Molecular cloning and expression of a human hST8Sia VI (α2,8-sialyltransferase) responsible for the synthesis of the diSia motif on O-glycosylproteins

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INTRODUCTION

Sialic acids are negatively charged monosaccharides found at the non-reductive terminal position of the carbohydrate groups of glycoconjugates or oligosaccharides. In mammals they are α2,3- or α2,6-linked to a β-D-galactopyranose (Gal) residue or α2,6-linked to a β-D-N-acetylglucosamine (GlcNAc) or a β-D-N-acetylgalactosamine (GalNAc) residue. Sialic acid residues can also form homopolymers of α2,8-linked sialic acids that are named according to their DP (degree of polymerization) disialic acid (disialic acid) for DP = 2, oligosialic acid (oligosia) for 3 ≤ DP ≤ 7 and PSA [polySia (polysialic acid)] for DP ≥ 8 [2]. Until now, PSA has been described on a small group of N-glycosylproteins including the extensively studied NCAM (neural cell adhesion molecule), the α-subunit of the voltage-sensitive sodium channels from Electrophorus electricus and most recently the O-glycosylprotein CD-36 of human milk [3–7]. PSAs confer anti-adhesive properties to the polysialylated glycoproteins that attenuate cell–cell interactions [8]. Associated with NCAM in the developing nervous system, PSA can be extended to as many as 60 sialic acid residues and is implicated in various biological processes such as neural cell migration, axonal growth or synaptogenesis [6,9,10]. diSia and oligoSia chains are commonly found on gangliosides of the b and c series that are known to play important roles in differentiation, signal transduction and cell adhesion [11–13]. More recently, these shorter sialic acid chains were also described on both N- and O-glycosylproteins. disia motifs were found on the N-glycans of Band 3 of human erythrocytes, fetuin and α2-macroglobulin of calf serum, and on the O-glycans of human glycoporphin, bovine adip-Q, CD-166 of Neuro2A cells and the bovine chromogranins [14–19]. OligoSia motifs were described on the N-glycans of ceruloplasmin and integrin α5 subunit [20,21] and on the O-glycans of megalin [22,23].

Until now, six members of the ST8Sia (α2,8-sialyltransferase) family have been described and cloned from various animal species. However, the role of each enzyme in the biosynthesis of diSia, oligoSia and PSA in vivo remains unclear. ST8Sia I and ST8Sia V are both involved in the α2,8-sialylation of gangliosides. ST8Sia I is responsible for the biosynthesis of G\textsubscript{D3}...
from G\textsubscript{M\textsubscript{1}} whereas ST8Sia V synthesizes mainly G\textsubscript{T\textsubscript{1}} [24,25]. ST8Sia II and ST8Sia IV are polysialyltransferases catalysing the polymerization of α2,8-sialic acid residues onto the Neu5Aca2-3(6)Galβ1-4GlcNAc structures found on the N-glycans of NCAM [26]. A third α2,8-sialyltransferase, ST8Sia III, transfers sialic acid residues to a terminal α2,3-linked sialic acid of glycoproteins and glycolipids [27]. Interestingly, h (human) ST8Sia III as well as ST8Sia II and ST8Sia IV are capable of autopolyGalactosylation [28,29]. This autopolyGalactosylation has been suggested to be implicated in the formation of active polysialyltransferases in vivo and in vitro [29–31]. However, nothing is known about the enzymes driving the synthesis of diSia, oligoSia or PSA chains on mammalian O-glycans.

In the present study, we describe the molecular cloning, genomic organization and the enzymatic characterization of the hST8Sia VI, which is orthologous to the recently cloned mouse enzyme [32]. The expression of an active, soluble recombinant enzyme clearly shows that the hST8Sia VI synthesizes mainly diSia motifs on the α2,3-sialylated core 1 structure (Neu5Aca2-3Galβ1-3GlcNAc 1-O-Ser) found in O-glycosylproteins. In contrast with the mouse enzyme, hST8Sia VI exhibits almost no activity towards either gangliosides or N-glycans.

EXPERIMENTAL

Materials

CMP-[\textsuperscript{14}C]Neu5Ac, CMP-[\textsuperscript{14}C]V-acetylenuraminic acid, 10.7 GBq/mmol) and First Strand cDNA Synthesis kit were from Amersham Pharmacia Biotech (Little Chalfont, U.K.). NucleoSpin® RNA II kit was from Macherey-Nagel (Düren, Germany). Oligo nts were synthesized and purified by Eurogentec (Seraing, Belgium) and dNTP were from Promega Biosciences (Son Luis Obispo, CA, U.S.A.). EXTRA-POL I DNA polymerase and DyNzyme Ext DNA polymerase were from Eurobio (Courtaboeuf, France) and Ozyme (Saint-Quentin-en-Yvelines, France). DMEM (Dulbecco’s modified Eagle’s medium) containing 4.5 g/l glucose without glutamine was from BioWhittaker Europe. TC100 medium, MEM (minimal essential medium), L-glutamine, antibiotics, geneticin G418, FCS (foetal calf serum) were a generous gift from J.-P. Zanetta (CNRS UMR 8576). Colon cancer cell lines HT-29 and Caco-2 (ATCC, CRL-1711) were cultured in TC100 medium supplemented with 5 % (v/v) FCS.

Bio-informatic analysis

BLAST algorithm (http://www.ncbi.nlm.nih.gov/genome/seq/Hsblast.html) [34] was used to retrieve nt sequences highly similar to hST8Sia I (GenBank\textsuperscript{®} accession number D26360), from the human HTG (High-Throughput Genomic) sequences division or the human and mouse EST (expressed sequence tag) divisions of the GenBank\textsuperscript{®}/EBI databases at the NCB (National Center for Biotechnology Information). The exon-intron junctions were analysed with the internet splice program (http://www.fruitfly.org/genii-bin/seqools splice.pl). The amino acid sequence analysis was performed using the program of translation for publication (http://www.infobiogen.fr/services/analyseq/cgi-bin/ forpub; Infobiogen, France). Hydrophathy analyses and determination of potential N-glycosylation sites were performed using the servers TM-Pred Prediction of Transmembrane Regions and orientation (http://www.ch.embnet.org/software/TMPRED_form) and the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc/) from Expert Protein Analysis System (Swiss Institute of Bioinformatics, Switzerland). Sequence alignments were performed using the LFASTAp program (http://www.infobiogen.fr/services/analyseq/cgibin/fastap) and clustalW algorithms (http://www.infobiogen.fr/services/analyseq/c8in-bin/ clustalw).

To examine hST8Sia VI gene expression in various human cell lines total RNA from various cell lines (MCF-7, MDA-MB-231, T47-D and NBEC) was extracted using the NucleoSpin® RNA II kit and cellular RNA was quantified by spectrophotometry at 260 nm. For subsequent PCR amplifications, first-strand cDNA was synthesized from total RNA using the First Strand cDNA Synthesis kit according to the manufacturer’s instructions.

A specific hST8Sia VI fragment of 834 bp was obtained after RT-PCR (reverse transcription-PCR) of RNAs isolated from various cultured cells, using 200 nM of sense (5′-GTAAGGACCAAC-TTTGCTTCCTG3′) and antisense (5′-TTTGACAGGTG-CATTTGCT 3′) primers, 200 µM dNTP and 1 unit of EXTRA-POL I DNA polymerase using the following conditions: 95 °C for 1 min, 38 cycles of 1 min at 95 °C for 2 min) and an extension step of 10 min at 72 °C.

Isolation of ST8Sia VI cDNA and construction of expression vectors

For subsequent plasmid constructions, restriction digestion and DNA sequencing (GenoScreen, France) confirmed the insert junctions and the total conservation between the inserted sequence and hST8Sia VI sequence (EMBL accession number: AJ621583).

To isolate cDNA clones, RT-PCR was performed using 150 ng of first-strand cDNA synthesized from MCF-7 total RNA. A first cDNA amplification was performed by PCR using a sense primer containing the restriction site NotI (5′-CATTTGCT 3′) and antisense (5′-CACTTTGCT 3′) primers, 200 µM dNTP and 1 unit of EXTRA-POL I DNA polymerase using the following conditions: 95 °C for 1 min, 38 cycles of 1 min at 95 °C, 1 min at 58 °C and 2 min at 72 °C, and 10 min at 72 °C.

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[26]
an antisense primer containing the restriction site XbaI (5'-3'
CTTGTGTTTGGAGACATCTAAGATCCACCTGTCG 3'). The RT-PCR amplified fragments (1285 bp) were subcloned into PCR2.1 TOPO TA cloning vector giving the TOPO-L-ST8Sia VI plasmid. The inserted fragment was cut out by digestion with NotI and XbaI and inserted into the NotI and XbaI sites of pRc-CMV expression vector. The resulting plasmid (pRc-CMV-L-ST8Sia VI) was further purified and used for stable transfection of MDA-MB-231 cells, as described below.

An expression vector was also prepared for subsequent transient transfection in animal cells. A cDNA encoding a truncated form of hST8Sia VI lacking the first 27 amino acids of the open reading frame was obtained by PCR amplification using the TOPO-L-ST8Sia VI as DNA template, the same antisense primer containing the restriction site XbaI (5'-3'
GGCGACTTCACATTTGCTAAA 3') and a sense primer containing the restriction site NotI (5'-3'
GACGCCGCCTCCGGGCGGCCAGCAGGATTCTGGTGGAG 3'). The resulting plasmid (pRc-CMV-L-ST8Sia VI) was further purified and used for stable transfection of MDA-MB-231 cells, as described below.

Production of soluble forms of hST8Sia VI
Qiagen-purified pFlag-S-ST8Sia VI (5 µg) or pFlag plasmids (5 µg) were transiently transfected into COS-7 cells in 100 mm diameter dishes using LIPOFECTAMINE™ Plus reagent, according to the manufacturer's instructions. The medium was harvested 48 h after transfection and the recombinant protein expressed in the medium was used as the enzyme source for sialyltransferase assays. Sf-9 cells were co-transfected by lipofection using LIPOFECTAMINETM. After transfections (2 days), the dishes were split at a 1:10 ratio and cells were cultured in MEM containing 5 % FCS and used for flow cytometric analysis.

Sialyltransferase assays and sialylated product characterization
Sialyltransferase assays were performed in 100 mM cacodylate buffer (pH 6.2) containing 10 mM MnCl₂, 0.2 % Triton CF-54, 40 µM CMP-[14C]Neu5Ac (1.94 Kb) and one of the acceptor substrates (2 mg/ml for glycoproteins, 1 mM for arylglycosides or glycolipids) and 23 µl of the enzyme source in a total volume of 50 µl. Unless stated otherwise, the reactions were performed at 32°C for 4 h. For glycoproteins, the reactions were stopped by addition of 2.5 x SDS/sample buffer and the reaction products were separated by SDS/PAGE. After transfer onto nitrocellulose membrane (Biotract, Pall Corporation) the radioactive products were detected and quantified by radio-imaging using a Personal Molecular Imager FX (Bio-Rad, France). For glycoproteins and arylglycosides, the incubation reaction was stopped with 1 ml of H₂O and products were applied onto C₁₈ Sep-Pak cartridges (Millipore Corp., Milford, MA, U.S.A.), eluted with 30 % CH₃OH and processed for scintillation counting.

[14C]Neu5Ac-labelled fetuin was produced using soluble recombinant hST8Sia VI in the incubation conditions described above. For linkage analysis of sialic acids, sialylated fetuin was treated with specific sialidases at 37°C for 1 h, according to the manufacturer's instructions (Glyko Inc.). For analysis of the glycan acceptor, sialylated fetuin was incubated with PNGase F at 37°C for 30–120 min according to the manufacturer’s instructions. In a second experiment, native fetuin was desialylated by Glyco® Sialidase S and was resialylated by the soluble recombinant hST8Sia VI. The resulting products were also resolved by SDS/PAGE and detected by radio-imaging. For analysis of the DP, sialylated fetuin and colominic acid were separately incubated for 2.5 h at 50°C in 50 µl of DMB reagent (2.7 mM DMB, 9 mM sodium hydrosulphite, 0.5 mM 2-mercaptoethanol in 20 mM trifluoroacetic acid) [38]. A volume (one-fifth) of 0.1 M NaOH was added and incubated for 1 h at room temperature to terminate the reaction and to eventually hydrolyse lactones [39]. DMB-tagged oligosialylated sequences from fetuin and colominic acid were co-injected onto a CarboPac PA-100 column (Dionex). Elution was performed at 0.8 ml/min with a concentration gradient of 0–32 % of 1 M KNO₃ in water. Elution was monitored by an on-line fluorescence detector set to 372 nm for excitation and 456 nm for emission. The samples were automatically collected and subsequently counted for radioactivity. The concentration of fetuin and asialofetuin were calculated on the basis of the number of O-glycosidically linked Galβ1-3GalNAc residues, determined by gas chromatography (61 nmol/mg).

Stable transfections
MDA-MB-231 cells were transfected with 20 µg of pRc-CMV or pRc-CMV-L-ST8SiaVI plasmids using LIPOFECTAMINE™. After transfections (2 days), the dishes were split at a 1:10 ratio and cells were cultured in MEM containing 5 % (v/v) FCS and genetin G418 (1 mg/ml). MEM and genetin were replaced every 2 days. After 15 days, the resistant colonies were well established, the cells selected and maintained with 1 mg/ml genetin and 10 % (v/v) FCS and used for flow cytometric analysis experiments.

Detection of α2,6-linked and α2,3-linked sialic acids in MDA-MB-231 transfected cells
Cell surface expression of sialic acids in two different types of linkages was quantified using SNA specific for α2,6-linked sialic acids and MAA specific for α2,3-linked sialic acids. Stably transfected cells were fixed with 4 % paraformaldehyde (30 min at 4 °C in the dark), quenched for 30 min with 50 mM NH₄Cl in PBS.
Figure 1   Genomic organization and exon/intron junctions of the hST8Sia VI gene

(A) The hST8Sia VI gene located on chromosome 10p12.31, spans 140 kb and contains 8 exons (labelled E1–E8). Black lines represent the DNA sequences identified in the human genomic databases. The entire genomic sequence of hST8Sia VI is included in the human contig: NT 008682.3.  (B) Schematic representation of the hST8Sia VI mRNA. The grey boxes represent the open reading frame and the open boxes the 5' and 3' untranslated regions respectively. The black lines represent the mouse (m) and human (h) EST identified in the public databases. (C) The nt sequences at the intron (lower case letters) and exon (upper case letters) junctions are shown. Exons are numbered from the 5' end with the initiator methionine denoted as +1.

RESULTS

Identification of hST8Sia VI gene

Similarity searches using the BLAST algorithms (BLASTn and tBLASTn) were conducted with the hST8Sia I cDNA (GenBank accession number: D26360) used as a bioinformatics probe in the human genomic databases (HTG division of NCBI). This study led us to the identification of two genomic clones AC016235 and AL158164 (Figure 1A), which partially aligned with the hST8Sia I cDNA and encompassed the signature motifs of mammalian sialyltransferases. In addition, these nt sequences were localized on human chromosome 10 where no sialyltransferase gene was described. Upon screening the human EST division of the NCBI databases, three ESTs, AV714822, BF678643 and AV724160 were retrieved, which corresponded to the 3' region of the putative gene whereas no human EST could be found to correspond with the 5' end of the gene (Figure 1B). However, BLAST analysis conducted in the mouse EST division of NCBI revealed the existence of two ESTs (BE686184 and BE633149) belonging to the same Integrated Molecular Analysis of Genome Expression (I.M.A.G.E.) clone (ID 34128403) that represented the mouse counterpart of the human sequence used for the search (Figure 1B) and which allowed us to identify the missing upstream sequences of the putative sialyltransferase in the human genomic clone, AL160289. The gene identification was completed at the Ensembl site (http://www.ensembl.org). As shown in Figure 1(A) the putative sialyltransferase gene spans over 140 kb of human genomic sequence (NT 008682) on human chromosome 10 (10p12.31). This nt sequence contains an open reading frame of 1197 bp (Figure 1B). In order to gain insights into the evolutional relationship of the hST8Sia VI with other sialyltransferase genes, the hST8Sia VI genomic organization was determined (Figure 1C) through the use of a specialized internet site (http://www.fruitfly.org/seq_tools/splice.html). The sequence of the splice junctions of the hST8Sia VI gene obey the AG/GT rule and the gene splits into eight coding exons in a genomic organization closely related to that of hST8Sia I and hST8Sia V (results not shown, see [40]).
Cloning and nt sequencing of hST8Sia VI

The nt sequences described above were used to design oligo nt primers for PCR amplification with MCF-7 first strand cDNA as template. However, probably because of the low level of expression of a long transcript and strong secondary RNA structures, we could not amplify the target sequence at once, so we amplified a full-length cDNA by nested PCR. Upon DNA sequencing of several of these MCF-7 PCR-amplified cDNA clones, we noticed various splicing variants of hST8Sia VI gene lacking 1 or 2 exons (results not shown). Indeed, theoretically, exons numbered 2, 3, 4 and 7 can be deleted without frame-shift in the open reading frame. The nt and deduced amino acid sequences of the full-length cDNA sequence obtained are shown in Figure 2. Hydropathy analysis of this protein indicated the presence of a hydrophobic sequence of 19 amino acids in the NH₂-terminal region, corresponding to the transmembrane domain characteristic of all sialyltransferases cloned to date (results not shown). The predicted protein consisted of 398 amino acids with a calculated molecular mass of 44 835 Da, contained in the catalytic domain the four sialylmotifs L, S, III and VS characteristic of all the animal sialyltransferases, and showed five potential N-glycosylation sites (Figure 2). The position of the initiation codon was estimated according to the Kozak consensus sequence [41] (http://www.hri.co.jp/atgpr/). In addition, the protein showed the (I/L)(F/Y)GFW(P/A)F sequence at the 3′ end of the sialylmotifs, which is conserved among the members of the α₂,8-sialyltransferase family. Besides, comparison of the amino acid sequence with those of the other human sialyltransferases indicated a phylogenetic linkage with the human sialyltransferases and particularly the greatest sequence identities with hST8Sia I (35 %) and hST8Sia V (32 %). Finally, hST8Sia VI is the human counterpart of a mouse sequence (GenBank® accession number AB059554) cloned during the time-course of our study [32]. The two sequences share 82.7 % sequence identity. Orthologues of the hST8Sia VI gene are also detected in several other vertebrate genomes such as Rattus norvegicus (AJ699423), Bos taurus (AJ868432), Gallus gallus (AJ699424), Takifugu rubripes (AJ715549, AJ715550) and Danio
amplified fragments are indicated on the right side of the gel. Dehydrogenase (GAPDH) was used as standard as previously described [50]. The sizes of the amplified fragments are indicated on the right side of the gel.

**hST8Sia VI gene expression in human tissues and cell lines**

In order to determine the expression pattern of hST8Sia VI gene and the size of hST8Sia VI mRNA, hybridization of a Northern blot of 12 human tissues and of an expression array of 72 different human tissues was performed with a full-length hST8Sia VI cDNA (1.27 kb). However, the expression levels of the ST8Sia VI gene were very low in all the tissues examined (results not shown). Thus we performed semi-quantitative RT-PCR analysis in several human cancer and normal cell lines. As shown in Figure 3, higher levels of expression of the hST8Sia VI gene were observed in breast cancer cell line MCF-7 and in Dami megakaryocyte cell line whereas very low levels of expression were observed in NBEC, in breast cancer cell line MDA-MB-231 and T47-D and in colon cancer cell lines HT-29 and Caco-2. No expression could be detected in the lung cancer cell line NCI.

**Decreasde expression of MAA-binding sites in the MDA-MB-231 cell line stably transfected with full-length hST8Sia VI**

In order to assess the function of hST8Sia in **in vivo**, a MDA-MB-231 cell line was stably transfected with the expression vector pRc-CMV-L-hST8Sia VI (full-length) as described in the Experimental section. Cell clones were selected by mRNA expression (Figure 4A) and by ST8Sia activity towards fetuin used as an acceptor substrate for **in vitro** assays (Figure 4B). Characterization of the sialylated carbohydrate profile of MDA-ST8Sia VI, as well as of the mock-transfected cells was performed by flow cytometry analysis of these cells using MAA and SNA lectins that are specific for α2,3- and α2,6-sialylated oligosaccharides respectively. As shown in Figure 4(C), in **in vivo** expression of hST8Sia VI led to a 4-fold decrease in MAA-binding sites in MDA-MB-231 transfected cells whereas very limited changes were observed for the SNA-binding sites in MDA-MB-231 transfected cells (Figure 4C). Our results suggest that in **in vivo** hST8Sia VI catalyses the transfer of the sialic acid residue onto the disaccharide structure Neu5Aca2-3Gal, but had very limited activity towards the disaccharide structures Neu5Aca2-6GalNac.

**Sialyltransferase activity of the hST8Sia VI**

In order to conclusively verify that the cloned DNA which we had obtained from MCF-7 cells indeed represented human ST8Sia VIII, and to facilitate functional analysis of the enzyme, we produced a soluble form of the enzyme with deleted cytoplasmic and transmembrane domains, using the baculovirus/SF-9 system. Thus a truncated cDNA lacking the first 24 amino acid residues of the N-terminus region was generated by PCR and used for the construction of a p119-hST8Sia VIII vector, as described in the Experimental section. The recombinant hST8Sia VIII enzyme produced in insect cells was used for **in vitro** sialyltransferase assays with various glycoconjugate acceptors (Table 1). This recombinant hST8Sia VIII enzyme exhibited highly decreased sialyltransferase activity towards asialoglycoproteins compared with the corresponding sialylated substrates indicating that the enzyme required a sialylated substrate. hST8Sia VIII exhibited strong sialyltransferase activity towards BSM (bovine submaxillary mucin) and OSM (ovine submaxillary mucin), which contains only O-glycans and towards fetuin, which contains both N- and O-glycans. On the contrary, it showed very low activity towards α1-acid glycoprotein, which contains only N-glycans. This set of results clearly showed that the recombinant hST8Sia VIII preferred sialylated O-glycans such as Neu5Aca2-3Galβ1-3GalNac-O-Ser/Thr or Neu5Aca2-6GalNac-O-Ser/Thr found on fetuin, to sialylated N-glycans as acceptor substrates. We next examined hST8Sia VIII activity towards various glycolipids such as GM3, GD3, or GD4 containing a sialylated lactosylceramide fraction (Table 1). hST8Sia VIII showed almost no activity ( < 11 %) towards these.
Various acceptor substrates were incubated in the standard assay mixture using transfected Sf-9 cells as an enzyme source. Each substrate was used at a concentration of 1 mM for arylglycosides, 2 mg/ml for glycoproteins and 1 mM for glycolipids. The specific $[^{14}C]$Neu5Ac incorporation resulting from recombinant hST8Sia VI activity was estimated by subtracting the background sialyltransferase activities of mock transfected cells from the radioactivity in the presence of exogenous acceptor. Relative rates were calculated as a percentage of the incorporation of sialic acid onto fetuin. A value of 0 indicates <1 %; nd, not determined; Cer, ceramide.

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* Data are from [32].
† Actual activities are shown in brackets in pmol/h per μL.

In order to confirm that hST8Sia VI preferentially sialylates O-glycans, α2,8-[14C]Neu5Ac-labelled sialylated fetuin was produced using a soluble recombinant hST8Sia VI (see the Experimental section) and was subjected to PNGase F, which hydrolyses N-glycans (Figure 5). After 30 min treatment, we observed a shift of mass corresponding to the loss of N-glycans but the intensity of the signal remained unchanged, suggesting that the sialic acid residue was transferred onto O-glycans of fetuin rather than onto N-glycans.

To characterize the linkage catalysed by hST8Sia VI, α2,8-[14C]Neu5Ac-labelled fetuin was produced using soluble recombinant hST8Sia VI. It was then subjected to linkage specific sialidases, Glyco® Sialidase S (specific for α2,3-linked sialic acid), Glyco® Sialidase C (specific for α2,3/6-linked sialic acid) and Glyco® Sialidase A™ (specific for α2,3/6/8-linked sialic acid) (Figure 6A). We observed that the treatment with the Glyco® Sialidase S and C, which hydrolyse the α2,3- and α2,6-linked sialic acid is ineffective on the signal. Conversely, this signal disappeared when labelled fetuin was treated with Glyco® Sialidase A™, which is specific for α2,3/6/8-linked sialic acid, indicating that the incorporated sialic acid was α2,8-linked. These results confirmed that the cloned hST8Sia VI belonged to the ST8Sia subfamily.

We next aimed to determine whether hST8Sia VI preferentially sialylated Neu5Acα2-3Galβ1-3GalNAc-R acceptor substrate or the Neu5Acα2-6GalNAc-R acceptor substrate that are both present on native bovine fetuin. Thus either native fetuin or α2,3-desialylated fetuin was incubated with recombinant hST8Sia VI. Indicative of the efficiency of the desialylation, a shift of mass was observed after Ponceau Red staining of the Western blot, when α2,3-sialic acid was removed before incubation (results not shown). As shown in Figure 6(B), hST8Sia VI did not mediate the efficient transfer of sialic acid residue onto α2,3-desialylated fetuin. This result suggested that the recombinant hST8Sia VI preferentially catalysed the transfer of a sialic acid residue to an α2,3-linked Neu5Ac rather than to an α2,6-linked Neu5Ac and...
confirmed the flow cytometric analysis of the transfected MDA- 
ST8Sia VI cells with the MAA lectin.

**DP analysis**

Based on the above results, we next addressed the question of whether hST8Sia VI may synthesize di-, oligo-, or PSA chains onto fetuin. For this purpose, native bovine fetuin was first incubated with hST8Sia VI and CMP-[14C]Neu5Ac. Sialylated sequences were then liberated from glycoproteins by mild acid hydrolysis, tagged with DMB and separated by HPLC on a CarboPac PA-100 column. Retention times for [Neu5Acα2-8]- Neu5Ac-DMB standard, as well as di-, tri- and tetra-sialylated sequences purified from a mild hydrolysate of colominic acid. As shown in Figure 7, in the hST8Sia VI incubation mixture, we clearly observed a radioactive peak with a retention time similar to Neu5Acα2-8Neu5Acα2-8DMB, strongly suggesting that synthesis of the di-sialylated epitope occurred from sialylated fetuin. Accordingly, such a peak was not observed in either the mock-transfected cell incubation mixture or native fetuin, indicating that hST8Sia VI catalysed the transfer of a single sialic acid residue onto native fetuin. Furthermore, a time course analysis of the sialylation of fetuin clearly confirmed the specificity of this enzyme for biosynthesis of diSia motifs.

**DISCUSSION**

In the present study, we report on the molecular cloning and enzymatic characterization of a human sialyltransferase gene that encodes an ST8Sia with preference for O-glycans. This gene spans over 140 kb of human chromosome 10 (10p12.31) and splits into eight coding exons with an organization closely related to that of the hST8Sia I and hST8Sia V genes. Phylogenetic analyses have shown that the ST8Sia gene family can be divided into two groups of genes, which evolved from a common ancestor and it was observed that the human sialyltransferases of each group showed distinct enzymatic properties [40]. In the first group, ST8Sia II, III and IV are known as oligo- or poly-sialyltransferases involved in the elongation of linear chains of sialic acids found mainly in glycoproteins. ST8Sia II and IV were clearly shown to be responsible for the elongation of polysialylated chains of NCAM [26] and even if the substrate specificity of ST8Sia III is not clearly demonstrated, this latter enzyme is capable of autopolydisialylation in vitro [29]. In the second group encompassing ST8Sia I, ST8Sia V and ST8Sia VI, ST8Sia I (G0 synthase) and ST8Sia V (GT3 synthase) are acting as di-sialyltransferases, transferring only one sialic acid residue onto G0 or G0, to convert these gangliosides into G0 or G0, respectively. It could be predicted that the enzymatic properties of hST8Sia VI would be those of a di-sialyltransferase. As hypothesized, we have clearly demonstrated that the human ST8Sia VI transferred only one sialic acid residue in α2,8-linkage. However, in contrast with its recently characterized mouse counterpart [32], which also acts on the Neu5Acα2-3Galβ1-4Glc sequence of G0, and on the Neu5Acα2-6Galβ sequences such as are found in N-glycosylproteins, the hST8Sia VI works almost exclusively on O-glycans.

Stable transfections of hST8Sia VI in the MDA-MB-231 cell line have shown that hST8Sia VI works preferentially on cell surface α2,3-sialylated structures. Our in vitro assays using a soluble form of the enzyme have demonstrated that hST8Sia VI preferentially uses the trisaccharide Neu5Acα2-3Galβ1-3GalNAcα1-0-Ser/Thr found in bovine fetuin, and to a lesser extent, to that found in gangliosides (G0). It shows also an activity towards the disaccharide Neu5Acα2-3GalNAcα1-0-Ser/Thr found on glycans of BSM and OSM. This discrepancy between the specific enzymatic activity of the mouse and the human enzymes could be related to inter-species enzymatic differences which indeed lead to species-specific glycosylation. Currently work is in progress to determine which O-glycosylprotein(s) might be disialylated in these stably-transfected MDA cells using specific monoclonal antibodies towards diSia, oligoSia or PSA [42].

The occurrence of diSia motifs in animal tissues was suggested a long time ago by Finne et al. [43] but indeed, Neu5Acα2-8 Neu5Acα2-3Galβ1-3GalNAcα1-0-O-Ser/Thr or Galβ1-3[Neu5-Aca2-8 Neu5Acα2-6]GalNAcα1-0-O-Ser/Thr oligosaccharides...
are not frequently described in mammalian glycoproteins. However, these diSia motifs were shown previously to occur in several embryonic and adult pig brain glycoproteins [44], in the murine CD-166 [18], on the N-glycans of Band 3 of human erythrocytes [14], on the N-glycans of fetuin and α2-macroglobulin of calf serum [17], on the O-glycans of human erythrocyte glycoporin [15], bovine adipo-Q [19] and chromogranin [16]. It has been suggested that the biosynthesis of these diSia residues on glycoproteins might be catalyzed by an α2,8-sialyltransferase distinct from the known polysialyltransferases ST8Sia II and ST8Sia IV and it was shown that a recombinant ST8Sia III could sialylate from the known polysialyltransferases ST8Sia II and ST8Sia IV towards cells [48] and displays unique ligand binding properties, different responses. Siglec-7 is the major siglec expressed by natural killer epitopes might be involved in the regulation of innate immune physiological phenomena in a way that remains to be determined [19]. During the adipocyte differentiation of murine fibroblastic 3T3-L1 preadipocytes, significant increases in diSia structures [18]. During the adipocyte differentiation of murine fibroblastic 3T3-L1 preadipocytes, significant increases in diSia structures were observed in fully differentiated adipocyte cells and among them the serum adipo-Q glycoprotein has been shown to have the diSia epitope [19]. Adipo-Q is considered to play an important role in energy homeostasis and it is believed that the diSia epitope could be functionally involved in these physiological phenomena in a way that remains to be determined [19]. Of the siglecs cloned so far, it is known that siglec-7 and siglec-11 have a high affinity for the diSia epitopes found on glycoproteins [47]. Siglec-11 shows a unique expression pattern on tissue macrophages and infiltrating mononuclear leukocytes in inflammatory tissues suggesting that this molecule and the diSia epitopes might be involved in the regulation of innate immune responses. Siglec-7 is the major siglec expressed by natural killer cells [48] and displays unique ligand binding properties, different from other members of the siglec family, towards α2,8-linked sialic acids over α2,6- and α2,3-linked sialic acids [49]. One can assume that glycan recognition by siglec-7 is likely to be directly linked to its function in modulating the activation of natural killer cells. The characterization of this new ST8Sia VI might help to determine the function of the diSia epitopes found on O-glycosylated proteins, although the presence of this structure remains to be demonstrated in vivo.

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REFERENCES

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28 Angata, K., Suzuki, M., McAuliffe, J., Ding, Y., Hindsaul, O. and Fukuda, M. (2000) Differential biosynthesis of polysialic acid on neural cell adhesion molecule (NCAM) and oligosaccharide acceptors by three distinct α2,8-sialyltransferases, STβSia IV (PST), STβSia II (STX), and STβSia III. J. Biol. Chem. 275, 18964–18961


