

The ST6GalNAc-I Sialyltransferase Localizes throughout the Golgi and Is Responsible for the Synthesis of the Tumor-associated Sialyl-Tn O-Glycan in Human Breast Cancer*

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The functional properties of glycoproteins are strongly influenced by their profile of glycosylation, and changes in this profile are seen in malignancy. In mucin-type O-linked glycosylation these changes can result in the production of mucins such as MUC1, carrying shorter sialylated O-glycans, and with different site occupancy. Of the tumor-associated sialylated O-glycans, the disaccharide, sialyl-Tn (sialic acid α 2,6GalNAc), is expressed by 30% of breast carcinomas and is the most tumor-specific. The ST6GalNAc-I glycosyltransferase, which can catalyze the transfer of sialic acid to GalNAc, shows a highly restricted pattern of expression in normal adult tissues, being largely limited to the gastrointestinal tract and absent in mammary gland. In breast carcinomas, however, a complete correlation between the expression of RNA-encoding ST6GalNAc-I and the expression of sialyl-Tn is evident, demonstrating that the expression of sialyl-Tn results from switching on expression of *hST6GalNAc-I*. Endogenous or exogenous expression of *hST6GalNAc-I* (but not *ST6GalNAc-II*) always results in the expression of sialyl-Tn. This ability to override core 1/core 2 pathways of O-linked glycosylation is explained by the localization of ST6GalNAc-I, which is found throughout the Golgi stacks. The development of a Chinese hamster ovary (CHO) cell line expressing MUC1 and ST6GalNAc-I allowed the large scale production of MUC1 carrying 83% sialyl-Tn O-glycans. The presence of ST6GalNAc-I in the CHO cells reduced the number of O-glycosylation sites occupied in MUC1, from an average of 4.3 to 3.8 per tandem repeat. The availability of large quantities of this MUC1 glycoform will allow the evaluation of its efficacy as an immunogen for immunotherapy of MUC1/STn-expressing tumors.

Post-translational modifications of proteins can strongly affect their function. In this context it is becoming clear that glycosylation plays an important role in eukaryotes, not merely in the stabilization of proteins *in vivo*, but in affecting cell-cell interactions and interactions with the matrix, as well as affecting the function of intracellular molecules such as transcription factors (1–3). In the development of malignancy, interactions of the cancer cell with other cells and matrices in different tissues are crucially important for survival and/or metastasis and interactions with immune effector cells can affect the immune response. In this context, cancer-associated changes in the glycosylation patterns of glycoproteins that transit the Golgi pathway and are either secreted or targeted to the membrane are of particular interest. In many carcinomas that express membrane and secreted mucins, changes in mucin-type O-glycosylation have been documented (4). In the change to malignancy in the breast, changes in the glycosylation of the MUC1 mucin has been extensively studied, following both the composition and density of the O-glycans added (5–8), and the profile of glycosyltransferases expressed (9–13). Because MUC1 is expressed by >90% of breast cancers, the carcinoma-associated glycoforms are being considered as targets both for exogenously administered antibodies (14) and for active specific immunotherapy using MUC1-based immunogens (15, 16).

Mucin type O-glycosylation is initiated by the addition of N-acetylgalactosamine (GalNAc) to serines and threonines, which generates the Tn antigen, and in the normal breast chains are extended by the addition of monosaccharides through the core 1/core 2 pathways. This results in the addition of linear and branched polylactosamine side chains to the core protein of MUC1 (see Fig. 1 and Ref. 8). In breast cancers and breast cancer cell lines, there is a shift toward the addition of shorter O-glycans, the degree to which this change is seen being dependent, to some extent, on the level of expression of the core 2 synthesizing enzyme, which can be totally absent (6, 9). In addition, sialylation of the shorter O-glycans is increased, thus terminating chain extension, and most breast cancers show an increase in the level of ST3Gal-I (10), which adds sialic acid to core 1 to give Neu5Ac α 2,3Gal β 1,3GalNAc or SialylT (Fig. 1). Although this O-glycan is found on many normal cells, including resting lymphocytes, it is not normally found on MUC1 in breast, and high expression is associated with aggressive disease and may be immune suppressive (10, 17).

Sialylation of the first sugar, GalNAc, leading to the product

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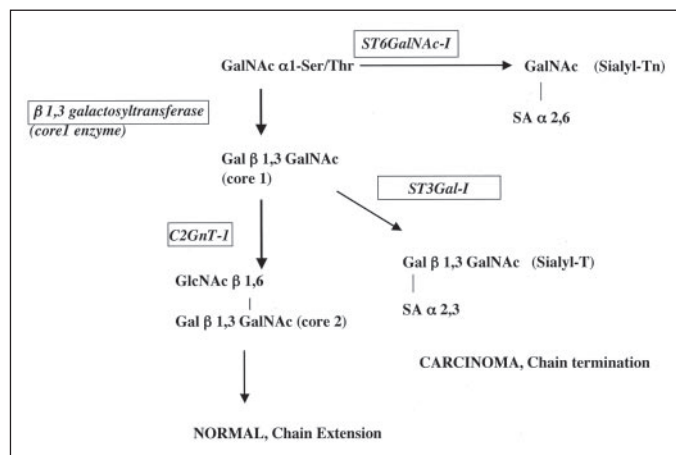


FIGURE 1. Pathways of O-glycosylation in normal breast tissue and carcinoma. O-Glycan structures on the normal epithelial cells of the breast are mainly core 2-based resulting from the action of C2GnT1 on core 1 O-glycans. In breast carcinoma shorter O-glycans are added, and sialylation of GalNAc and/or core 1 by ST6GalNAc-I or ST3Gal-I, respectively, results in chain termination. Although some breast cancers and breast cancer cell lines lose the C2GnT1 enzyme, others continue to express this glycosyltransferase.

Neu5Ac α 2,6GalNAc or Sialyl-Tn (STn)⁶ is a less common event, and immunohistochemical analysis detected this O-glycan on 25–30% of breast cancers (18, 19). Expression of STn in breast cancer correlates with poor prognosis (20) and predicts a poor response to chemotherapy (19), whereas expression of STn on normal tissues is highly restricted. Thus the STn O-glycan has been seen as a possible target for therapeutic intervention (21–24). In human cells, the enzyme ST6GalNAc-I has been identified as a glycosyltransferase able to add sialic acid in α 2,6 linkage to GalNAc linked to serine or threonine, thus creating the STn epitope (25). Other sialyltransferases active in the pathway of mucin type O-glycosylation, including hST6GalNAc-II, can add sialic acid in α 2,6 linkage to GalNAc *in vitro* (26) but show a preference for GalNAc residues already modified by the addition of galactose or galactose followed by sialic acid in α 2,3 linkage (26, 27).

Although STn has a highly restricted expression on normal tissues (28–30), it is found on several types of carcinomas (19, 28, 31). The mechanisms underlying the appearance of this O-glycan are not totally clear and may vary with the tissue. One obvious mechanism is an increase in the level of activity of the enzyme(s), which adds sialic acid to GalNAc. Studies in colon carcinoma have demonstrated that this is not the case in this malignancy (32), because the level of α 2,6-sialyltransferase enzyme activity is similar in normal and cancerous colonic tissue, whereas STn is only detectable in the cancers. The difference in colon carcinoma appears to be due to a lack of detection of the STn on most normal tissue, because of a modification of the sialic acid resulting in it not being recognized by the STn-specific antibodies. However, as we report here, although the hST6GalNAc-I mRNA, which synthesizes STn, is found in normal tissues of the gastrointestinal tract, it is not present in the normal breast. Therefore there is a strong possibility that the expression of STn in breast cancer relates to the *de novo* expression of hST6GalNAc-I.

Here we have examined this hypothesis and find a complete correlation between the expression of the STn epitope in breast cancers and the expression of hST6GalNAc-I mRNA. Significantly, there was no correlation of STn expression with expression of hST6GalNAc-II mRNA. Transfection studies show that expression of hST6GalNAc-I can override the core 1/core 2 pathway in breast cancer cell lines and that the enzyme appears to be found throughout the Golgi. Thus stable expression of the enzyme has allowed the production of large quantities of a MUC1 glycoform carrying STn as the dominant O-glycan.

MATERIALS AND METHODS

Cell Culture—The breast cancer cell line T47D was cultured in E4, 10% FCS, and the E2J cell line, derived from the transfection of T47D with C2GnT-1 (13), was cultured in E4, 10% FCS with 500 μ g/ml G418. The myelogenous leukemia cell line, K562, was cultured in RPMI, 10% FCS. CHO MUC1 cells were developed by transfection of CHO cells with a MUC1-murine IgG2a fusion cDNA construct containing 16 tandem repeats (36) and were initially cultured in Iscove's modified Dulbecco's medium, 10% FCS with 600 μ g/ml G418. For large scale production, they were adapted to serum-free medium (see below).

Cloning of hST6GalNAc-I by RT-PCR—Total cellular RNA was prepared from K562 cells using TRIzol (Invitrogen) as per the manufacturer's instructions. First strand cDNA synthesis was performed with oligo(dT) primers from 3 μ g of RNA in a 20- μ l reaction volume using a superscript pre-amplification system (Invitrogen). The amplification of cDNA was performed with the Expand Long template PCR system (Roche Applied Science) in a total volume of 50 μ l containing 0.3 μ M of a primer set, 5'-GCTAGCCGACCCACCACCATGAGG-3' (corresponding to bp (–12 to –6) of the ST6GalNAc-I sequence) and 5'-CTCAGTCAATTACCAAGACGATTCATTTCAATATCAGT-ATAAAGTTGACGATTGGGCGGGTCTTGGCTTTGGCAGTT-C-3' (corresponding to bp 1761–1800 of the ST6GalNAc-I sequence). The 5'-end of the published cDNA sequence (accession number Y11339 (25)) was found to disagree with the genomic ST6GalNAc-I (accession number AC005837). The sequence of the 5'-primer was taken from the genomic sequence, because use of the published 5' cDNA sequence did not result in a product. The primers were designed to generate NheI and XhoI sites at the 5'- and 3'-ends, respectively. The 3'-oligonucleotide also generates a mutated stop codon in the ST6GalNAc-I sequence followed by 2 proline residues, the vsv-g tag sequence, and a TGA stop codon. PCR buffer 1 was used at a final MgCl₂ concentration of 1.75 mM. After initial denaturation for 3 min at 94 °C, 30 cycles of PCR were performed, each cycle comprising 30 s at 94 °C, 30 s at 60 °C, and 2 min at 68 °C. The PCR product was cloned into pCR2.1 using the Original TA cloning kit (Invitrogen), sequenced and subcloned into pcDNA3.1-hygro (Invitrogen) using NheI/XhoI restriction sites.

Cell Transfections—Cells were transiently transfected with 2 μ g of ST6GalNAc-I-vsv or ST6GalNAc-II-FLAG expression plasmids using 10 μ l of the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. T47D-ST3 (12) and CHO MUC1 cells were stably transfected with 30 μ g of ST6GalNAc-I-vsv-g pcDNA3.1hygro vector by electroporation using the following conditions: T47D-ST3, 250 V at 960 microfarads and CHO MUC1 450 V at 250 microfarads. Three days post-transfection, cells were split 1:10 into E4, 10% FCS with 500 μ g/ml G418 and 300 μ g/ml hygromycin (T47D-ST3-STn) and 1:20 into Iscove's modified Dulbecco's medium, 10% FCS with 600 μ g/ml hygromycin, and 600 μ g/ml G418 (CHO MUC1 STn). Media was changed every 3 days until selection of clones was isolated and expanded.

⁶ The abbreviations used are: STn, Sialyl-Tn; T, Thomas-Friedreich antigen; ST6GalNAc, CMP-Neu5Ac:GalNAc-R α 2,6-sialyltransferase; ST3Gal-I, CMP-Neu5Ac:Gal β 1,3GalNAc-R α 2,3-sialyltransferase; C2GnT1, UDP-N-acetyl- α -D-glucosamine- β Gal/ α GalNAc β 1,6-N-acetylglucosaminyltransferase; core 1 enzyme, core 1 β 1,3-galactosyltransferase; PNA, peanut agglutinin; vsv-g, vesicular stomatitis-g protein; KLH, keyhole limpet hemocyanin; CHO, Chinese hamster ovary cells; FITC, fluorescein isothiocyanate; LC-ESI, liquid chromatography-electrospray ionization; MS, mass spectrometry; FCS, fetal calf serum.

Immunofluorescence Staining of Cultured Cells—Cells on coverslips, fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized by 0.1% Triton-X for 5 min at room temperature, were washed and then incubated with the mouse monoclonal antibody TKH2 (reactive with STn) or with the anti-vsv-g antibody (P5D4) for 1 h at room temperature. Following three washes in phosphate-buffered saline, rabbit anti-mouse FITC (Dako) was added for 1 h at room temperature at a dilution of 1:40. For the detection of FLAG-tagged ST6GalNAc-II expression, cells were incubated with a FITC-labeled FLAG-M2 antibody (Sigma) for 1 h at room temperature. The cells were washed, mounted in CITIFLUOR and viewed on a fluorescence microscope.

Flow Cytometric Analysis—Cells were stained with monoclonal antibody SM3 (MUC1) or TKH2, 30 min on ice, washed, and incubated with FITC-conjugated rabbit anti-mouse immunoglobulins (Dako) for 30 min on ice. When biotinylated antibodies were used, binding was detected with streptavidin-FITC (Dako). For internal staining of the vsv-g tag, cells were incubated on ice with the anti-vsv-g antibody (P5D4) in 0.1% saponin for 30 min followed by FITC-conjugated rabbit anti-mouse immunoglobulins (Dako) for 30 min on ice. Cells were stained with peanut agglutinin before and after sialidase treatment as previously described (17). Cells were fixed in 1% formaldehyde and analyzed using an XL Flow Cytometer (Beckman-Coulter, High Wycombe, UK) and WinMDA software. Dead cells were excluded on the basis of forward and side light scatter.

Immunoelectron Microscopy—Cells were fixed for 1 h at room temperature in 0.1% glutaraldehyde/4% paraformaldehyde before being scraped, spun down, and stored overnight in 2% paraformaldehyde at 4 °C. The grids were incubated overnight at 4 °C with 20 μ g/ml P5D4 (reactive with the vsv-g tag), followed, after washing, with rabbit anti-mouse IgG1 for 30 min. After further washing, grids were incubated at room temperature for 30 min with protein A coupled to 10-nm gold particles. The grids were embedded in 1.8% methyl cellulose/0.4% uranyl acetate before examination using a Jeol 1010 transmission electron microscope with a Gatan 2K digital camera for image capture.

RT-PCR of Glycosyltransferases—The generation of cDNA was performed as described above for K562 cells. The cDNA was amplified using 2.5 units of Taq polymerase in a total volume of 50 μ L. The following oligonucleotides were used for each glycosyltransferase: Core 1, β 1,3-galactosyltransferase-specific primers; 5'-ACAGAAATACACTTTCGGGAAATG-3' and 5'-TCAAGGATTCCTAAGTTCACCTT-3'. These primers generate the full-length cDNA resulting in the amplification of a 1112-bp fragment. The C2GnT-1-specific primers used were 5'-ATGCTGAGGACGTTGCTG-3' and 5'-TCAGTGTTTAAATGTCTCCAA-3'. These primers generate the full-length C2GnT-1 cDNA, amplifying a 1286-bp fragment. ST6GalNAc-I oligonucleotides used were 5'-AAAGGAGTGACCACAGCAG-3' and 5'-TCAGTTCTTGGCTTTGGCAGTTC-3'. These primers generate a 1155-bp fragment, which represents the message for the active full-length form of ST6GalNAc-I. The MUC1-specific oligonucleotides used were 5'-CTTGCCAGCCATAGCACCAAG-3' and 5'-CTCCACGTCGTGACATTGATG-3'. These primers result in the amplification of a region between the end of exon 2 and the beginning of exon 4 and would result in the generation of a 341-bp fragment from RNA and a 590-bp fragment from genomic cDNA. PCR samples were run on an agarose gel containing 1 μ g/ml ethidium bromide, and the gel was viewed under a long wave UV transilluminator.

Northern Blotting—Total RNA of primary breast carcinoma tissue was generated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions following mechanical disaggregation of samples

using a dismembrator on dry ice. Northern blots were performed as previously described (13). ST6GalNAc-I mRNA was detected using a full-length probe, and ST6GalNAc-II mRNA was detected using a 1-kb HindIII-XbaI fragment. ST6GalNAc-I mRNA expression in normal human tissues was analyzed on a multiple tissue expression array (Clontech, Basingstoke, UK) containing normal human tissue poly(A)+ RNA, using the above full-length ST6GalNAc-I probe.

Western Blotting—CHO MUC1 STn cell culture supernatants were collected for analysis of the secreted MUC1 protein. 20 μ L of supernatant was separated on a 10% SDS-polyacrylamide gel and then blotted onto nitrocellulose membrane overnight. The blot was blocked overnight at 4 °C in phosphate-buffered saline, 0.2% Tween 20, 1% bovine serum albumin (PTB). For detection of the STn-carrying glycoprotein, the blot was incubated at room temperature for 1 h with biotinylated TKH2 (4 μ g/ml) in PTB. Following three washes in PTB the blot was incubated at room temperature for 1 h with streptavidin-horseradish peroxidase (1/1500 dilution in PTB). For the detection of total MUC1-IgG in the supernatants, the blot was incubated directly with rabbit anti-mouse serum (1/1500 dilution in PTB). After three additional washes the bound antibody-streptavidin horseradish peroxidase complex was detected using enhanced chemiluminescence (ECL) and light-sensitive film.

Large Scale Production of MUC1-IgG-STn—CHO MUC1-ST6GalNAc-I cells were grown in suspension in the serum-free medium ProCHO4-CDM (BioWhittaker Europe). Continuous perfusion fermentations with cell retention by spin filtration were done in a standard stirred vessel bioreactor with a working volume of 1L (Applicon Biotek, Knuellwald, Germany) under controlled conditions as described elsewhere (37). Cells were removed from supernatant by filtration through a Sartobran 300 capsule with a 0.2- μ m cut-off (Sartorius, Gottingen, Germany), and the cell-free supernatant was concentrated by ultrafiltration and rebuffed in 200 mM Tris/HCl buffer, pH 7.2, using an Ultrasette tangential flow device (Pall, Dreieich, Germany) with a 100-kDa cut-off. The whole downstream processing was done at a temperature of 0–4 °C.

O-Glycan Structure and Site Occupancy Determination by Liquid Chromatography-Electrospray Ionization Mass Spectrometry—O-glycans from 20 μ g of purified MUC1-IgG were released by reductive β -elimination and analyzed by LC-ESI MS (36). The relative amounts of the different oligosaccharides were obtained from the integrated peak areas in the chromatogram corresponding to each of the molecular ions (Neu5Ac α 2–6)GalNAc α 1 (m/z 513), Neu5Ac α 2–3Gal β 1–3GalNAc α 1 and Gal β 1–3(Neu5Ac α 2–6)GalNAc α 1 (m/z 675), and Neu5Ac α 2–3Gal β 1–3(Neu5Ac α 2–6)GalNAc α 1 (m/z 966 and 483). The occupancy of the O-glycosylation sites in MUC1 was determined as described previously (36), except that the treatment with *Vibrio cholerae* neuraminidase was performed at pH 4.5–5.0 and the glycopeptides generated by Clostripain digestion were analyzed by nano-LC-ESI MS using a Finnegan LTQ mass spectrometer (Thermo Electron, Waltham, MA). Integration of peaks in mass chromatograms was performed using XCalibur software.

RESULTS

Expression of the STn O-Glycan Correlates with Expression of hST6GalNAc-I but Not with hST6GalNAc-II in Primary Breast Cancer—Unlike the situation with cells of the normal gastrointestinal tract, mRNA coding for the hST6GalNAc-I enzyme is not expressed in the normal human mammary gland (Fig. 2, G9).

To examine the correlation between STn expression and the expression of hST6GalNAc-I, we analyzed a series of 29 primary breast cancers

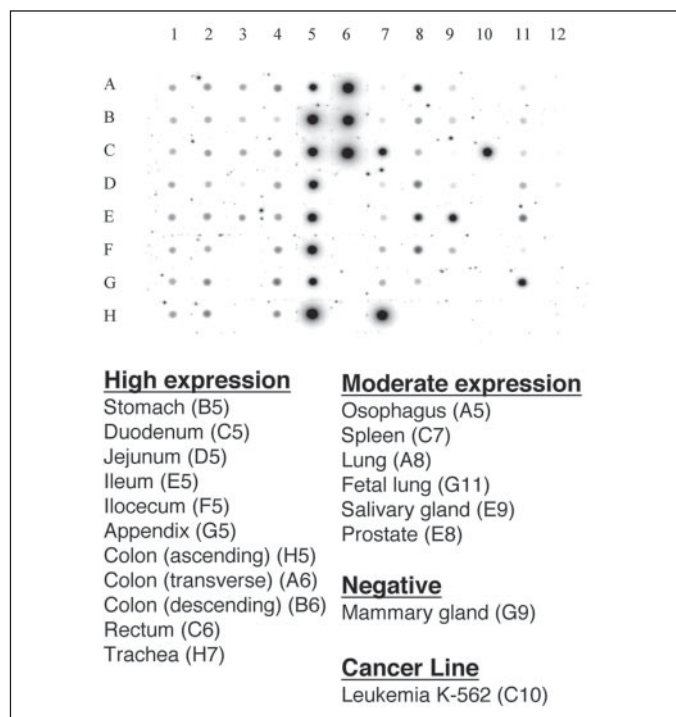


FIGURE 2. *ST6GalNAc-I* mRNA shows a restricted pattern of expression in human tissues. A dot blot of human tissues was analyzed using a full-length *hST6GalNAc-I* probe. Tissues expressing high and moderate levels of *hST6GalNAc-I* are listed. Normal mammary gland (G9) is negative for *ST6GalNAc-I* mRNA.

TABLE 1

Correlation between expression of STn and *ST6GalNAc-I* in primary breast cancers

Case number	STn status	<i>ST6GalNAc-I</i> Northern analysis	<i>ST6GalNAc-I</i> RT-PCR analysis
26	+	—	+
23	+	—	+
27	+	—	+
4	+	—	+
1	++	—	+
28	+++	+	NA ^a
29	+++	+	NA
22	+++	+	+
10	+++	+	+
24	+++	+	+
13, 3, 20, 2, 12, 14	—	—	—
21, 18, 7, 5, 19, 9, 8	—	—	—
15, 16, 6, 11, 17, 25	—	—	—

^a NA, tissue not available.

for expression of *hST6GalNAc-I* mRNA and of STn. Immunohistochemical analysis of the breast cancers was previously performed using the monoclonal antibody TKH2, which recognizes single as well as clustered STn O-glycans (38). Of the 29 breast carcinomas analyzed, 10 were found to express STn (Table 1). Six were highly positive while four cases showed weaker staining. Using Northern blot analysis and a full-length probe (Fig. 3a), it appeared that some of the tumors expressing STn did not express detectable *hST6GalNAc-I* message. These were tumors showing a weaker expression of STn (see Table 1), but by using RT-PCR, expression of the *hST6GalNAc-I* mRNA was detected (Fig. 3b, upper panel).

Two isoforms of the *hST6GalNAc-I* mRNA have been described, a functional full-length form and a shorter form, which has 250 bp of sequence deleted and codes for an inactive protein (25). As the Northern analysis was performed with a full-length *hST6GalNAc-I* probe,

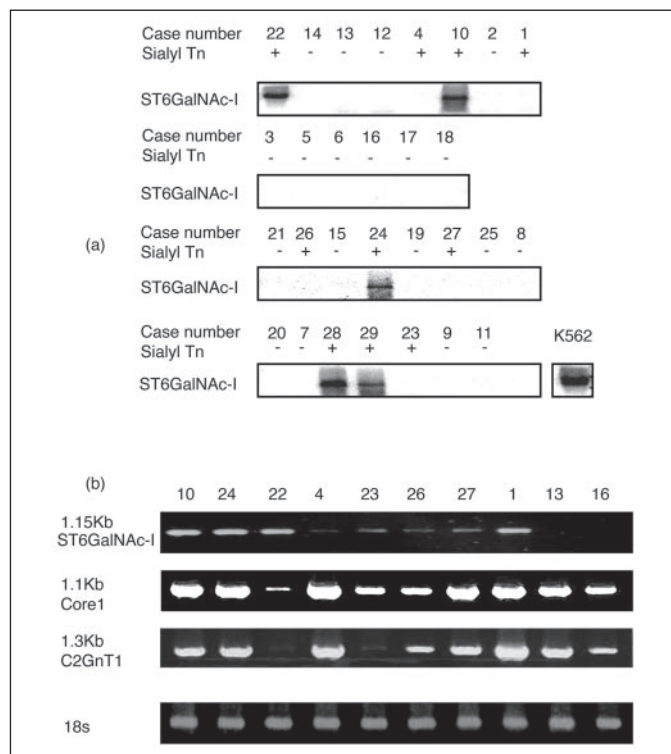


FIGURE 3. *ST6GalNAc-I* mRNA correlates with STn expression in primary breast cancers. a, Northern blot analysis of 29 breast carcinomas using a full-length *ST6GalNAc-I* probe. K562 was used as a positive control. STn status is recorded as + or - for each case, determined by immunohistochemical staining with antibodies to STn. b, RT-PCR analysis of STn-positive breast carcinomas detecting the functional form of *ST6GalNAc-I* (upper panel), core 1 β 1,3-GalT (second panel), and C2GnT1 (third panel) was performed. Cases 13 and 16 are STn-negative and were used as negative controls as they had previously been shown not to express *ST6GalNAc-I* by RT-PCR.

which would not discriminate between the two transcripts, primers for RT-PCR analysis of *hST6GalNAc-I* were designed to detect the long form of the message (see "Materials and Methods"). This gave rise to a 1.15-kb PCR product that represents the message coding for the active form of *hST6GalNAc-I* (see Fig. 3b, upper panel). All of the STn-positive tumors were found to express the full-length transcript, whereas none of the tumors that showed negative staining with the TKH2 expressed *hST6GalNAc-I* mRNA (Cases 28 and 29 were not analyzed because tumor samples were no longer available). The combined data from both the Northern and RT-PCR analysis demonstrate a complete correlation between *hST6GalNAc-I* expression and expression of STn in primary breast cancer (Table 1).

In breast, the normal pathway for O-glycan synthesis follows the core 1/core 2 route (see Fig. 1), with shorter core 1-based O-glycans being more dominant in many breast cancers (6, 11). RT-PCR analysis of STn-positive and -negative breast cancers showed that cDNAs coding for the core 1-synthesizing enzyme (Fig. 3b, second panel) and the core 2-synthesizing enzyme C2GnT1 (Fig. 3b, third panel) were present at various levels. This implies that *hST6GalNAc-I*, when present, can compete with core 1 for the GalNAc substrate, at least to some extent, to synthesize the STn O-glycan.

Although both the mouse and human *ST6GalNAc-II* have been shown to synthesize STn *in vitro* (26, 39), a secreted form of human *ST6GalNAc-II* apparently did not synthesize this disaccharide (27). To analyze the expression of the human *ST6GalNAc-II* enzyme in breast cancer 8 cases were selected from the 29 analyzed for expression of STn, representing tumors with high, low, or no expression of this O-glycan. (see Table 1). As illustrated in Fig. 4a, Northern blot analysis showed

FIGURE 4. ST6GalNAc-II does not correlate with STn expression in breast cancer and does not direct synthesis of STn in transfected cells. *a*, Northern blots using a 1-kb *ST6GalNAc-II* cDNA probe for RNA extracted from primary breast cancers (upper panel), and breast cell lines (lower panel). K562 was used as a control for a cell line expressing the STn O-glycan. *b*, T47D cells were transiently transfected with either myc-tagged *hST6GalNAc-I* cDNA (upper panels A–E) or FLAG-tagged *hST6GalNAc-II* cDNA (lower panels F–J). 48 h later, cells were fixed and stained with an anti-myc antibody (panels A and C for *ST6GalNAc-I* transfectants), an anti FLAG antibody (panels F and H for *ST6GalNAc-II* transfectants), or the TKH2 antibody recognizing the STn O-glycan (panels E and J). Panels B, D, G, and I show the phase contrast corresponding to panels C, E, H, and J, respectively. Magnifications: A and F, $\times 400$; B, C, G, and H, $\times 1260$; and D, E, I, and J, $\times 630$.

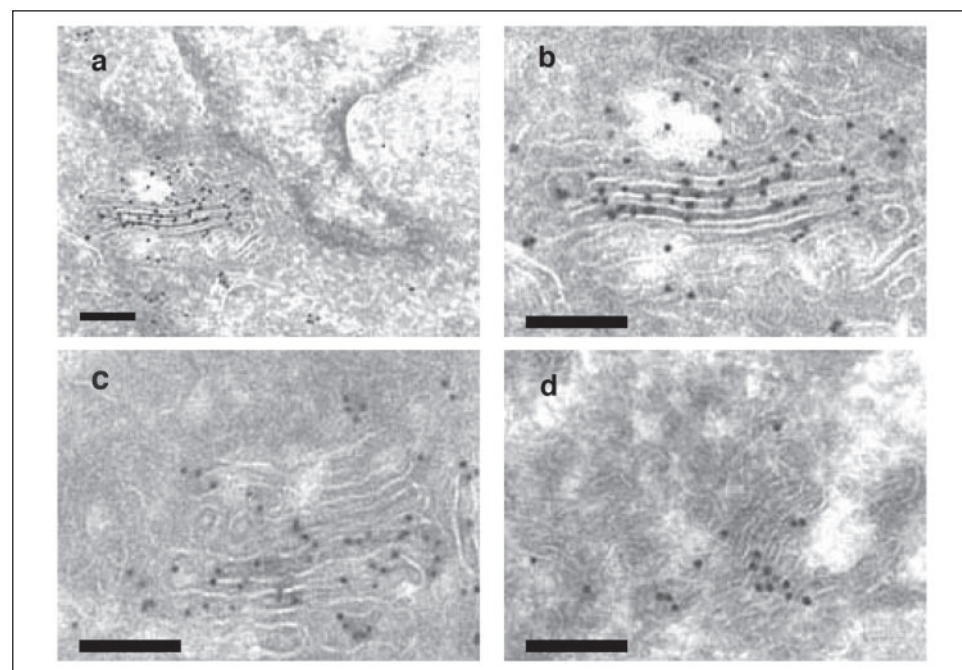
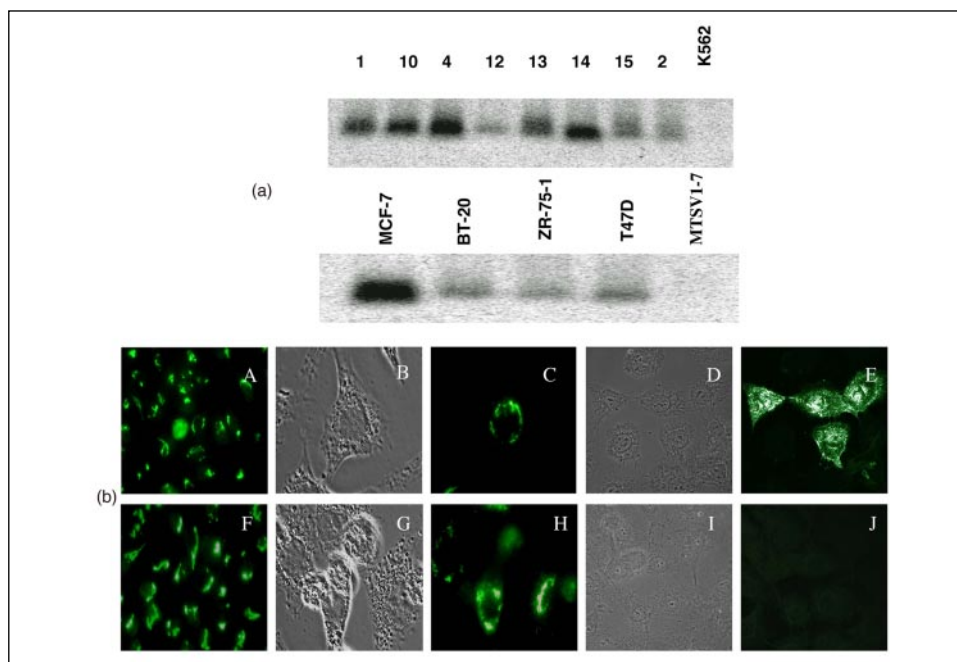


FIGURE 5. Immunoelectron microscopy of T47D-ST3 cells permanently transfected with *hST6GalNAc-I-vsv-g* cDNA. Sections prepared as described under "Materials and Methods" were stained with the P5D4 antibody followed by a rabbit anti-mouse antibody and then protein A coupled to 10-nm gold particles. All photographs show the distribution of the vsv-g-tagged *hST6GalNAc-I* throughout the Golgi. *a* and *b* represent the same Golgi at different magnifications (*c* and *d* show different Golgi apparatus). Bar, 0.2 μ m.

that *hST6GalNAc-II* mRNA was expressed in all of the 8 breast cancers regardless of their STn status. Furthermore, four STn-negative breast cancer cell lines were found to express *hST6GalNAc-II* mRNA (see also Ref. 27), whereas K562 cells, which express STn and *hST6GalNAc-I* (Fig. 3*a*), did not express *hST6GalNAc-II* (Fig. 4*a*). Interestingly, the non-malignant breast epithelial cell line, MTSV1-7, did not express *ST6GalNAc-II*. These results suggest that *hST6GalNAc-II* does not play a role in STn synthesis in breast cancer.

Transfection of *hST6GalNAc-I* into Breast Epithelial Cell Lines Results in the Expression of the STn O-Glycan—Most breast cancer cell lines, including T47D, do not express the STn O-glycan or *hST6GalNAc-I* mRNA (data not shown). To directly demonstrate that

hST6GalNAc-I can override existing pathways of O-glycosylation in breast cancer cells (and direct the synthesis of STn), cDNA coding for the human functional enzyme was cloned by RT-PCR from K562 cells and transiently transfected into the human breast cancer cell line T47D. T47D cells express core 1-based but not core 2 O-glycans (6), and the transfected *hST6GalNAc-I* was able to compete with the core 1 enzyme as demonstrated by the expression of STn seen by staining with the anti-STn monoclonal antibody, TKH2 (Fig. 4*b*, panel E). As predicted, the STn O-glycan was not synthesized in T47D cells transfected with cDNA coding for the *hST6GalNAc-II* enzyme (Fig. 4*b*, panel J), confirming the key role of *hST6GalNAc-I* in the expression of STn in breast cancer cells. Significantly, expression of both *ST6GalNAc-I* and

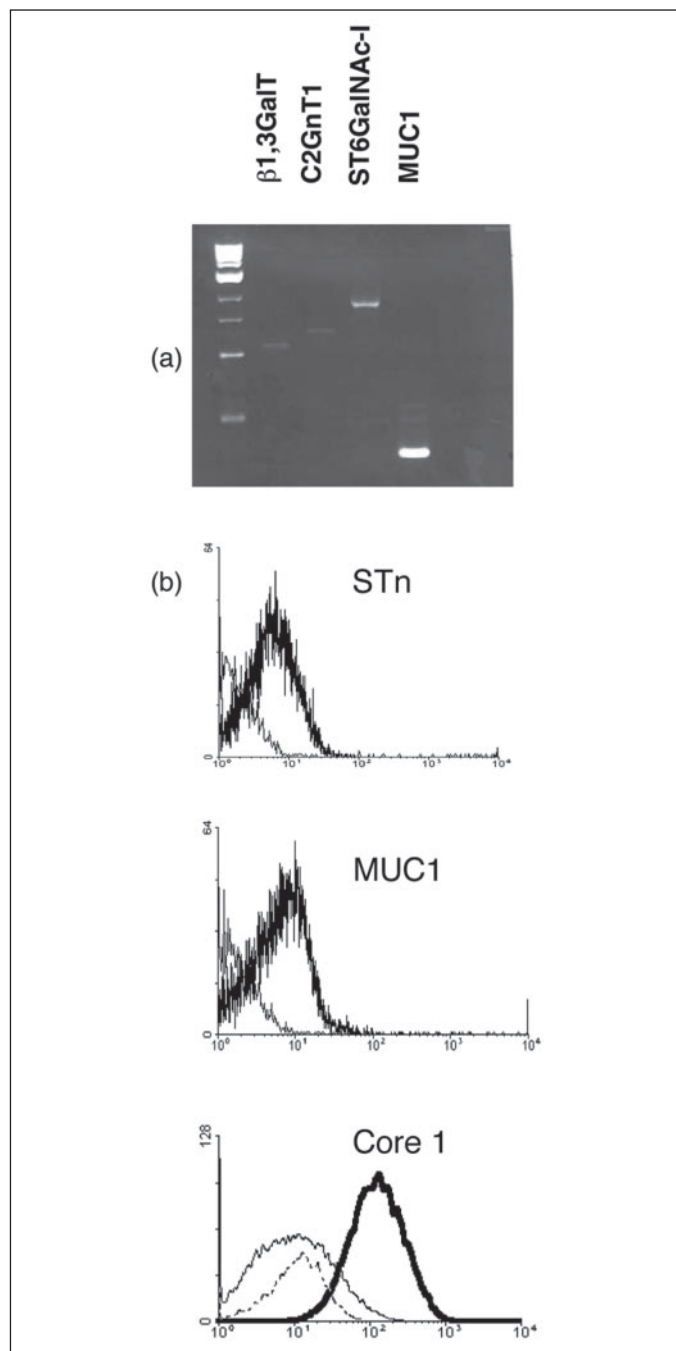


FIGURE 6. Expression of glycosyltransferases, O-glycans, and MUC1 in K562 cells. *a*, RT-PCR was performed on mRNA from K562 cells using the primers for MUC1 and the C2GnT1, core 1, and ST6GalNAc-1 glycosyltransferases, as described under "Materials and Methods." *b*, FACS analysis of K562 cells. *Top panel*: expression of STn. Cells were incubated with TKH2 (thick line) followed by FITC-conjugated rabbit anti-mouse immunoglobulins or with secondary antibody alone (thin line). *Middle panel*: expression of MUC1. K562 cells were incubated with SM3 (thick line) followed by FITC-conjugated rabbit anti-mouse immunoglobulins or secondary antibody alone (thin line). *Bottom panel*: expression of Core1 structures. Cells were incubated with FITC-conjugated peanut agglutinin before (thin continuous line) or after (thick line) treatment with neuraminidase; cells alone (thin dotted line).

ST6GalNAc-II could be detected using antibodies to the tags in a number of cells (Fig. 4*b*, panels A and F, respectively). Higher magnification shows that both enzymes were expressed in a Golgi localization (Fig. 4*b*, panels B, C, G, and H).

T47D cells have previously been transfected with the core 2 enzyme, C2GnT-1 to develop the E2J cell line (13). This resulted in the expres-

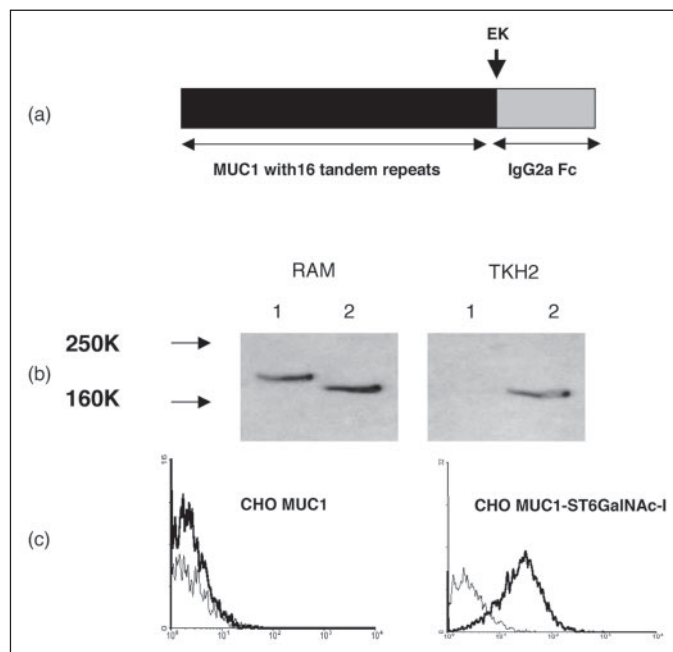


FIGURE 7. Analysis of CHO cells expressing a MUC1 fusion protein carrying STn. *a*, schematic representation of the MUC1-IgG2a fusion protein, EK, enterokinase cleavage site. *b*, Western blot of secreted proteins from supernatants of CHO MUC1 cells (lane 1) and the CHO MUC1 ST6GalNAc-I transfectant (lane 2) cells. Blots were probed with rabbit anti-mouse horseradish peroxidase (left) or biotinylated TKH2 followed by Streptavidin-horseradish peroxidase (right). *c*, CHO MUC1-STn cells express the ST6GalNAc-I-vsv-g fusion protein. CHO MUC1 cells (left) or CHO MUC1 ST6GalNAc-I cells (right) were permeabilized and stained with biotinylated anti vsv-g antibody P5D4 followed by FITC-labeled streptavidin (thick line) or FITC-labeled streptavidin alone (thin line).

sion of core 2 O-glycans on MUC1 as demonstrated by the loss of reactivity with the SM3 monoclonal antibody, which is blocked by core 2 structures (13, 17). Transfection of ST6GalNAc-I into E2J cells showed that expression of this sialyltransferase also resulted in the expression of STn in breast epithelial cells expressing core 2-based O-glycans (data not shown).

ST6GalNAc-I Transferase Is Found throughout the Golgi Stacks in Transfected Cells—The position of the core 1 enzyme in the Golgi pathway has not been mapped, but the C2GnT1 (core 2 enzyme) is found mainly in the *cis* Golgi (13). To be able to override the core 1/core 2 pathway, the hST6GalNAc-I enzyme would therefore be expected to be either in the *cis* Golgi or throughout the Golgi stacks, as has been found for some polypeptide GalNAc transferases (41). The vsv-g-tagged hST6GalNAc-I construct was transfected into T47D-ST3 (previously used to map the ST3Gal-I enzyme (12)) and a stable cell line selected (T47D-ST3-STn), which expresses both STn and hST6GalNAc-I (data not shown). Immunoelectron microscopy was used to position the location of the enzyme within the Golgi. Sections of the transfected cells were labeled with the monoclonal antibody (P5D4) to the vsv-g tag and binding detected with rabbit anti-mouse antiserum, followed by 10-nm gold-labeled protein A (see "Materials and Methods"). The Golgi stacks in breast cancer cell lines such as T47D are not all as structurally well organized as in normal cells. However, where they could be seen to be linearly stacked, the enzyme appeared to be distributed throughout the stacks, and some examples are shown in Fig. 5. Fig. 5*a* demonstrates that there was no background labeling, because all the labeling is confined to the Golgi and none is present in the nucleus. The micrographs in Fig. 5 (*b–d*) show in more detail the distribution of the hST6GalNAc-I enzyme, which is found throughout the Golgi of T47D cells.

TABLE 2

Comparison of O-glycans and O-glycosylation site occupancy on MUC1-IgG produced in wild-type CHO cells (CHO MUC1) and in CHO MUC1 cells transfected with hST6GalNAc-I (CHO MUC1 ST6GalNAc-I)

	CHO MUC1 ST6GalNAc-I	CHO MUC1 (36)
O-Glycans	Relative amounts (%) ^a	
Galβ1-3GalNAc-	0	2
(Neu5Acα2-6)GalNAc-	83	0
Galβ1-3(Neu5Acα2-6)GalNAc-	1	1
Neu5Acα2-3Galβ1-3GalNAc-	4	84
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc-	12	13
Number of glycosylated sites (n) in the MUC1 tandem repeat	% Peptides with n sites ^b	
5 (fully glycosylated)	25	52
4	39	30
3	25	18
2	12	0
1	0	0
Sites per tandem repeat	3.8	4.3

^a Mean of two separate sample preparations and analyses. Integrated peak areas in mass chromatograms for each oligosaccharide were used to calculate the relative amounts of each structure. Areas were corrected for the different signal intensities obtained by neutral, mono-sialylated, and di-sialylated structures as previously described (50).

^b Mean of three (CHO MUC1 ST6GalNAc-I) or two (CHO MUC1) separate sample preparations and analyses. Peaks in mass chromatograms representing MUC1 tandem repeat glycopeptides with n GalNAcs were integrated and expressed as a percentage of the area for all MUC1 tandem repeat glycopeptides.

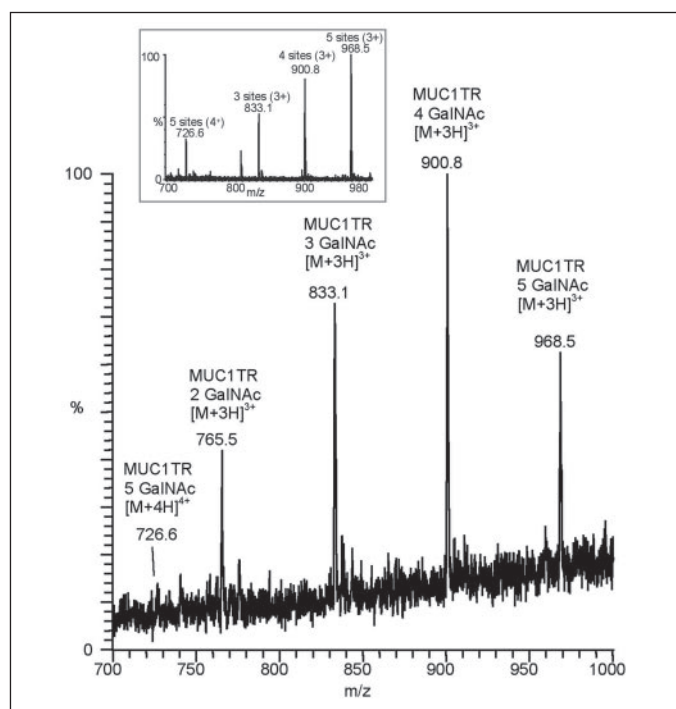


FIGURE 8. Combined nano-LC-ESI mass spectra of MUC1 glycopeptides obtained after neuraminidase and β -galactosidase treatment and Clostripain digestion of a secreted MUC1 from CHO cells transfected with hST6GalNAc-I. Peaks correspond to MUC1 tandem repeat peptides with two to five GalNAcs. Inserted for comparison is the corresponding spectrum for wt CHO MUC1.

Endogenous Expression of hST6GalNAc-I in K562 Cells Is Associated with Expression of the STn O-Glycan—The above data show that in breast cancer cells hST6GalNAc-I can compete with existing glycosylation pathways when expressed from the strong cytomegalovirus promoter. The results from the analysis of primary breast cancers suggest that hST6GalNAc-I can also access the GalNAc substrate when expression is driven by its own promoter. To confirm this, we analyzed the human leukemia cell line K562, which expresses the STn O-glycan (Fig. 6b, top panel). RT-PCR was used to demonstrate that mRNA transcripts coding for the hST6GalNAc-I, core 1 and core 2 enzymes were all present (Fig. 6a), although ST6GalNAc-II is not (see Fig. 4). The expression

of MUC1 was also observed at the level of mRNA (Fig. 6a) and at the protein level as demonstrated by reactivity with the SM3 monoclonal antibody (Fig. 6b, middle panel). The presence of core 1 structures was demonstrated by binding of peanut agglutinin lectin but only after treatment with neuraminidase, indicating that the core 1 structures are predominantly sialylated (Fig. 6b, bottom panel). The level of core 2 structures was not directly analyzed. However, MUC1 reacted with SM3, the binding of which is blocked by core 2 structures. This suggests that the dominant O-glycans synthesized by K562 cells are sialylated core 1 structures and sialylated Tn and that the levels of endogenous hST6GalNAc-I are sufficient to result in the expression of STn. It is theoretically possible, that an as yet unidentified sialyltransferase is responsible for STn synthesis in K562 cells. Transfectants with constitutively reduced ST6GalNAc-I mRNA (developed using by RNA interference) could be developed to eliminate this possibility.

Stable Expression of hST6GalNAc-I in CHO Cells Modifies the O-Glycans of a Secreted MUC1 Glycoprotein—The STn O-glycan is relatively tumor-specific, and clinical studies using STn coupled to KLH as an immunogen have suggested that there may be clinical benefit for breast cancer patients (23). It is possible that STn might be a more effective immunogen if carried on a natural substrate such as the MUC1 mucin, expressed in most breast cancers. A qualitative assessment of MUC1-expressing T47D cells transfected with hST6GalNAc-I, indicated that along with the STn O-glycans, some sialylated core 1 structures were also present (data not shown). A quantitative analysis, however, requires more material and this is often not possible to acquire with a membrane-associated glycoprotein. Instead we turned to the production of a secreted MUC1 protein in CHO cells. The construct that was transfected into the CHO cells contains most of the extracellular domain of MUC1, including 16 tandem repeats, fused to mouse IgG2a Fc to aid secretion (36, 37) (Fig. 7a). This secreted glycoprotein (MUC1-IgG2a) carries over 80% mono-sialylated core 1 structures (Neu5Acα2-3Galβ1-3GalNAc or ST) but no STn O-glycans. To produce a MUC1 glycoprotein carrying STn O-glycans (MUC1-IgG2a-STn), CHO cells expressing the MUC1-IgG2a fusion protein were transfected with the vsv-g-tagged hST6GalNAc-I cDNA.

Supernatants from selected clones were subjected to Western blot analysis to detect total secreted MUC1 glycoprotein and MUC1-carrying STn (Fig. 7b). A secreted MUC1 product was observed at ~170 kDa, similar but slightly lower in molecular mass to the product produced by

the parental CHO MUC1 cell line (Fig. 7b, left panel, lanes 1 and 2). This indicated that the hST6GalNAc-I-transfected cells secrete a MUC1 glycoprotein carrying shorter O-glycans. The presence of STn was demonstrated by probing the blot with a biotinylated TKH2 antibody followed by horseradish peroxidase-conjugated streptavidin (Fig. 7b, right panel, lane 2). Expression of hST6GalNAc-I was demonstrated by intracellular FACScan analysis using a biotinylated anti vsv-g antibody (Fig. 7c).

Chemical release of the O-glycans from MUC1-IgG2a-STn followed by LC-ESI MS analysis showed that 83% of the released oligosaccharides were Neu5Ac α 2-6GalNAc (STn) O-glycans along with some mono-sialylated T (Neu5Ac α 2-3Gal β 1-3GalNAc, ST 4%) and di-sialylated T (Neu5Ac α 2-3Gal β 1-3(Neu5Ac α 2-6)GalNAc, di-ST 12%) (Table 2). Thus, hST6GalNAc-I was able to compete very effectively with the core 1 enzyme to produce STn.

Stable Expression of hST6GalNAc-I in CHO Cells Reduces the O-Glycosylation Site Occupancy in a Secreted MUC1 Glycoprotein—We also investigated the effect of hST6GalNAc-I on the number of O-glycans added to the secreted MUC1 glycoprotein. There are five potential O-glycosylation sites in each tandem repeat of MUC1. In the parental CHO-MUC1 cells, approximately half of the tandem repeats are fully glycosylated and the rest carry either three or four O-glycans, giving an average occupancy of 4.3 sites per tandem repeat (36). The presence of hST6GalNAc-I significantly reduced this number to an average occupancy of 3.8, with only 25% of the tandem repeats being fully glycosylated, with the repeats having four O-glycans being the most common (39%) (Fig. 8 and Table 2). A significant number of repeats (12%) had only two sites occupied by O-glycans, which was in contrast to wt CHO MUC1 where peptides with only two GalNAcs were not detected. Thus the expression of hST6GalNAc-I had two major effects on the glycosylation of MUC1 in CHO cells. Firstly, the majority of the O-glycans added were STn instead of di- or mono-sialylated T, and secondly, there was a reduced occupancy of the five possible O-glycosylation sites in the tandem repeat of MUC1.

DISCUSSION

The simple disaccharide Neu5Ac α 2,6GalNAc linked to serine or threonine, the STn epitope, has a highly restricted expression on normal tissues (28–30) but is found on several carcinomas, including breast (19, 28, 31). The STn epitope is synthesized through the pathway of mucin type O-glycosylation, where sugars are added individually and sequentially, each addition being catalyzed by a glycosyltransferase, or in some cases by a family of enzymes. There are for example multiple enzymes (polypeptide GalNAc transferases), which catalyze the addition of the first sugar N-acetylgalactosamine to serines and threonines (42), and these are localized throughout the Golgi pathway (41). Thus mucin-type O-glycosylation can be initiated in all compartments of the Golgi. Glycoproteins carrying GalNAc are substrates for any enzyme that adds a second sugar in the synthetic pathway. For the synthesis of the STn disaccharide in breast cancer cells, our results indicate that the enzyme responsible is hST6GalNAc-I. Thus we have demonstrated a complete correlation between the expression of hST6GalNAc-I in breast carcinomas and the expression of STn and shown expression of STn in breast cancer cells transfected with the enzyme. This is in contrast to the total lack of correlation between hST6GalNAc-II and STn expression and the lack of expression of STn in cells transfected with this enzyme. Because there is a lack of detectable hST6GalNAc-I mRNA in the normal human mammary gland (see Fig. 2), we conclude that in breast cancer the presence of STn in 30% of these tumors is the result of the switching on of the hST6GalNAc-I gene.

Our findings are in contrast to the data relating to the appearance of

STn in colon cancers, where no correlation was found between the activity of α 2,6-sialyltransferase(s) adding sialic acid to GalNAc and STn expression in cancer tissues (32). The explanation for the apparent *de novo* expression of STn in colon cancer relates to a modification of the sialic acid in the cells of normal colonic tissues. The sialic acid added to GalNAc in the normal colonic epithelium is O-acetylated and not recognized by the antibodies used to detect STn, whereas in colon cancer cells, O-acetylation is reduced and the STn epitope is unmasked and recognized by the antibodies (38, 43). This reduction in sialic acid O-acetylation appears to be an early event in the process of malignant transformation in human colorectal cancer (44). However, in hepatocellular carcinoma, the underlying mechanism for the expression of STn is not due to a loss of O-acetylation of sialic acid (45) and could relate to changes in sialyltransferase expression as we have observed in breast cancer.

Although levels of expression of glycosyltransferases can give an indication of the potential pathways of glycosylation, the final composition of the O-glycan is also crucially dependent on the position of the enzyme in the Golgi stacks. This is particularly relevant to those steps in glycan synthesis where enzymes producing different products are competing for the same substrate. The enzyme responsible for initiation of chain extension in breast cancer is the core 1 enzyme that adds galactose to GalNAc and thus potentially can compete with hST6GalNAc-I for the GalNAc substrate. The core 1-synthesizing enzyme is very widely distributed, but its position in the Golgi pathway has not yet been determined. Here we show that the transfected hST6GalNAc-I enzyme, like the polypeptide GalNAc transferases, appears to be distributed throughout the Golgi stacks (Fig. 5), which would allow access to the GalNAc substrate even in the *cis* Golgi. This positioning would explain our observation that, even when a functional core 1 (and even core 2) enzyme is expressed, expression of hST6GalNAc-I can override this pathway to a large extent. Indeed, when ST6GalNAc-I was expressed in CHO cells, which express core 1 but no core 2 enzymes, 83% of the O-glycans added to MUC1 were STn. Other sialyltransferases have been localized toward the *trans* compartment of the Golgi, but these enzymes are adding sialic acid to glycans that have been extended in passing through the Golgi (12). Confirmation of the Golgi localization of endogenous ST6GalNAc-I awaits the development of a specific monoclonal antibody.

The expression of functional ST6GalNAc-I not only altered the O-glycan structures but also reduced the site occupancy of the potential five O-glycosylation sites in the MUC1 tandem repeat. It appears that the addition of sialic acids to the core GalNAcs inhibits, to some degree, the subsequent action of polypeptide-GalNAc transferases with the result that fewer GalNAcs are added to the protein. This may be due to sialic acid interfering with the binding of the lectin domains of polypeptide GalNAcT, which is required for their action (46), and is possible because ST6GalNAc-I, like the GalNAc transferases, is found throughout the Golgi. Reducing the number of sites glycosylated leads to exposure of antigenic epitopes in the protein, which, combined with the multiple novel STn epitopes, will radically change the antigenicity of the MUC1 glycoform expressed in ST6GalNAc-I-expressing breast cancers.

STn expression is associated with poor prognosis in breast (20, 47) and other carcinomas (48, 49). Moreover, in node-positive breast cancer patients, STn expression is correlated with a lack of response to adjuvant chemotherapy (19). The expression of this carbohydrate antigen therefore appears to be identifying a particular group of patients that may benefit from some other form of treatment. An immunotherapeutic approach could be one such treatment, and we have developed a CHO

cell line that secretes large quantities of MUC1-expressing STn (Fig. 7 and Table 2). Indeed, the first experiments using this product in a mouse model where MUC1 is expressed as a self-antigen demonstrate that specific antibodies can be induced, which recognize the STn glycoform of MUC1 but not other glycoforms.⁷ The availability of MUC1-STn as produced by the modified CHO cells will now allow the evaluation of this glycoprotein as an immunogen for effects on STn-expressing tumors.

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⁷ R. Sewell, unpublished observations.