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ABSTRACT

The Sialyl-Tn antigen (Neu5Acα2–6GalNAc-O-Ser/Thr) is highly expressed in several human carcinomas and is associated with carcinoma aggressiveness and poor prognosis. We characterized two human sialyltransferases, CMP-Neu5Ac:GalNAc-R α2,6-sialyltransferase (ST6GalNAc-I) and ST6GalNAc-II, that are candidate enzymes for Sialyl-Tn syntheses. We expressed soluble recombinant hST6GalNAc-I and hST6GalNAc-II and characterized the substrate specificity of both enzymes toward a panel of glycopeptides, glycoproteins, and other synthetic glycoconjugates. The recombinant ST6GalNAc-I and ST6GalNAc-II showed similar substrate specificity toward glycoproteins and GalNAc-O-Ser/Thr glycopeptides, such as glycopeptides derived from the MUC2 mucin and the HIVgp120. We also observed that the amino acid sequence of the acceptor glycopeptide contributes to the in vitro substrate specificity of both enzymes. We additionally established a gastric cell line, MKN45, stably transfected with the full-length cDNA of ST6GalNAc-I or ST6GalNAc-II and evaluated the carbohydrate antigens expression profile induced by each enzyme. MKN45 transfected with ST6GalNAc-I showed high expression of Sialyl-Tn, whereas MKN45 transfected with ST6GalNAc-II showed the biosynthesis of the Sialyl-T antigen (Galβ1–3 (Neu5Acα2–6)GalNAc-O-Ser/Thr).

In conclusion, although both enzymes show similar in vitro activities when Tn antigen alone is available, whenever both Tn and T antigens are present, ST6GalNAc-I acts preferentially on Tn antigen, whereas the ST6GalNAc-II acts preferentially on T antigen. Our results show that ST6GalNAc-I is the major Sialyl-Tn synthase and strongly support the hypothesis that the expression of the Sialyl-Tn antigen in cancer cells is due to ST6GalNAc-I activity.

INTRODUCTION

The modification of cellular glycosylation is a common phenotypic alteration observed in cancer, and the aberrant expression of sialylated carbohydrate structures on carcinoma cells has been widely reported. The Sialyl-Tn antigen (Neu5Acα2–6GalNAc-O-Ser/Thr) is a simple mucin-type carbohydrate antigen that has attracted much attention for several reasons. First, Sialyl-Tn is rarely observed in normal tissues but highly expressed in most gastric (1–3), colorectal (4), ovarian (5), breast (6, 7), and pancreatic (8) carcinomas. Second, Sialyl-Tn has been associated with carcinoma aggressiveness and poor prognosis. Sialyl-Tn expression has been shown to be an independent indicator of poor prognosis in carcinomas and in sera of patients with gastric (9–11), colorectal (4), and ovarian cancer (5). Third, Sialyl-Tn expression is aberrantly observed in premalignant lesions of the gastrointestinal tract, such as intestinal metaplasia (2), adenomatous polyps (12), and chronic ulcerative colitis (13). Taken together these observations suggest an important role for Sialyl-Tn in the biology of carcinoma cells. In addition, the expression of Sialyl-Tn antigen in carcinomas has been associated with the aberrant glycosylation of the MUC1 mucin, with both Sialyl-Tn and MUC1 being used as targets for cancer immunotherapy in preclinical and clinical studies (14–18).

Whereas the clinical significance of Sialyl-Tn has been characterized, a functional role for this glycan in cancer cells remains to be elucidated. A number of publications have pointed out that the Sialyl-Tn structure may be implicated in cell-cell interactions and recognition. The biosynthesis of Sialyl-Tn blocks additional elongation of the oligosaccharide chain and induces additional modification of the cell glycosylation profile due to the exposure or masking of underlying sugar structures (19, 20). These glycosylation alterations induce or prevent the recognition by lectin-like molecules, such as selectins (21), siglecs (22), and galectins (23). In addition, Sialyl-Tn antigen may also play a role in cancer cell recognition by the immune system and protecting metastatic cells from degradation in the blood stream (19, 24).

A family of enzymes named sialyltransferases is responsible for the biosynthesis of sialylated structures (25–27). To date 20 human sialyltransferase genes have been cloned (28). These genes encode type II transmembrane proteins with a short NH2-terminal cytoplasmic tail, a 16–24 amino acid signal anchor domain, and a large COOH-terminal catalytic domain in the Golgi apparatus lumen (29). They all catalyze the transfer of a sialic acid residue from cytidine 5′-monophospho-N-acetylanaminc acid (CMP-Neu5Ac) to oligosaccharide chains of glycoproteins and glycolipids. The linkage formed can be α2–3 or α2–6 to Galactose (Gal) residue, α2–6 to N-acetylgalactosamine (GalNAc) residue, or α2,8 to another sialic acid residue. Each enzyme can catalyze only one linkage and has its own substrate specificity (28). Six α2,6-sialyltransferase members have been shown to catalyze the transfer of Neu5Ac to GalNAc. Among them only CMP-Neu5Ac:GalNAc-R α2,6-sialyltransferase (ST6GalNAc-I), -IL, and -IV are active on glycoproteins, and it has been shown that ST6GalNAc-IV is unable to sialylate the Tn antigen (GalNAc-O-Ser/Thr; ref. 30). Both ST6GalNAc-I and ST6GalNAc-II have been shown to sialylate the T antigen (Galβ1–3GalNAc-O-Ser/Thr) and the Sialyl-T antigen (Neu5Acα2,3Galβ1–3GalNAc-O-Ser/Thr), respectively (31, 32), but the identification of the enzyme responsible for the α6 sialylation of the Tn antigen (GalNAc-O-Ser/Thr) remains to be clarified. ST6GalNAc-I enzyme shows an efficient transfer of sialic acid to the GalNAc residue of [α2,3GalNAc]-Aβn polymer (31), whereas ST6GalNAc-II was first described as showing no activity toward asialo-ovine submaxillary mucin, a mucin rich in the GalNAc-O-Ser/Thr structure (32). However, a recent study showed that the murine ST6GalNAc-II is also able to sialylate the GalNAc-O-Ser/Thr present in asialo-ovine submaxillary mucin, and the authors suggest that the murine ST6GalNAc-II may also function as a Sialyl-Tn synthase (33). To elucidate the sialyltransferase responsible for the biosynthesis of...
the Sialyl-Tn in cancer cells we investigated the hypothesis that both human ST6GalNAc-I and ST6GalNAc-II may function as Sialyl-Tn synthases. For this purpose we characterized for the first time the substrate specificity of purified recombinant ST6GalNAc-I and ST6GalNAc-II toward a panel of well-defined glycopeptides. We additionally established a human gastric carcinoma cell line model stably transfected with either ST6GalNAc-I or ST6GalNAc-II to evaluate the carbohydrate antigens expression profile induced by each enzyme.

**MATERIALS AND METHODS**

**Expression of Soluble Recombinant ST6GalNAc-I and ST6GalNAc-II.**

Expression constructs of soluble human ST6GalNAc-I and ST6GalNAc-II were designed to encode enzymes lacking the cytoplasmic and transmembrane regions, determined according to the Hoop and Woods hydrophobicity plot. The ST6GalNAc-I soluble construct encoded amino acid residues 43–601 and was prepared by PCR using human cDNA (GenBank accession no. Y11339) obtained from cell line HT-29-MTX (34) with the forward primer 5′-CA GCG GAT CCT TCT AGG CAT CAA CGC A-3′, which introduced a BamHI restriction site, and the reverse primer 5′-AGC GCC GCG GCC GCG GGT CTG GCG GCC TGC TTT GGC-3′, which removed the stop-codon and introduced a NorI restriction site. The ST6GalNAc-II soluble construct encoded amino acid residues 34–375 and was prepared by PCR using human cDNA (GenBank accession no. A251053) obtained from cell line MDA-MB-231 (32) with the forward primer 5′-GA GCG GCC GAT CCC TAC CGG GGG CCA GCC GGC-3′, which introduced a BamHI restriction site, and the reverse primer 5′-ACG GCC GGC GCC GCG CTG GTA CAG CTT AA-3′, which removed the stop-codon and introduced a NorI restriction site. Each PCR product was cloned directionally between the BamHI and NorI site of the pAcGP67 vector (PharMingen, San Diego, CA) containing a myc-tag upstream the NorI site. Both constructs were fully sequenced (ABI Prism 377; Applied Biosystems, Foster City, CA).

Spodoptera frugiperda (Sf9) cells (Invitrogen Life Technologies, Inc, Carlsbad, CA) were cotransfected with plasmids pAcGP67/ST6GalNAc-I (sol) or pAcGP67/ST6GalNAc-II (sol) and Baculo-Gold DNA (PharMingen, San Diego, CA) containing a myc-tag upstream the NorI site. Both constructs were fully sequenced (ABI Prism 377; Applied Biosystems, Foster City, CA).

**Purification of Recombinant ST6GalNAc-I and ST6GalNAc-II.**

Purification of recombinant soluble ST6galNAc-I and ST6GalNAc-II was performed by a stirring incubation with Amberlite (Sigma, St. Louis, MO) for 2 hours. Samples were filtered and dialysed against buffer A (ST6GalNAc-I) or buffer B (ST6GalNAc-II). Dialyzed samples were submitted to a cation exchange chromatography in a SP-Sepharose (Pharmacia Biotech, Stockholm, Sweden) column equilibrated with the respective buffer. The samples were eluted with a gradient of NaCl (from 10 mmol/L to 1 mol/L). Fractions of the eluted samples were analyzed for sialyltransferase activity as described below, and the active fractions were concentrated using an Ultrafree-15 Centrifuge Filter Device with 30,000 cutoff (Millipore, Bedford, MA) and with a Micro-ProDiCon Dialysis system using a membrane with 30,000 cutoff (Spectrum, Rancho Dominguez, CA). The purity and protein concentration of final fractions of the ST6GalNAc-I and ST6GalNAc-II were assessed by SDS-PAGE and Coomassie staining or by Western Blotting using an anti-myc MAb.

**Sialyltransferase Assay.**

Standard sialyltransferase assays were performed in 50 µL of total reaction mixture containing 20 mmol/L Bis-Tris (pH 6.5), 20 mmol/L EDTA (pH 5.9), 1 mmol/L DTT, 0.2 mmol/L CMP-Neu5Ac (3,000 cmc/µmol), 10 mg/mL asialofetuin, and 10 µL of supernatant culture. Sialyltransferase activity was measured after incubation at 37°C for 1 hour. The reaction was terminated with the addition of 1 mL of 2.5% phosphotungstic acid in 1 mol/L HCl at 4°C, followed by filtration on glass fiber filter (Whatman GF/A, Clifton, NJ). Precipitates were washed with 1 mL of 2.5% phosphotungstic acid in 1 mol/L HCl at 4°C and 1 mL of EtOH 1% at 4°C, and the membranes were dried at 60°C for 10 min and processed for scintillation counting. One unit of enzyme is defined as the amount of enzyme that transfers 1 µmol of Neu5Ac from CMP-Neu5Ac in 1 minute using the standard reaction mixture with Asialofetuin as acceptor substrate.

Enzymatic assays for ST6GalNAc-I and ST6GalNAc-II were performed using a panel of different substrates (see Table 2 for structure). Polyacrylamide glycoconjugates Tn-polyacrylamide (GalNAc-α-polyacryl- amide) and T-polyacrylamide (Galβ1–3GalNAc-α-polyacrylamide) were obtained from Syntosome (Munch, Germany). Peptides and glycopeptides were synthesized by ourselves and by Neosystems: GalNAc-glycopeptides MUC1 (7-mer), MUC2, and HVI120gp (17-mer) were chemically synthesized as described previously (37); glycopeptides MUC1 (25-mer), MUC1 (60-mer), MUC4, and HVI120gp (21-mer), were enzymatically GalNAc-glycosylated using recombinant UDP-N-acetyl-α-D-galactosamine-polypetide N-acetylgalactosaminyltransferases as described previously (38–40). Glycoproteins Asialofetuin, Fetuin, and arylglycosides (GalNAc1–O-benzyl, GalNAc1–O- pNP, and Galβ1–3GalNAc1–O-benzyl) were obtained from Sigma. Substrate specificity assays were performed as described above but with 1 µL of purified/concentrated ST6GalNAc-I or ST6GalNAc-II and the acceptor substrate in the following concentrations: 100 µmol/L for GalNAc-peptides, 10 mg/mL for glycoproteins, 10 mg/mL for glyco-polyacrylamide substrates (Tn-polyacrylamide and T-polyacrylamide), and 1 mmol/L for arylglycosides (GalNAc1–O-benzyl, GalNAc1–O-pNP, and Galβ1–3GalNAc1–O-benzyl).

Table 1: Purification of recombinant ST6GalNAc-I and ST6GalNAc-II

<table>
<thead>
<tr>
<th>Purification step</th>
<th>ST6GalNAc-I</th>
<th>ST6GalNAc-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>Activity (mUnits)</td>
<td>Yield (%)</td>
</tr>
<tr>
<td>Cell culture supernatant</td>
<td>400</td>
<td>1012</td>
</tr>
<tr>
<td>Amberlite</td>
<td>50</td>
<td>951</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>50</td>
<td>542</td>
</tr>
<tr>
<td>Concentration</td>
<td>5</td>
<td>398</td>
</tr>
</tbody>
</table>

* One unit of enzyme is defined as the amount of enzyme that transfers 1 µmol of Neu5Ac from CMP-Neu5Ac in 1 minute using the standard reaction mixture as described in Materials and Methods with Asialofetuin as acceptor substrate.

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Table 2 Comparison of acceptor substrate specificity of recombinant human ST6GalNAc-I and ST6GalNAc-II

<table>
<thead>
<tr>
<th>Acceptor substrate*</th>
<th>Structure/amino acid sequence†</th>
<th>ST6GalNAc-I</th>
<th>ST6GalNAc-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asialofetuin</td>
<td>Galβ1–3GalNAc1-O-Ser-Thr</td>
<td>3.0 (5.49)</td>
<td>3.0 (9.22)</td>
</tr>
<tr>
<td></td>
<td>Galβ1–4GlcNAc-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin</td>
<td>NeuAcβ2–3Galβ1–3GalNAc1-O-Ser/Thr</td>
<td>84</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>NeuAcβ2–3Galβ1–3[NeuAcβ2–6]GalNAc1−O−Ser/Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuAcβ2–3Galβ1–4GlcNAc-R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tn-PAA</td>
<td>GalNAcα-PAA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Tn-benzyl</td>
<td>GalNAcα1-O-Bn</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tn-nitrogenyl</td>
<td>GalNAcα1-O-pNP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T-PA</td>
<td>Galβ1–3GalNAcα-PAA</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>T-benzyl</td>
<td>Galβ1–3GalNAcα1-O-Bn</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MUC1 (25-mer)</td>
<td>T/APP/AGVT/TS/ADPT/RPA/AGPS/TT/APP</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>MUC1 (60-mer)</td>
<td>V/T/4ADP/TR/AGPS/TT/APP/AGHS</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>MUC1 (7-mer)</td>
<td>ADPT/RPA</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>MUC2</td>
<td>PT/TT/TP/TS/TT/TM</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>MUC4</td>
<td>CPLVTD/TSSASTGHT/PLPV</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>HIV120gp (23-mer)</td>
<td>CIRIQRG/PR/AFVT/IK/IGNM</td>
<td>62</td>
<td>58</td>
</tr>
<tr>
<td>HIV120gp (17-mer)</td>
<td>GRAFVT/IK/G/NGMRQAC</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>R5M§</td>
<td>PTDTST/TT/PAPTNT</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

* Acceptor substrates were incubated in the standard assay mixture as described in Materials and Methods using purified recombinant human ST6GalNAc-I and ST6GalNAc-II. Concentration of the substrates: peptides were used at 100 μM; Asialofetuin, Fetuin, and glyco-PAA at 10 mg/mL.
† Amino acid is GalNAc glycosylated.
‡ The relative rate is presented in percentage of sialyltransferase activity using Asialofetuin as standard. Actual activities are shown in parentheses in pmol/hour/μL.
§ Rat submandibular mucin.

Monoclonal Antibodies. Tn antigen (GalNAcThr/Ser) was detected by HB-Tn (DAKO, Glostrup, Denmark), diluted 1:60, and IEX, diluted 1:2; Sialyl-Tn antigen (NeuAc2–6GalNAcThr/Ser) was detected by HB-STn (DAKO), diluted 1:100, and TKH2 (41), diluted 1:2; T antigen (Galβ1–3GalNAcThr/Ser) was detected by HB-T (DAKO), diluted 1:100, and HH8 (42), diluted 1:2.

Product Development Monitored by Enzyme-Linked Immunosorbent Assay. The synthetic MUC2 glycopeptide (0.25 mg) containing GalNAc (sequence shown in Table 2) was sialylated in vitro using 4 μM of either ST6GalNAc-I or ST6GalNAc-II under the standard sialyltransferase assay described above. Sialylated glycopeptide was purified by C-18 reverse-phase high-performance liquid chromatography as described previously (38, 39). ELISA was performed in Maxisorp plates (Nalge Nunc International, Roskilde, Denmark) coated with peptide diluted in bicarbonate-carbonate buffer (pH 9.6) overnight at 4°C. Samples designed for desialylation were incubated for 2 hours at 37°C with neuraminidase from Clostridium perfringens type IV (Calbiochem, La Jolla, CA) diluted to 0.1 units/mL in 0.1 mol/L sodium acetate buffer (pH 5.5). Plates were blocked for 30 minutes with 5% bovine serum albumin diluted in PBS and incubated for 1 hour with MAbbs diluted in PBS. Plates were washed and incubated with a peroxidase-conjugated rabbit antimouse antibody (DAKO) diluted 1:500 in PBS. Plates were washed and developed with 0.4 mg/mL of 1.2-phenylenediamine tablets (Sigma) in 0.1 mol/L citric acid-phosphate buffer (pH 5.0) using 1 μL of H2O2/mL plates. Plates were read in an ELISA reader at 492 nm.

Stable Transfections of Full-Length ST6GalNAc-I and ST6GalNAc-II. Full-length human ST6GalNAc-I and ST6GalNAc-II vectors were prepared by PCR using human cDNA from cell lines HT-29-MTX (34) and MDA-MB-231 (32), respectively, as a template. The PCR was performed with Pfu DNA polymerase native (Fermentas, Vilnius, Lithuania) using, for ST6GalNAc-I, the forward primer 5′-C GCA TCC ATG TCA ATA GGA CTC CAG AT-3′, which introduced a BamHI restriction site, and the reverse primer 5′-C AGC CTC TGC TCA GTC TCT GCC TTT TT-3′, which introduced a XhoI restriction site; for ST6GalNAc-II, the forward primer was 5′-C AGC GGA TCC ATG GGG CTC CCG CGC G-3′, which introduced a BamHI restriction site, and the reverse primer was 5′-C AGC CTC TGC GAG TCA GCC CTG TGA CAG-3′, which introduced a XhoI restriction site. For each enzyme gene, the PCR product was cloned directionally between the BamHI and XhoI site of the pcDNA3.1 vector (Invitrogen Life Technologies, Inc.) and was fully sequenced (ABI Prism 377, Applied Biosystems) to verify the correct sequence and insertion.

Gastric carcinoma cell line MKN45 (ref. 43; American Type Culture Collection, Manassas, VA) was transfected with plasmid pcDNA3.1/ST6GalNAc-I (full) or pcDNA3.1/ST6GalNAc-II (full) using the TFX-50 reagent (Promega, Madison, WI). Cells were selected in the presence of 0.3 mg/mL Geneticin in RPMI 1640 with Glutamax supplemented with 10% inactivated fetal calf serum for 2 weeks. Resistant cells were cloned by the limiting dilution method (0.3 cell/well), and single-cell wells were identified and propagated with medium changes every 3 days. The clones were screened by mRNA expression.

RNA and Multiplex Reverse Transcription-PCR. Total RNA was extracted using the Purescript RNA isolation kit (Gentra Systems, Minneapolis, MN), according to the manufacturer’s protocol. RNA yield and quality were determined spectrophotometrically, and 3.0 μg of total RNA was reverse transcribed using the Superscript III RNase H–Reverse Transcriptase kit (Invitrogen Life Technologies, Inc.) according to the manufacturer’s instructions. To control the yield of cDNA synthesis, as well as any genomic DNA contamination, a PCR with primers in different exons for amplification of the hypoxantine phosphoribosyltransferase mRNA was performed.

Primer pairs used were as follows: hypoxantine phosphoribosyltransferase was amplified using the forward primer 5′-TTC CTC CTC CTC AGC AGT CAG C-3′ and the reverse primer 5′-GCC ATG TCA ATA GGA CTC CAG AT-3′ (amplification length 756 bp); ST6GalNAc-I was amplified using the forward primer 5′-GCA ACC ACA GCC AAG ACG CTC ATT CCC AA-3′ and the reverse primer 5′-TGT CAC GAC TCT CAC GAA GGA GT-3′ (amplification length 455 bp); ST6GalNAc-II was amplified using the forward primer 5′-AAG CTC CTA CAT CCG GAC TTT AAC CTT CAG-3′ (amplification length 242 bp).

The multiplex PCR of ST6GalNAc-I and hypoxantine phosphoribosyltransferase was done as follows: after preheating for 4 minutes at 94°C, 35 cycles were performed with each cycle of 30 seconds at 92°C, 1 minute at 64°C and 3 minutes at 72°C, followed by a final extension of 10 minutes at 72°C.

The multiplex PCR of ST6GalNAc-II and hypoxantine phosphoribosyltransferase was done as follows: after preheating for 4 minutes at 95°C, 40 cycles were performed with each cycle of 45 seconds at 95°C, 1 minute and 30 seconds at 63°C, and 2 minutes and 30 seconds at 67°C, followed by a final extension of 10 minutes at 67°C.

Both PCR reaction mixtures contained 4.0 μL sample cDNA, 5.0 μL PCR buffer [500 mmol/L KCl, 15 mmol/L MgCl2, and 100 mmol/L Tris-HCl (pH 9.0)], 3.0 μL of 10 mmol/L deoxynucleotide triphosphates, 0.4 μL TaqDNA polymerase, 2.0 μL of each 3 pm hypoxantine phosphoribosyltransferase primer, 4.0 μL of each 3 pm ST6GalNAc-I or ST6GalNAc-II primers, and H2O to a total volume of 25 μL. In all of the experiments, negative control reactions were performed by replacing cDNA template with sterile water.

Reaction products obtained were submitted to electrophoresis in 2% agarose gels containing ethidium bromide.

Reference:
—H. Clausen and S. Hakomori, unpublished observations.
Preparation of Cell Lysates and α2,6-Sialyltransferase Activity of Full-Length Transfectants. Cells were harvested by trypsinization and washed twice with PBS. Cells were counted, and 2.5 × 10^6 cells were centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in 100 µL of 50 mM NaCl, 4-morpholinepropanesulfonic acid buffer containing 1% Triton X-100 and incubated overnight at 4°C. The lysates were centrifuged at 1000 rpm for 10 minutes to remove cell debris. Supernatants were transferred to fresh tubes and stored at −20°C.

Enzymatic assays were performed as described above for the standard sialyltransferase assay and incubated for 4 hours at 37°C with 10 mg/mL Asialofetuin as an acceptor substrate.

**Immunofluorescence.** The MAb and dilutions used for immunodetection of antigens Tn, Sialyl-Tn, and T are described above. Detection of antigen Sialyl-6T was done using either the anti-T antibodies HH8 or HB-T after neuraminidase treatment. Cell lines were fixed in cold acetone for 5 minutes before immunofluorescence. Samples designed for neuraminidase treatment were incubated with neuraminidase from C. perfringens type IV diluted in 0.1 mol/L sodium acetate buffer (pH 5.5) to a final concentration of 0.1 unit/mL. The incubation was carried out for 2 hours at 37°C and washed three times with cold water.

Immunofluorescence was performed as described previously (44), using different MAb diluted in Tris-buffered saline containing 5% of bovine serum albumin. As a secondary antibody samples were incubated with FITC-conjugated rabbit antimouse immunoglobulin (DakoCytomation, Glostrup, Denmark) diluted in 1:70 in Tris-buffered saline containing 5% of bovine serum albumin.

A semiquantitative approach was used to score the immunofluorescence labeling. Percentage of positive cells was scored as the following: <25%, 25%–50%, 50%–75%, and >75%, based on the analysis of ~1 × 10^4 cells. Intensity of immunofluorescence labeling was graded as: −, negative; +, weakly positive; ++, moderately positive; ++++, strongly positive. Results are based on three independent assays analyzed by two independent observers.

**Fluorescence-Activated Cell-Scanning Analysis.** The MAb and dilutions used for immunodetection by fluorescence-activated cell-scanning analysis (FACS) of antigens Sialyl-Tn and Sialyl-6T are listed above. Detection of antigen Sialyl-6T was done using the anti-T antibody HH8 after neuraminidase treatment of the cells. Confluent cells were trypsinized and washed twice with PBS. After being centrifuged at 1,000 rpm for 10 minutes, cells were resuspended at a concentration of 2 × 10^6 cells/mL in PBS. Aliquot cells of 1 mL were fixed in 70% EtOH at 4°C for 15 minutes. After washing with PBS, samples designed for neuraminidase treatment were incubated as described above for immunofluorescence. The incubation was carried out for 1 hour at 37°C and washed with cold PBS. Cells were incubated with MAb diluted 1:2 in PBS for 1 hour on ice. The cells, once washed with PBS, were stained with FITC-conjugated AfinityPure immunoglobulin antitoxin IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:50 in PBS and then subjected to FACS in a Counter Epics XL-MCL (Coulter).

**RESULTS**

Expression and Purification of Secreted Recombinant ST6GalNAc-I and ST6GalNAc-II. Insect Sf9 cells were transfected with each of the vectors, pAcGP67/ST6GalNAc-I (sol) or pAcGP67/ST6GalNAc-II (sol), and recombinant baculoviruses were obtained after four successive amplifications in Sf9 cells. Soluble ST6GalNAc-I and ST6GalNAc-II have been produced in HighFive and Sf9 cells. We have found that the HighFive cells produced 3-fold higher amounts of the two enzymes (data not shown). Protein production was monitored for 4 days after infection of the cells by Western blot using an anti-myc tag antibody and by enzyme activity. It was found that the amount of active protein secreted increased until the third day after infection; after this period of time no significant increase was observed. Thus, the supernatants were collected on the third day after infection. The large-scale expression of recombinant sialyltransferases in serum-free medium yielded 1.2 to 2.0 units/500 mL in shaker flasks. Recombinant soluble ST6GalNAc-I and ST6GalNAc-II have been partially purified by a combination of ion exchange chromatography and concentration in size-exclusion devices with final recoveries of 39% for ST6GalNAc-I and 37% for ST6GalNAc-II (Table 1); the purified enzymes had activities of 79.6 and 52.5 mM/mL, respectively. They appeared in SDS-PAGE gels after staining with Coomassie Blue R-250 at apparent molecular masses of 65 kDa and 39 kDa for ST6GalNAc-I and ST6GalNAc-II, respectively (Fig. 1). These results agreed with the predicted molecular masses calculated from their primary amino acid sequences. The proteins stained positively with the anti-myc tag antibody indicating that they have not been proteolytically cleaved at their COOH-terminus.

**Substrate Specificity of ST6GalNAc-I and ST6GalNAc-II.** Comparison of the acceptor substrate specificity of ST6GalNAc-I and ST6GalNAc-II toward a panel of glycoconjugates is shown in Table 2. ST6GalNAc-I and ST6GalNAc-II efficiently mediated the transfer of sialic acid to O-linked oligosaccharide of fetuin and asialofetuin. On the contrary, synthetic glycoconjugates such as GalNAc1-O-polyacylamide and Galβ1–3GalNαc1–O-polyacylamide, and arylglycosides, such as GalNαc1–O-Bn, GalNαc1–O-pNP, and Galβ1–3GalNαc1–O-Bn (Table 2), did not serve as acceptor substrate for the soluble ST6GalNAc-I and ST6GalNAc-II. Glycopeptides containing different amino acid sequences, lengths, number, and density of potential sites for sialylation were used as acceptor substrates. ST6GalNAc-I and ST6GalNAc-II showed some incorporation in the glycopeptide derived from the intestinal mucin MUC2, containing multiple potential acceptor sites (Table 2), and also to the glycopeptide derived from MUC4, with a single acceptor site. Low activities were observed toward all of the glycopeptides derived from the mucin MUC1. The highest activity was observed toward glycopeptides derived from the glycoprotein 120 from the HIV (HIV120gp). Two different designs of the HIV120 glycopeptides, HIV120gp (21-mer) and HIV120gp (17-mer), containing the single threonine glycosylated with GalNAc but encompassing different NH2- and COOH-terminal sequences were tested. Both
ST6GalNAc-I and ST6GalNAc-II showed identical rates of incorporation toward HIV120gp (17-mer; Table 2).

The kinetic parameters of the recombinant human ST6GalNAc-I and ST6GalNAc-II were determined using CMP-Neu5Ac as the donor substrate and asialofetuin as acceptor substrate. The $K_m$ value of CMP-Neu5Ac for ST6GalNAc-I and ST6GalNAc-II was 108 μmol/L ($V_{max}$ 16.41 pmol/hour/μL) and 154 μmol/L ($V_{max}$ 12.42 pmol/hour/μL), respectively. ST6GalNAc-I and ST6GalNAc-II showed apparent $K_m$ values for asialofetuin of 81 μmol/L ($V_{max}$ 5.87 pmol/hour/μL) and 242 μmol/L ($V_{max}$ 9.84 pmol/hour/μL).

Characterization of Product Development by Enzyme-Linked Immunosorbent Assay. The immunochemical characterization of the products of the MUC2 glycopeptide sialylated with either ST6GalNAc-I or ST6GalNAc-II showed that both enzymes induced the synthesis of the Sialyl-Tn structure. The anti-Sialyl-Tn monoclonal antibody reacted with the MUC2 glycopeptide sialylated with either ST6GalNAc-I or ST6GalNAc-II (Fig. 2A). Neuraminidase treatment of the MUC2 glycopeptide sialylated with either ST6GalNAc-I or ST6GalNAc-II abolished the Sialyl-Tn immunoreactivity (Fig. 2A) and re-established the Tn immunoreactivity (Fig. 2B).

Characterization of Gastric Cell Lines Transfected with Full-Length ST6GalNAc-I and ST6GalNAc-II. The MKN45 cell line was stably transfected with the plasmids pcDNA3.1/ST6GalNAc-I (full) or pcDNA3.1/ST6GalNAc-II (full), respectively. Transfected cells were cloned by the limiting dilution method. Clones were selected by mRNA expression. As shown in Fig. 3, analysis of clones from MKN45 transfected with ST6GalNAc-I (Fig. 3A) or ST6GalNAc-II (Fig. 3B) showed that clone 5 of MKN45-ST6GalNAc-I and clone 2 of MKN45-ST6GalNAc-II had the highest mRNA expression. The clones showing the highest mRNA expression were also the ones with the highest sialyltransferase activity as measured by the standard assay using asialofetuin as acceptor substrate (Fig. 3C). Clone 5 of ST6GalNAc-I and clone 2 of ST6GalNAc-II will be referred from this point on as MKN45-ST6GalNAc-I and MKN45-ST6GalNAc-II, respectively.

Characterization of carbohydrate antigen profile was performed on the MKN45-ST6GalNAc-I and MKN45-ST6GalNAc-II, as well as on the mock-transfected cells, and results are summarized in Table 3. Lower expression of Tn antigen was observed by immunocytochemistry in MKN45-ST6GalNAc-I when compared with MKN45-Mock. The expression of T antigen was negative for all of the cells. The expression of Sialyl-Tn (Neu5Ac α2–6GalNAc-O-Set/Thr) and Sialyl-6T [Gal β1–3 (Neu5Ac α2–6) GalNAc-O-Set/Thr] antigen was determined by immunofluorescence (Fig. 4; Table 3) and FACS analysis (Fig. 4). MKN45-ST6GalNAc-I showed high levels of expression of Sialyl-Tn in >75% of the cells as shown in Fig. 4, A and B. MKN45-ST6GalNAc-I also showed low levels of expression of Sialyl-6T in <25% of the cells (Fig. 4, E and H). On the contrary, MKN45-ST6GalNAc-II showed high levels of expression of Sialyl-6T in 50%–75% of the cells (Fig. 4, B and G).
F and H) and also showed low levels of expression of Sialyl-Tn in <25% of the cells (Fig. 4, C and G). Analysis of other clones transfected with either ST6GalNac-I or ST6GalNac-II revealed the same patterns of expression of carbohydrate antigens, although in lower amounts, proportional to mRNA expression levels of each enzyme (data not shown). In all of the experiments MKN45-Mock showed the same results as MKN45wt.

**DISCUSSION**

The synthesis of the cancer-associated simple mucin-type carbohydrate antigen Sialyl-Tn (Neu5Ac\(^{-6}\)GalNAc-O-Ser/Thr) depends on the activity of an \(\alpha_{2,6}\)-sialyltransferase that catalyzes the transfer of sialic acid from CMP-Neu5Ac to GalNAc \(\alpha\)-linked on proteins. The initial step of mucin \(O\)-glycosylation is the linkage of the initial

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**Table 3** Expression of carbohydrate antigens in MKN45 cells mock-transfected and transfected with ST6GalNAc-I and ST6GalNAc-II

<table>
<thead>
<tr>
<th>Intensity*localization</th>
<th>Percentage of positive cells</th>
<th>Intensity*localization</th>
<th>Percentage of positive cells</th>
<th>Intensity*localization</th>
<th>Percentage of positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tn</strong></td>
<td>MKN45-Mock</td>
<td>MKN45-ST6GalNAc-I</td>
<td>MKN45-ST6GalNAc-II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+/D,M</td>
<td>50–75</td>
<td>+/+/D,M</td>
<td>25–50</td>
<td>+/+/D,M</td>
<td>50–75</td>
</tr>
<tr>
<td>Sialyl-Tn</td>
<td>NA</td>
<td>+/+/M,PN</td>
<td>&gt;75</td>
<td>+/M,PN</td>
<td>&lt;25</td>
</tr>
<tr>
<td>T</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sialyl-6T</td>
<td>NA</td>
<td>+/+/M,PN</td>
<td>&gt;75</td>
<td>+/M,PN</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

* Intensity grading: -, negative; +, weakly positive; ++, moderately positive; ++++, strongly positive.

† Sialyl-6T antigen was evaluated using anti-T antigen antibodies after neuraminidase treatment as described in Materials and Methods.

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Fig. 4. Analysis of the expression of Sialyl-Tn and Sialyl-6T in MKN45 cells transfected with ST6GalNAc-I or ST6GalNAc-II. Detection of Sialyl-Tn was done using MAb TKH2, and detection of Sialyl-6T was done using the anti-T MAb HH8 after neuraminidase treatment. MKN45-ST6GalNAc-I cells showed high levels of expression of Sialyl-Tn in >75% of the cells (B) and low levels of expression of Sialyl-6T in <25% of the cells (E). MKN45-ST6GalNAc-II cells showed low levels of expression of Sialyl-Tn in <25% of the cells (C) and high levels of expression of Sialyl-6T in 50–75% of the cells (F). The control MKN45-Mock cells expressed no positive cells for Sialyl-Tn (A) and Sialyl-6T (D). Images were obtained at ×400 magnification. Flow cytometry analysis of the expression of Sialyl-Tn (G) and Sialyl-6T (H) was evaluated in clones ST6GalNAc-I (dark filled peak), ST6GalNAc-II (light filled peak), Mock (dark line), and negative control (dotted line). ST6GalNAc-I- and ST6GalNAc-II-transfected clones showed 88% and 15% of Sialyl-Tn-positive cells, respectively. In contrast, ST6GalNAc-I- and ST6GalNAc-II-transfected clones showed 17% and 65% of Sialyl-6T-positive cells, respectively.
GalNAc residue to serine or threonine residues of the polypeptide under the control of polypeptide:GalNAc transferase(s) (45). Once synthesized, GalNAc-O-Ser/Thr can be additionally processed by different glycosyltransferases forming different core structures such as Core1 (Galβ1–3GalNAcα-O-Thr/Ser) and Core2 [GlcNAcβ1–6 (Galβ1–3)GalNAcα-O-Thr/Ser]. Alternatively, GalNAcα-O-Ser/Thr can be sialylated leading to formation of the Sialyl-Tn antigen, which cannot be additionally processed and, therefore, stops elongation of the O-linked oligosaccharide chain (46).

The characterization of the human sialyltransferases using acceptor substrate glycopolymers such as fetuin, asialofetuin, agalactoasialofetuin, and other substrates has proposed that ST6GalNAc-I and ST6GalNAc-II have similar substrate specificity (31, 32). The main difference in acceptor substrate specificity was described for the Tn antigen (GalNAcα-O-Thr/Ser); however, some controversy still persists (31–33).

To identify the role of the human ST6GalNAc-I and ST6GalNAc-II in the synthesis Sialyl-Tn antigen we have produced active recombinant human ST6GalNAc-I and ST6GalNAc-II, which were purified with high yields and with few contaminants (Table 1; Fig. 1). Analysis in vitro showed that both enzymes efficiently mediated the transfer of sialic acid to O-linked oligosaccharides of fetuin and asialofetuin (Table 2). Both ST6GalNAc-I and ST6GalNAc-II sialylated the intestinal MUC2 mucin glycopeptide (Table 2) forming the Sialyl-Tn antigen as monitored by ELISA (Fig. 2). ST6GalNAc-I and ST6GalNAc-II showed no activity toward the synthetic glycoconjugates and arylglycosides. Theses results are in agreement to those reported previously for both enzymes (31, 32) and differ from other glycosyltransferases that use arylglycosides and polyacrylamide acceptor substrates (36, 47, 48). These observations support the hypothesis that the aglycone part in the acceptor substrate is crucial for the in vitro activity of both enzymes.

Detailed analysis of substrate specificity of recombinant ST6GalNAc-I and ST6GalNAc-II using a panel of GalNAc-glycopeptides containing different amino acid sequences, lengths, number, and density of potential sites for sialylation showed marked differences in rate of incorporation between different glycopeptides. Both enzymes showed some activity toward the glycopeptide derived from the intestinal mucin MUC2 containing multiple potential acceptor sites and also to the glycopeptide derived from MUC4 with a single acceptor site. Low activities were observed toward glycopeptides derived from the membrane-associated mucin MUC1 independent from the number and density of potential acceptor sites. The highest sialyltransferase activities of ST6GalNAc-I and ST6GalNAc-II were observed toward both glycopeptides derived from the glycoprotein 120 from HIV. We tested two different designs of the HIV120 glycopeptides, HIV120gp (21-mer) and HIV120gp (17-mer), containing the same single threonine substituted with GalNAcα-O-Thr but encompassing slightly different NH2- and COOH-terminal sequences. Both ST6GalNAc-I and ST6GalNAc-II showed identical rates of incorporation toward HIV120gp (21-mer) and half-fold rates of incorporation toward HIV120gp (17-mer; Table 2). Taken together, our observations show for the first time that apart from ST6GalNAc-I, the human ST6GalNAc-II is also capable of synthesizing the Sialyl-Tn antigen in vitro. The detailed analysis of our results also allowed us to conclude that among the characteristics of the acceptor substrate that could influence the enzyme activity (such as number/density of acceptor GalNAc and length of the peptide), only the primary sequence of the glycopeptides appears to be a determinant for the substrate specificity of the enzymes.

Glycosyltransferases are arranged in the Golgi apparatus in a spatial and functional hierarchy. We cannot rule out that, in vivo, activities shown by ST6GalNAc-I and ST6GalNAc-II may be influenced from different intra-Golgi localization of the two enzymes. In fact, previous studies have shown that cell lines lacking Sialyl-Tn expression may still show low levels of ST6GalNAc-I mRNA (44). This is the case of the gastric carcinoma cell line MKN45, which shows low levels of ST6GalNAc-I and ST6GalNAc-II mRNA (Fig. 3, A and B), and shows no expression of Sialyl-Tn and Sialyl-6T (Table 3; Fig. 4). These observations may stem from substrate competition between the sialyltransferases with other glycosyltransferases, namely Core1Gal-T. Factors such as substrate affinity, enzyme intra-Golgi localization, and/or overexpression may explain these different glycosylation outcomes (19).

To elucidate if both human ST6GalNAc-I and ST6GalNAc-II may function in vivo as Sialyl-Tn synthases, we established a human gastric carcinoma cell line model transfected with the full length of either ST6GalNAc-I or ST6GalNAc-II. MKN45 was chosen according to the fact that this cell line does not express the Sialyl-Tn antigen (44). The transfected clones that expressed the highest amounts of mRNA transcripts of ST6GalNAc-I, clone 5 (Fig. 3A), and ST6GalNAc-II, clone 2 (Fig. 3B), also showed the highest sialyltransferase activity toward asialofetuin (Fig. 3C). ST6GalNAc-I clone 5 and ST6GalNAc-II clone 2 were used for additional analysis and designated as MKN45-ST6GalNAc-I and MKN45-ST6GalNAc-II, respectively.

A detailed characterization of antigenic profile of MKN45-ST6GalNAc-I and MKN45-ST6GalNAc-II using flow cytometry and immunocytochemistry showed altered carbohydrate expression. MKN45-ST6GalNAc-I showed high levels of expression of Sialyl-Tn in >75% of the cells and showed low levels of expression of Sialyl-6T in <25% of the cells (Table 3; Fig. 4). These results are in agreement with previous reports showing that the transfection with ST6GalNAc-I of HCT human colon cancer cells (31) and breast cancer cells (34) induced Sialyl-Tn expression. It is worth noticing that a decrease in Tn antigen was also observed, which is probably due to the processing of this structure for sialylation. Contrarily, MKN45-ST6GalNAc-II expressed high levels of Sialyl-6T in 50–75% of the cells and low levels of expression of Sialyl-Tn in <25% of the cells (Table 3; Fig. 4). The reproducibility of these observations was confirmed among different clones transfected with either ST6GalNAc-I or ST6GalNAc-II. Although the mRNA expression levels of these clones were lower than MKN45-ST6GalNAc-I or MKN45-ST6GalNAc-II, the pattern of expression of carbohydrate antigen expression was the same but in lower levels. This was observed for both enzymes and reflects directly the amounts of each enzyme present. These observations strongly support the hypothesis that ST6GalNAc-I is the main enzyme involved in the Sialyl-Tn biosynthesis, whereas ST6GalNAc-II is an enzyme involved in the synthesis of more complex structures such as Sialyl-6T. Therefore, these results show that the two enzymes play distinct roles in the biology of cells.

Our in vitro observations using GalNAc-glycosylated peptides allowed us to determine enzyme affinity for the Tn antigen substrate alone. Both ST6GalNAc-I and ST6GalNAc-II are active toward Tn antigen, forming the Sialyl-Tn antigen. The cell line model offers a diversity of substrates, resembling more closely the in vivo conditions, where both Tn and T antigens are available. Our observations show that ST6GalNAc-I synthesizes primarily Sialyl-Tn, whereas ST6GalNAc-II synthesizes primarily Sialyl-6T.

In conclusion, we have shown that both human ST6GalNAc-I and ST6GalNAc-II are capable of sialylating both Tn and T antigens but with clear differences in affinities toward each of these substrates. Although both enzymes show similar in vitro activities when Tn antigen alone is available, whenever both Tn and T antigens are present ST6GalNAc-I acts preferentially on Tn antigen, whereas ST6GalNAc-II acts preferentially on T antigen. Our results also
demonstrate that the amino acid sequence of the acceptor substrate, besides being a requirement for the sialyltransferase activity, is also a determinant for the substrate specificity of the enzymes. Our results show that ST6GalNAc-I is the major Sialyl-Tn synthase and strongly support the hypothesis that the expression of the Sialyl-Tn antigen in cancer cells is due to ST6GalNAc-I activity.

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