consisting of a short N-terminal cytoplasmic domain, a transmembrane domain and a large C-terminal active domain. This enzyme expressed in COS-7 cells exhibited transferase activity toward NeuAc α2,3Gal β 1,3GalNAc, fetuin and GM1b, although the activity toward the latter is very low, no significant activity being detected toward Gal β 1,3GalNAc or asialofetuin, the other glycoprotein substrates tested. The $^{14}$C-sialylated residue of fetuin sialylated by this enzyme with CMP-[$^{14}$C]NeuAc was sensitive to treatment with α2,3-, α2,6- and α2,8-specific sialidase of *Vibrio cholerae* but resistant to treatment with α2,3-specific sialidase (NANase I), and α2,3- and α2,8-specific sialidase of Newcastle disease virus. These results clearly indicated that the expressed enzyme is a type of GalNAc α2,6-sialyltransferase like mST6GalNAc IV, which requires sialic acid residues linked to Gal β 1,3GalNAc-residues for its activity.

**Key words**: Sialyltransferase, cDNA, human fetal liver, substrate specificity

†Corresponding author
Phone: 82-51-200-7591, Fax: 82-51-200-6993
E-mail: yclee@mail.donga.ac.kr

# Introduction

Sialic acid residues occur at the terminal positions of the carbohydrate groups of three types of glycoconjugates (N- and O-glycosidically linked oligosaccharides of glycoproteins, and glycosphingolipids) and play important roles in a variety of biological processes, such as cell-cell communication, cell-matrix interaction, cell differentiation, invasiveness of a number of pathogenic organisms, clearance of asialo glycoproteins from circulation, protein targetting and adhesion of leukocytes to endothelial cells mediated by selectins [1]. The transfer of sialic acid from CMP-sialic acid (CMP-NeuAc) to these glycoconjugates is catalyzed by a family of glycosyltransferases called sialyltransferases [2]. Precise molecular knowledge of sialyltransferases is vital for understanding the regulatory mechanism for the sialylation of glycoconjugates. To date, eighteen members of the sialyltransferase gene family have been cloned from several animals by PCR and expression cloning methods (Table 1) [3-48]. They have a domain structure similar to that of other glycosyltransferases: a short N-terminal cytoplasmic tail, a signal anchor domain, and an extended stem region followed by a large C-terminal active domain. Comparison of these sequences has revealed highly conserved regions named sialylmotif in the active domain of these enzymes, which are not found in other glycosyltransferases [15]. From the conservation of this sialylmotif it was expected that other members of sialyltransferase gene family might have the same motif. The PCR-based approach with degenerate primers based on the conserved sequence in the sialylmotif has resulted in the isolation of several new members of the sialyltransferase gene family [15].

Recently we cloned a cDNA encoding mouse brain NeuAc α2,3Gal β 1,3GalNAc GalNAc α2,6-sialyltransferasemouse (mST6Gal IV) by a PCR method as described above [18]. mST6GalNAc IV exhibits the most restricted substrate

**Table 1.** So far cloned sialyltransferases (STs).

| Enzymes | Abbreviation | Sources |
|---|---|---|
| Gal $\beta$ 1,3GalNAc $\alpha$ 2,3-ST | ST3Gal I | Mouse[11], Pig[20], Chick[21], Human[22] |
| Gal $\beta$ 1,3GalNAc $\alpha$ 2,3-ST (2nd-type) | ST3Gal II | Mouse[10], Rat[10], Human[6] |
| Gal $\beta$ 1,3(4)GlcNAc $\alpha$ 2,3-ST | ST3Gal III | Mouse[23], Rat[24], Human[25] |
| Gal $\beta$ 1,4(3)GlcNAc $\alpha$ 2,3-ST | ST3Gal IV | Mouse[23], Human[26, 27] |
| GM3 synthase | ST3Gal V | Mouse[28,29], Human[30,31] |
| Gal $\beta$ 1,4GlcNAc $\alpha$ 2,3-ST | ST3Gal VI | Human[32] |
| Gal $\beta$ 1,4GlcNAc $\alpha$ 2,6-ST | ST6Gal I | Mouse[3], Rat[33], Chick[34], Human[35] |
| GalNAc $\alpha$ 2,6-ST | ST6GalNAc I | Chick[8], Mouse[36] |
| Gal $\beta$ 1,3GalNAc $\alpha$ 2,6-ST | ST6GalNAc II | Mouse[37], Chick[9] |
| NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc $\alpha$ 2,6-ST | ST6GalNAc III | Mouse[18] |
| NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc $\alpha$ 2,6-ST (2nd-type) | ST6GalNAc IV | Mouse[18], Human[19, in this study] |
| GD1 $\alpha$ synthase | ST6GalNAc V | Mouse[38] |
| GD1 $\alpha$ /GT1a $\alpha$ /GQ1b $\alpha$ synthase | ST6GalNA VI | Mouse[39] |
| GD3 synthase | ST8Sia I | Rat[40], Human[13,41,42] |
| Polysialic acid synthase | ST8Sia II | Mouse[43,44], Rat[12] |
| NeuAc $\alpha$ 2,3Gal $\beta$ 1,4GlcNAc $\alpha$ 2,8-ST | ST8Sia III | Mouse[45], Human[46] |
| Polysialic acid synthase | ST8Sia IV | Mouse[17], Hamster[47], Human[48] |
| $\alpha$ 2,8-ST | ST8Sia V | Mouse[7], Human[5] |

*Underlines* indicate works performed by author group or co-works.

specificity, only utilizing the trisaccharide sequence NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc, which is found on either O-glycosyl proteins or ganglioside GM1b.

On the other hand, since cDNAs encoding sialyltransferases obtained from non-human species would not suffice to study their biological functions, including the expression of that gene at the mRNA level under various physiological and pathologic conditions in human, ST6GalNAc IV obtained from human species is necessary for that purpose.

In this regard, we have attempted to isolate a cDNA encoding hST6GalNAc IV using the PCR-based approach and the results are described in this paper

# Materials and Methods

## Materials

Fetuin, asialofetuin, $\alpha$ 1-acid glycoprotein, CMP-NeuAc, Gal $\beta$ 1,3GalNAc, Gal $\beta$ 1,3GlcNAc, Gal $\beta$ 1,4GlcNAc and Triton CF-54 were purchased Sigma (St. Louis, USA). CMP-[$^{14}$C]NeuAc (11GBq/mmole) was from Amersham Pharmacia Biotech. NeuAc $\alpha$ 2,3Gal $\beta$ 1,3 (NeuAc $\alpha$ 2,6)GalNAc was from Seikagaku Company (Japan). Gal $\beta$ 1,3GalNAc was sialylated using the secreted form of ST3Gal I expressed in COS-7 cells [17]. Then NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc was purified by preparative TLC (ethanol/1-butanol/pyridine/water/ acetic acid=100/10/10/30/3) and subsequent DEAE-Sephadex A-25 anionic exchange chromatography. NeuAc $\alpha$ 2,3Gal $\beta$ 1,3 (NeuAc $\alpha$ 2,6)GalNAc-ol was prepared by the reduction of NeuAc $\alpha$ 2,3Gal $\beta$ 1,3 (NeuAc $\alpha$ 2,6)GalNAc [49]. NANase I was purchased from Oxford Glycosystems (U.K.), and *Vibrio cholerae* sialidase and Newcastle disease virus sialidase were from Boehringer Mannheim (Germany).

## Polymerase chain reaction (PCR) cloning

For preparation of a probe for screening a cDNA library,

PCR was performed with degenerate primers (5'-primer, 5'-AG(C/T)TG(C/T)GC(C/T)GT(G/T)GT(G/T)TC(C/T)AG (C/T)TC(C/T)GG(C/G)-3'; 3'-primer, 5'-(C/G)CC(A/G)TA (C/G)AC(C/G)ACGAT(C/T)TC(C/T)TC(A/G)CA(C/G)AG -3') deduced from the sialylmotifs of mouse ST6GalNAc IV [18]. The cDNA isolated from human fetal liver cDNA library (Clontech) was used as a template for PCR. PCR amplification was carried out by 30 cycles consisting of 94 ℃ for 1 min, 37℃ for 1 min and 72℃ for 2 min. The 0.5 kb PCR products (HR1) were subcloned into pT7Blue -TA cloning vector (Novagen) and sequenced.

## Screening of the cDNA library

Human fetal liver cDNA library in $\lambda$ TriplEx (Clontech) was used for the cloning of human ST6GalNAc IV cDNA. About 10$^6$ plaques were screened by plaque hybridization [50] with the cloned PCR fragment (0.5 kb) as a probe. Two positive clones were plaque-purified, and phage DNAs were digested with EcoRI/XbaI. The EcoRI/XbaI fragments of cDNA inserts were isolated and subcloned for DNA sequencing.

## Preparation of soluble hST6GalNAcIV fused with protein A

A truncated form of hST6GalNAcIV, which lacks 36 amino acids from the N-terminus of the open reading frame, was prepared by PCR amplification using a 5'-primer containing an in-frame EcoRI site (5'-CGCG*AATTC*TGCCTGGACCA CCACTTCCC-3';nucleotides 109-128) and a 3'-primer with XhoI site (5'-GCGC*TCGAG*AAGCTACTCAGTCCTCCA GG-3'; complementary to the coding strand, nucleotide 893-911). PCR reaction was carried out with Taq polymerase by 94℃ for 40 seconds, 55℃ for 30 seconds and 72℃ for 1 min. The amplified PCR fragment (803 bp) was cut out

by digestion with EcoRI and XhoI, and then inserted into EcoRI/XhoI sites of expression vector pcDSA [51] to yield pcDSA-hST6GalNAc IV. The insert junction was confirmed by restriction enzyme and DNA sequencing. The pcDSA-hST6GalNAc IV consisted of the IgM signal peptide sequence, the protein A IgG binding domain, and the truncated form of hST6GalNAc IV. Each 20 $\mu$g of pcDSA-hST6 GalNAc IV and pcDSA was transfected into COS-7 cells on 150-mm plate using LipofectAMINE™ reagent (Life Technologies, Inc.) and cultured for 16 h in Dulbecco's Modified Eagle's Medium (DMEM) containing 2% fetal calf serum. The medium was then replaced with serum-free medium (Macrophage-SFM medium, Life Technologies, Inc.), and the cells were cultured for another 32 h. After 48 h transfection, the cell culture media was collected and protein A-fused hST6GalNAc IV expressed in the medium was absorbed to IgG-Sepharose gel (Amersham Pharmacia Biotech; 50 $\mu\ell$ of resin/50 ml of culture medium) at 4℃ for 16 h. The complex of the IgG-Sepharose gel and the enzyme fused with protein A was used as the enzyme source.

## Sialyltransferase assays

Sialyltransferase assays and linkage analysis were performed as described previously [18]. In brief, enzyme activity was measured in 50 mM MES buffer (pH 6.0), 1 mM MgCl₂, 1 mM CaCl₂, 0.5% Triton CF-54, 100 $\mu$M CMP-[14C] NeuAc (10.2 KBq), an acceptor substrate, and an enzyme preparation, in a total volume of 10 $\mu\ell$. As acceptor substrates, 10 $\mu$g of proteins, 5 $\mu$g of glycolipids, or 10 $\mu$g of oligosaccharides were used. The enzyme reaction was performed at 37℃ for 2 h. After the enzyme reaction, the oligosaccharide products were separated from the substrate, CMP-[14C]Neu Ac, by HPTLC with a solvent system of ethanol/pyridine/1-butanol/acetate/water=100/10/10/3/30. For glycolipid acceptors, the reaction mixtures were applied on a C-18 column, which was washed with water and 0.1 M KCl. After washing the column with water, the glycolipids were eluted with methanol, dried, and then subjected to HPTLC with a solvent system of chloroform/methanol/0.2% CaCl₂=55/45/10. For glycoprotein acceptors, the reaction was terminated by adding the loading buffer for SDS-PAGE and then the mixture was subjected to SDS-PAGE. The radioactivity was visualized with a BAS2000 radioimage analyzer (Fuji Film, Tokyo)

## Linkage analysis and identification of sialylated products

For linkage analysis of sialic acids, [14C]NeuAc-incorporated fetuin was synthesized with hST6GalNAc IV. To obtain oligosaccharide portion of 14C-sialylated fetuin, the sialylation of fetuin was carried out essentially as described, but on a 10-fold larger scale. To maximize the product yield, the incubation period was extended to 24 h. The incubation mixture was then treated with 0.1 N NaOH/1 M NaBH₄ at 37℃ for 48 h, and neutralized by the gradual addition of acetic acid in an ice bath. A sample was desalted by

Sephadex G-25 chromatography (1.3 × 25 cm). The 14C-sialylated oligosaccharide alditol and reference oligosaccharide NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol were treated with a linkage-specific sialidase, NANase I (specific for $\alpha$ 2,3-linked sialic acids), Vibrio cholerae sialidase (specific for $\alpha$ 2,3-, $\alpha$ 2,6- and $\alpha$ 2,8-linked sialic acids), or Newcastle disease virus sialidase (specific for $\alpha$ 2,3- and $\alpha$ 2, 8-linked sialic acids) [18]. A radioactive sample containing at least 10,000 cpm or a sialylated sample containing at least 5 $\mu$g of sialic acid was spotted onto a TLC plate (Merck, Darmstadt, Germany) and then developed with ethanol/1-butanol/pyridine/water/acetic acid=100/10/10/30/3 or 1-propanol/aqueous ammonia/water=6/1/2.5. The chromatogram was visualized with a BAS2000 radioimage analyzer (Fuji Film, Tokyo). A nonradioactive sample containing sialic acid was visualized by spraying the plate with the resorcinol reagent and heating at 120℃ for 20 min.

## Results

### Isolation of the human ST6GalNAc IV cDNA

Previously cloned sialyltransferases have showed highly conserved regions named sialylmotifs L and S, which are not found in other glycosyltransferases [15]. Using human fetal liver cDNA as a template, PCR experiments with degenerate primers deduced from the sialylmotifs L and S of mouse ST6GalNAc IV [18] resulted in the amplification of the expected 0.5 kb band which was subcloned and sequenced. Among several clones, six clones had the same nucleotide sequence and proved to be the human homolog of the mouse ST6GalNAc IV. In order to isolate the entire coding sequence, the 0.5 kb fragment (HR1) was used as a probe to screen a human fetal liver cDNA library. One positive clones with insert size of 1.2 kb was obtained and sequenced. The nucleotide sequence analysis of this cDNA clone revealed that this cDNA was 1,165 bp in length and included the complete open reading frame of sialyltransferase. It consists of a 49 bp of 5'-untranslated region, an open reading frame encoding a protein of 302 amino acids, and a 205 bp of 3'-untranslated region (Fig. 1). Comparison of the coding sequence of hST6GalNAc IV with that of mST6 GalNAc IV revealed that there are 85% identity on the nucleotide sequence levels. On the amino acid level, 88% identity between hST6GalNAc IV and mST6GalNAc IV is observed (Fig. 2). Analysis of the deduced primary sequence of this enzyme showed a type II transmembrane topology with a predicted N-terminal cytoplasmic domain (7 residues), a hydrophobic transmembrane domain (29 residues) and an active domain (266 residues). Sialylmotifs L and S conserved highly in sialyltransferases cloned previously are found in this sequence.

### Expression of an active form of hST6GalNAc IV and enzyme assay

To verify that our cloned cDNA code for hST6GalNAc IV

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                                    TCCAGCCCAGTCGGCCCGGCCCG        -241
GGGGCCATGGAGCTCCGAGCGGCGGATCGCGAGCCTCCTGCGAACCCCAGCCTGCACGCC
CGGTTAGCATTCGGCCGGGAGATGCGGCAGTGGAATCTGGAAGGGCGGTGAAAAACCTAC   -121
GTCCTGCCCTCGCCCGGCCTCTCCATTCGTCCCCCGGGTAGAGAGGTGCCCGGCTCCCAC
CCCTTCCCAGCCCCAGCCCTGGAGACAGCAGCCCCTAGACTACTGAGGGACAGCGACAGC    -1

ATGAAGGCTCCGGGTCGGCTCGTGCTCATCATCCTGTGCTCCGTGGTCTTCTCTGCCGTC    60
M  K  A  P  G  R  L |V  L  I  I  L  C  S  V  V  F  S  A  V |    20

|TACATCCTCCTGTGCTGCTGGGCCGGCCTGCCCCTCTGCCTGGCCACCTGC|TGGACCAC   120
|Y  I  L  L  C  C  W  A  G  L  P  L  C  L  A  T  C |L  D  H     40

CACTTCCCCACAGGCTCCAGGCCCACTGTGCCGGGACCCCTGCACTTCAGTGGATATAGC   180
H  F  P  T  G  S  R  P  T  V  P  G  P  L  H  F  S  G  Y  S      60

AGTGTGCCAGATGGGAAGCCGCTGGTCCGCGAGCCCTGCCGCAGCTGTGCCGTGGTGTCC   240
S  V  P  D  G  K  P  L  V  R  E  P  C  R  S  C  A  V  V  S      80

AGCTCCGGCCAAATGCTGGGCTCAGGCCTGGGTGCTGAGATCGACAGTGCCGAGTGCGTG   300
S  S  G  Q  M  L  G  S  G  L  G  A  E  I  D  S  A  E  C  V     100

TTCCGCATGAACCAGGCGCCCACCGTGGGCTTTGAGGCGGATGTGGGCCAGCGCACGACC   360
F  R  M  N  Q  A  P  T  V  G  F  E  A  D  V  G  Q  R  T  T     120

CTGCGTGTCGTCTCACACACAAGCGTGCCGCTGCTGCTGCGCAACTATTCACACTACTTC   420
L  R  V  V  S  H  T  S  V  P  L  L  L  R  N  Y  S  H  Y  F     140

CAGAAGGCCCGAGACACGCTCTACATGGTGTGGGGCCAGGGCAGGCACATGGACCGGGTG   480
Q  K  A  R  D  T  L  Y  M  V  W  G  Q  G  R  H  M  D  R  V     160

CTCGGCGGCCGCCACCTACCGCACGCTGCTGCAGCTCACCAGGATGTACCCCGGCCTGCAG   540
L  G  G  R  T  Y  R  T  L  L  Q  L  T  R  M  Y  P  G  L  Q     180

GTGTACACCTTCACGGAGCGCATGATGGCCTACTGCGACCAGATCTTCCAGGACGAGACG   600
V  Y  T  F  T  E  R  M  M  A  Y  C  D  Q  I  F  Q  D  E  T     200

GGCAAGAACCGGAGGCAGTCGGGCTCCTTCCTCAGCACCGGCTGGTTCACCATGATCCTC   660
G  K  N  R  R  Q  S  G  S  F  L_S_T_G_W_F_T_M_I_L              220

GCGCTGGAGCTGTGTGAGGAGATCGTGGTCTATGGGATGGTCAGCGACAGCTACTGCAGG   720
A_L_E_L_C_E_E_I_V_V_Y_G_M_V_S_D_S_Y_C_R                        240

GAGAAGAGCCACCCCTCAGTGCCTTACCACTACTTTGAGAAGGGCCGGCTAGATGAGTGT   780
E  K  S  P  S  V  P  Y  H  Y  F  E  K  G  R  L  D  E  C       260

CAGATGTACCTGGCACACGAGCAGGCGCCCCGAAGCGCCCACCGCTTCATCACTGAGAAG   840
Q  M  Y  L  A  H  E  Q  A  P  R  S  A  H  R  F  I  T  E  K    280

GCGGTCTTCTCCCGCTGGGCCAAGAAGAGGCCCATCGTGTTCGCCCATCCGTCCTGGAGG   900
A  V  F  S  R  W  A  K  K  R  P  I  V  F  A  H  P  S  W  R    300

ACTGAGTAGCTTCCGTCGTCCTGCCAGTCGCCATGCCGTTGCGAGGCCTCCGGGATGTCC   960
T  E  *

CATCCCAAGCCATCACACTCCACAAAAACATTTAATTTATGGATCCTGCCTCCTGCCACG  1020
TGCTGGGTGGACCTAAGGTTCCTTTCCACTCCCTGAGTAATTCACGGCATTTGGGGGCTC  1080
ACCCCACCTCCAGGTCTGTCAAGTGGCCTTTGTCCC                          1116
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Fig. 1. Nucleotide and deduced amino acid sequences of human ST6GalNAc IV. The nucleotide and amino acid sequences of hST6GalNAc IV are numbered from the presumed start codon and initiation methionine, respectively. The boxed amino acids correspond to a putative transmembrane domain. Sialylmotifs L and S are boxed by solid and dashed lines, respectively. The nucleotide sequence of hST6GalNAc IV has been deposited in the GeneBank/EMBL data bank with accession number AF 127142.

and not for other closely related proteins, we constructed the expression plasmid, pcDSA-hST6GalNAc using the expression vector, pcDSA [51]. In this expression system, a soluble form of the protein was designed to be generated by replacing the first 36 amino acids including the N-terminal cytoplasmic and the transmembrane domains with the cleavable immunoglobulin signal peptide and protein A IgG binding domain on pcDSA.

After transfecting pcDSA-R1 into COS-7 cells, the proteins secreted into the medium was bound to IgG-Sepharose, and used as the enzyme source. The bound fusion protein was assayed for sialyltransferase activity using a variety of acceptor substrates.
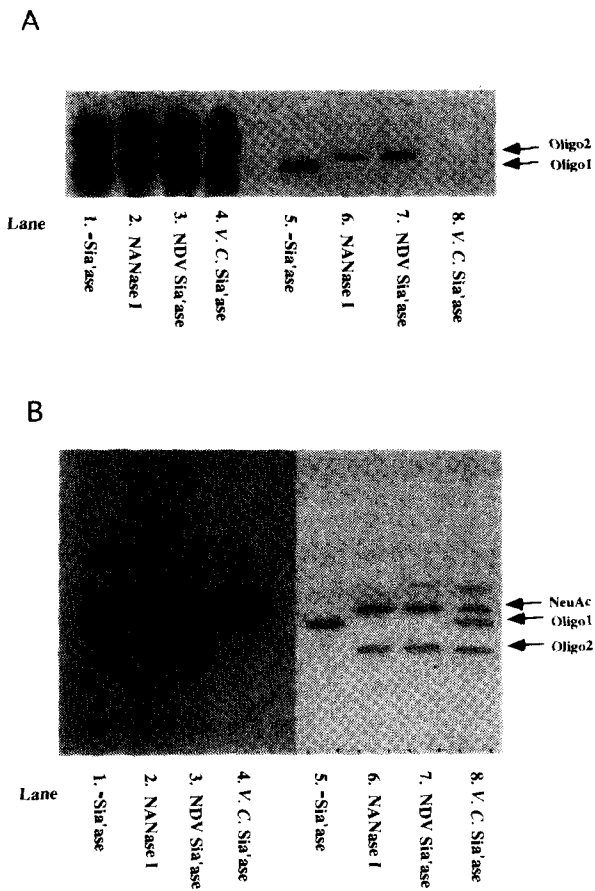
As shown in Table 2, when fetuin was used as an acceptor substrate, a strong sialyltransferase activity was detected,

while asialofetuin was not an acceptor substrate for this enzyme. Among the glycolipids exmained in this study, only GM1b, i.e. not asialoGM1, served as an acceptor substrate for this enzyme. These results suggested that hST6GalNAc IV requires the NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc-residue in fetuin and GM1b, just like mST6GalNAc IV [18]. In addition, as expected, the oligosaccharide, NeuAc $\alpha$ 2,3Gal $\beta$ 1,3GalNAc, was a good acceptor substrate for hST6GalNAc IV, while nonsialylated Gal $\beta$ 1,3GalNAc and disialylated NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc were not (Table 2).

## Analysis of linkage specificity of hST6GalNAc IV

For linkage analysis, [$^{14}$C]NeuAc-incorporated fetuin was synthesized with this enzyme, and a series of linkage-specific sialidases were used. First of all, the $^{14}$C-sialylated oligosaccharide alditol was prepared by $\beta$ -elimination of the sialylated fetuin. A desalted sample was then spotted onto a TLC plate and developed with ethanol/1-butanol/ pyridine/water/acetic acid=100/10/10/30/3. All of the radioactive product migrated as a low molecular compound and no radioactivity remained at the origin, suggesting that $^{14}$C-sialylation occurred exclusively on O-linked glycan chains of fetuin (data not shown). Furthermore, more than 70% of the radioactivity comigrated with the reference oligosaccharide, NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol (data not shown). This radioactive material was isolated by preparative TLC and used for linkage analysis (Fig. 3). The reference oligosaccharide, NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol, was also used for comparison. The $^{14}$C-sialylated oligosaccharide alditols were detected by BAS 2000 radioimage analyzer (Fig. 3, lanes 1-4). In case of reference oligosaccharide, resorcinol reagent was used for detection (Fig. 3, lanes 5-8). On NANase I ( $\alpha$ 2,3 linkage-specific sialidase) or Newcastle disease virus sialidase ( $\alpha$ 2,3 and $\alpha$ 2,8 linkage-specific sialidase) digestion, NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol (oligo 1, lane 5) is converted to Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol (oligo 2, lane 6 and 7). With these sialidase treatments, the radioactive band that co-migrated with oligo 1 (lane 1) migrated to the same position as oligo 2 (lanes 2 and 3). It should be noted that the digestion with NANase I and Newcastle disease virus sialidase was partial for some reason. When NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6)GalNAc-ol (oligo 1) was treated with Vibrio cholerae sialidase ( $\alpha$ 2,3, $\alpha$ 2,6 and $\alpha$ 2,8 linkage-specific sialidase), it was converted to Gal $\beta$ 1,3GalNAc-ol, which was not detectable with the resorcinol method (lane 8). With this treatment, the radioactive band comigrated with neither oligo 1 nor oligo 2, but with NeuAc, indicating that the linkage type of the [$^{14}$C]NeuAc residue in the oligosaccharide alditol is $\alpha$ 2,6 (lane 4). Taken together, the results strongly suggest that the $^{14}$C-sialylated oligosaccharide alditol derived from fetuin is NeuAc $\alpha$ 2,3Gal $\beta$ 1,3(NeuAc $\alpha$ 2,6) GalNAc-ol. Therefore, these results indicate that the cloned enzyme is human ST6GalNAc IV with the same substrate specificity as mouse ST6GalNAc IV [18].

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hSTGalNA6IV : 1    [black boxed sequence]SVV[black]IL[black]G[black]TGS[black] : 60
mSTGalNA6IV : 1    [black boxed sequence]LT[black]ILT[black]CVF[black]C[black]AAP[black] : 60

hSTGalNA6IV : 61   [black boxed sequence]VPP[black]R[black]M[black]S[black] A[black] : 120
mSTGalNA6IV : 61   [black boxed sequence][black]G[black]D[black] : 120
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*Sialylmotif L*

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hSTGalNA6IV : 121  [black boxed sequence]VS[black]KA[black]M[black]M[black] : 180
mSTGalNA6IV : 121  [black boxed sequence]I[black]H[black]V[black]M[black] : 180

hSTGalNA6Iv : 181  [black boxed sequence]TFTERMM[black]WF[black]M[black]R : 240
mSTGalNA6IV : 181  [black boxed sequence]TT[black]MM[black]S : 240
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*Sialylmotif S*

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hSTGalNA6IV : 241  EKSHPS[black sequence]LA[black]WR : 300
mSTGalNA6IV : 241  EKSPRS[black sequence]RL[black]WR : 300

hSTGalNA6IV : 301  TE : 302

mSTGalNA6IV : 301  AK : 302
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Fig. 2. Comparison of the deduced amino acid sequences of the human and mouse ST6GalNAc IV. Identical residues between the two sequences are *boxed* in *black*. Sialylmotifs L and S are *underlined*.

Table 2. Acceptor substrate specificity of human ST6GalNAc IV

| Acceptor and Representive Structures of Carbohydrates | Activity Units (pmol/h/ml medium) | Relative Activity (%) |
|---|---|---|
| Fetuin | 21.5 | 100 |
|    NeuAc α 2,3Gal β 1,3GalNAc-Ser/Thr | | |
|    NeuAc α 2,3Gal β 1,3(NeuAc α 2,6)GalNAc-Ser/Thr | | |
| Asialo-fetuin | 0 | 0 |
| α 1-Acid glycoprotein | 0 | 0 |
|    NeuAc α 2,6(3)Gal β 1,4GlcNAc-R | | |
| Ovomucoid | 0.7 | 3.3 |
|    NeuAc α 2,3Gal β 1,4GlcNAc-R | | |
| Asialo-ovomucoid | 0 | 0 |
| GM3 | 0 | 0 |
| GM1b(NeuAc α 2,3Gal β 1,4GalNAc β 1,4Gal β 1,4Glc β 1-Cer) | 1.9 | 8.8 |
| AsialoGM1 | 0 | 0 |
| GD1a | 0 | 0 |
| Paragloboside | 0 | 0 |
| Gal β 1,3GlcNAc | 0 | 0 |
| Gal β 1,4GlcNAc | 0 | 0 |
| Gal β 1,3GalNAc | 0.1 | 0.4 |
| NeuAc α 2,3Gal β 1,3GalNAc | 54.8 | 255 |
| NeuAc α 2,3Gal β 1,3(NeuAc α 2,6)GalNAc | 1.2 | 5.6 |

[a]R represents the remainder of the N-linked oligosaccharide chains

## Discussion

In this study, we have isolated the human ST6GalNAc IV cDNA by the PCR-based approach using sequence infor-

mation of a highly conserved regions, sialylmotifs L and S, in the mouse ST6GalNAc IV (mST6GalNAc IV) cloned previously. The sialylmotifs L and S shared by all sialyl-

A



B



**Fig. 3.** Thin layer chromatography of the oligosaccharide alditols derived from fetuin ^{14}C-sialylated by hST6GalNAc IV. The TLC plates were developed in ethanol/1-butanol/pyridine/water/acetic acid=100/10/10/30/3 (A) or 1-propanol/aqueous ammonia/water= 6/1/2.5 (B). The oligosaccharide alditols derived on 0.1 N NaOH/ 1 M NaBH₄ treatment of ^{14}C-sialylated fetuin (lanes 1-4) and NeuAc α 2,3Gal β 1,3(NeuAc α 2,6)GalNAc-ol, as a reference (lanes 5-8), were, respectively, treated in absence of sialidase (lanes 1 and 5) or the presence of α 2,3-linkage specific-NANase I (lanes 2 and 6), α 2,3- and α 2,8-specific Newcastle disease virus sialidase (lanes 3 and 7), or α 2,3-, α 2,6- and α 2,8-specific Vibrio cholerae sialidase (lanes 4 and 8). Each sample was then chromatographed on a TLC plate and visualized with a BAS2000 radioimage analyzer for ^{14}C-sialylated fetuin products (lanes 1-4) or with resorcinol reagent for reference oligo-saccharide products (lanes 5-8). Oligo 1, NeuAc α 2,3Gal β 1,3 (NeuAc α 2,6)GalNAc-ol; Oligo 2, Gal β 1,3(NeuAc α 2,6)Gal NAc-ol.

transferases could principally contain either a CMP-NeuAc binding site or catalytic site, or both. Datta et al. demonstrated that the sialylmotif L is involved in the binding of the CMP-NeuAc, a common donor substrate for all sialyl-transferases [52], and that the sialylmotif S participates in the binding of both donor and acceptor substrates [53]. Very recently, the results of site-directed mutagenesis of ST6Gal I suggested that the invariant cysteine residues in the

sialylmotifs L and S participate in the formation of an intradisulfide linkage that is essential for proper conformation and activity of ST6Gal I [54]. While this work was in progree, the human ST6GalNAc IV cDNA was cloned by means of an EST screening procedure and its enzymatic activity was characterized [19]. Comparison of two cDNAs showed that the predicted coding region contains the same sequence in both nucleotide and amino acid levels, although the lengths of 5'- and 3'-UTR are different. In addition, their enzymatic activities showed the same specificity for various acceptor substrates except GM1b. Similar to other glycosyl-transferases, hST6GalNAc IV has a type II membrane protein topology with a distinct domain structure and is the smallest protein (302 amino acid) among all cloned sialyltransferases.

Previously we reported that the mouse ST6GalNAc III and IV almost have the same substrate specificities, but they differ in substrate preference [18]. mST6GalNAc III preferred glycolipid as substrate over glycoprotein, while mST 6GalNAc IV preferred glycoprotein as substrate over glycolipid. The present results indicate that hST6GalNAc IV preferred glycoprotein as substrate over glycolipid, and its acceptor specificity is restricted to the trisaccharide NeuAc α 2,3Gal β 1,3GalNAc, just like mST6GalNAc IV.

It is very interesting that there are two different kinds of sialyltransferase with the same acceptor substrate specificity. Previously we demonstarated the existence of another group of sialyltransferase (ST3Gal I and II) with the same acceptor substrate specificity and different preference for glycolipids and glycoproteins [10,11]. The existence of these groups, or subfamilies is probably important for fine control of the expression of sialyl-glycoconjugates. In vivo expression using the two enzyme genes and mammalian cell lines will provide clear information on the preference and specificities of the two enzymes.

## Acknowledgments

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