The Relative Activities of the C2GnT1 and ST3Gal-I Glycosyltransferases Determine O-Glycan Structure and Expression of a Tumor-associated Epitope on MUC1*

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In breast cancer, the O-glycans added to the MUC1 mucin are core 1- rather than core 2-based. We have analyzed whether competition by the glycosyltransferase, ST3Gal-I, which transfers sialic acid to galactose in the core 1 substrate, is key to this switch in MUC1 glycosylation that results in the expression of the cancer-associated SM3 epitope. Of the three enzymes known to convert core 1 to core 2, by the addition of GlcNAc to GalNAc in core1 C2GnT1 is the dominant enzyme expressed in normal breast tissue. Expression of C2GnT1 is low or absent in around 50% of breast cancers, whereas expression of ST3Gal-I is consistently increased. Mapping of ST3Gal-I and C2GnT1 within the Golgi pathway showed some overlap. To examine functional competition, the enzymes were overexpressed in T47D cells, which normally make core 1-based structures, to see if the switch to core 2 structures was controlled by C2GnT1. Core 3 epitope expression was lost in the presence of ST3Gal-I, accompanied by a decrease in the GalNAc/GlcNAc ratio, indicative of a switch to core 2 structures. Transfection of a C2GnT1 expressing line with ST3Gal-I restored SM3 binding and reduced GlcNAc incorporation into MUC1 O-glycans. Thus, even when C2GnT1 is expressed, the O-glycans added to MUC1 become core 1-dominated structures, provided expression of ST3Gal-I is increased as it is in breast cancer.

Interest has been focused on the MUC1 mucin as a target antigen for immunotherapy of breast cancer, since the cancer-associated mucin is overexpressed and aberrantly glycosylated (1). The MUC1 mucin is a membrane glycoprotein with an extracellular domain, which contains potential O-glycosylation sites (2, 3). The aberrant glycosylation has been well documented in breast cancers where it has been shown (4, 5) that the addition of shorter O-glycans leads to exposure of peptide epitopes in the tandem repeats, which are normally masked, and the appearance of novel carbohydrate epitopes. Recent data suggest that not only the composition but the number of O-glycans added to MUC1 is altered in breast cancer (6). Thus, the cancer-associated glycoprotein is antigenically distinct from the normally processed mucins (7). This difference in antigenic profile is detected by the antibody SM3 (8, 9), which recognizes a core protein epitope selectively exposed in the tandem repeat of the cancer-associated MUC1 mucin (10).

Analysis of the O-glycans attached to the mucin produced by the normal lactating breast and by breast cancer cell lines has shown that the oligosaccharides added to the normal mucin are core 2-based structures (11), whereas in the cancer-associated mucin, shorter core 1-based structures dominate (4, 5, 12). As illustrated in Fig. 1, core 1 (Galβ1,3GalNAc-Ser/Thr) can be a substrate for enzymes adding GlcNAc in the β1,6 linkage to GalNAc to form core 2 or for sialyltransferases adding sialic acid in α2,3 linkage to galactose. These early steps in O-glycan synthesis are crucial to the final definition of O-glycan structure, since the conversion to core 2 promotes chain branching and elongation, whereas the addition of sialic acid terminates chain growth.

In considering the potential competition of glycosyltransferases for the core 1 substrate, the level of activity of a specific glycosyltransferase and their relative positions in the Golgi pathway need to be considered. It now appears that glycosyltransferases are not confined exclusively to specific Golgi compartments but show a diffuse distribution among compartments (13). For example, although the expression of the sialyltransferase ST3Gal-I is highest in the medial/trans-Golgi, some expression can be seen in the cis Golgi (14).

Another important finding is that most glycosidic linkages are in fact formed by multiple glycosyltransferase isoenzymes that have different kinetic properties and expression patterns and possibly different subcellular distributions. Thus there are several transferases that are able to add sialic acid in α2,3 linkage to the galactose moiety in core 1 (15). However, of the cloned α2,3-sialyltransferases enzymes, ST3Gal-I appears to

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1 ST3Gal-I (SiaT-4a) refers to a sialyltransferase with GenBank™ accession number L13872. C2GnT1 (C3GnT, C2GnT-I), C2GnT2 (C2/ C4GnT, C2GnT-M), and C2GnT3 refer to human β6GlcNAc-transferases with GenBank™ accession numbers M97347, AF038650/ AF102542, and AF132035, respectively.
display the major role in the sialylation of core 1 structures on MUC1 in breast cancer. As deduced from enzyme specificity and tissue distribution (16–18). Significantly, the ST3Gal-I enzyme has been found to be elevated in breast cancer cell lines and primary breast cancers (14, 19).

Similarly, formation of the branched complex type O-glycans (core 2) may be controlled by at least three β6GlcNAcT, which show different patterns of expression in tissues. The enzymes C2GnT1 and C2GnT3 (20, 21) act only on core 1 to produce core 2. On the other hand, C2GnT2 or C4GnT (22, 23) is able to catalyze the formation of core 4 from core 3 as well as core 2 from core 1. Of the C2GnTs identified to date by cloning, it appears that C2GnT1 is the enzyme most commonly expressed in breast tissue and tumors, although C2GnT3 can occasionally be expressed (this manuscript).

Although the expression of the C2GnT1 enzyme has been found to be decreased or absent in most breast cancer cell lines (24, 14), reasonable levels of the mRNA coding for this enzyme can be found in a high proportion of primary breast cancers (19). This is in contrast to the level of expression of ST3Gal-I mRNA, which is consistently elevated in primary breast cancers (19). Moreover, the level of expression of the mRNA coding for ST3Gal-I correlates with the level of expression of the SM3 epitope with tumor grade (19). Since core 2 branching by β6GlcNAc-transferases is inhibited by α2,3 sialylation of core 1 in vitro (25, 26, 27), it follows that ST3Gal-I could be a key controlling enzyme leading to simple mucin type core 1 glyco-sylation pattern in cancer cells. The question, however, is whether ST3Gal-I can effectively compete with C2GnT1 in vivo, leading to an inhibition in the formation of core 2 structures on MUC1 and whether this relates to exposure of the cancer-associated SM3 epitope.

For competition to be effective, the enzymes must overlap in the compartments of the Golgi apparatus. We have therefore examined in detail the level of expression of C2GnT1 throughout the Golgi pathway and compared this profile with the profile of expression of ST3Gal-I in the same Golgi compartments. We find that although the peak expression of C2GnT1 is in the cis and ST3Gal-I peaks in the medial/ trans, the enzymes did show partial overlap. To directly examine if this degree of overlap is sufficient to allow competition between the two enzymes, we transfected the cDNAs encoding C2GnT1 and ST3Gal-I into the breast cancer cell line T47D (28). Our data clearly show that despite the later peak of localization in the Golgi, the ST3Gal-I enzyme is able to compete effectively with C2GnT1 for the core 1 substrate in vivo, when the sialyltras-ferase is highly expressed. The results show that the relative levels of the competing enzymes affect both the structure of the O-glycans attached to MUC1 and the exposure of the SM3 epitope. Thus, the SM3 epitope was exposed in breast cancer cells expressing C2GnT1, providing the ST3Gal-I enzyme was elevated as it is in breast cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Biotinylated *Arachis hypogaea* Peanut agglutinin (PNA) was from Vector laboratories Ltd. (Peterborough, UK). Streptavidin-horseradish peroxidase, 0.1 M Kglucosamine hydrochloride (26 Ci/mmol), MegaPrime kit and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech. pcDNA3 vector was from Invitrogen. P.Babe-puro retroviral vector was from Promega. *Clostridium perfringens* and *Vibrio cholerae* sialidases were from Oxford Glycosciences (Abingdon, UK). [α-32P]dCTP (5,000 Ci/mmol) was from ICN.

**Antibodies**— Supernatants of the mouse monoclonal antibodies HMF-1, HMF-2, and SM-3, reactive with MUC1, (29, 8) were used for FACS analyses. CT-1 polyclonal rabbit antibody was raised against the cytoplasmic tail of MUC1 (30).

**Cell Culture**—The human mammary epithelial cell line MTSV1−7 was derived by SV40 immortalization of normal mammary epithelial fundus isolated from human milk (31) and grown in E4, 10% FCS with 10 μg/ml insulin, 5 μg/ml hydrocortisone, and 0.3 μg/ml glutamine. The cell line T47D was derived from breast carcinoma cells isolated from a pleural effusion (28) and was cultured in E4, 10% FCS. HPAF, a human pancreatic carcinoma line, was grown in E4, 10% FCS.

**Breast Tumors**—20 frozen breast cancer tumor tissues were provided by Dr. Andrew Hanby, Imperial Cancer Research Fund Breast Pathology Group. All samples were classified as invasive ductal carcinoma grade 3 showing positive staining with the SM3 antibody. Before RNA extraction (which was done as described for the cell lines; see above), samples were kept in liquid nitrogen and then subject to mechanical dis-aggregation using a dis-membranator.

**DNA**—The cDNA encoding the human C2GnT1 was obtained from Dr. R. McEver (W. K. Warren Medical Research Institute, University of Oklahoma). A 950-base pair EcoRI fragment containing the open reading frame from +710 to +1,660 was used as a probe for Northern analysis. The cDNA encoding the human C2GnT1 (32) was obtained from Dr. J. Lau (Roswell Park Cancer Institute, Buffalo, NY). A 1,121-base pair open reading frame fragment released from a pCR2.1 vector (Invitrogen) by EcoRI digestion was used as a Northern probe. C2GnT2 (22) probe was a 1,227-base pair EcoRI digest of the expression vector pAAGP67/C2/ 4GnT-oligomer spanning the open reading frame from +91 to +1,318. The C2GnT3 (21) probe was a 1,254-base pair EcoRI/BamHI digest containing the open reading frame from +114 to +1,368.

**cDNA Constructs**—The full-length C2GnT1 cDNA-coding region was ligated into the EcoRI site of pcDNA3 expressing the neomycin-selectable marker (Invitrogen). For Golgi localization experiments, C2GnT1 cDNA was tagged with six 9E10 Myc epitopes and cloned into the retroviral vector, pBabe-puro. The production of amphotrophic retrovirus was essentially as previously described (14). Briefly, ST3Gal-I DNA (tagged with an Myc epitope at the C terminus) and C2GnT1 cDNA, subcloned into the pBabe puro vector, was transfected into the amphotrophic packaging cell line AM12. 48 h after transfection, cells were split 1:10 into growth medium containing 2 μg/ml puromycin for selection. Medium was changed every 3 to 4 days for 4 weeks until selection was complete. The retrovirus producer cell lines were grown to 70% conflueny, and the spent medium was replaced with a half-volume of fresh medium. 3 days later, the virus-containing medium was removed, filtered, quick frozen in dry ice, and stored at -70°C.

**Transfection of T47D Cells with C2GnT1**—T47D cells were transfected with pcDNA3-neo expressing C2GnT1 or with the empty vector to give the T47 E2B and T47D E2J cell lines expressing C2GnT and the T47D neo cell line expressing only the selectable marker. To ensure efficient transfection of cells and the isolation of stable clones, a modified method of electroporation in the presence of Me2SO was used,
which improves the efficiency of transfection of epithelial cell lines (33). 1.25% MeSO was included in both the electroporation cuvette and also after the pulse in the culture medium. Three days post-transfection T47D cells were split 1:10 into growth medium containing 50 µg/ml G418. Media were changed every 3 days until selection was complete and stable clones were expanded.

Transduction of T47D C2GnT1 Clones and the MTSV1–7 Cell Line—For transduction of T47D-E2B, T47D-E2J, and MTSV1–7 cell lines with the ST3Gal-I and C2GnT1 retroviruses, cells were seeded at ~1 × 10^5 cells/well on a 6-well plate overnight, then 2 ml of retroviral supernatant (derived from transfection of packaging cell line above) was added along with sterile Polybrene at 8 µg/ml and incubated for 7 h. Medium was then changed to E4, 10% FCS for T47D and E4, 10% FCS + 10 µg/ml insulin and 5 µg/ml hydrocortisone for MTSV1–7, and cells were left for 24 h. At this time the medium was changed to contain the appropriate antibiotic for selection. Selection was continued for 7 days, after which individual clones were isolated and expanded.

Localization of C2GnT1 within the Golgi Apparatus—MTSV1–7 cells (34) were transduced with the Myc-tagged C2GnT1 retrovirus (34), and clones were isolated. Cells were fixed for 1 h at room temperature in 0.1% glutaraldehyde, 4% paraformaldehyde before being scraped, spun, and stored overnight at 4 °C. Pellets were then stained with the 9E10 monoclonal antibody followed by goat anti mouse Ig coupled to 10-nm gold particles and processed for electron microscopy as described by Whitehouse et al. (14). The orientation of the Golgi was determined by reference to the position of β1–4 galactosyltransferase (GalT1) using rabbit antibody to GalT1 (a generous gift from Dr. Eric Berger) and followed by protein A coupled to 5-nm gold particles. Quantitation was performed as described by Whitehouse et al. (14).

Assays of Glycosyltransferase Activities—Cells were harvested and washed three times in phosphate-buffered saline. 1 ml of 0.25% sucrose was added to 10^7 cells, and the cells were hand-homogenized and stored at −80 °C. The activities of βGlcNAc transferases in cell homogenates were measured using Galβ1,3GalNAc-nitrophenol as substrate, and the product was estimated by high performance liquid chromatography, as described by Brockhausen et al. (24). Activities were expressed as nmol/h/mg.

Fluorescence-activated Cell-scanning Analysis (FACScan)—Cells were trypsinized and washed once in growth medium, and a minimum of 5 × 10^6 cells were resuspended in 100 µl of the appropriate specific antibody diluted in growth medium, neat tissue culture supernatant, or in 100 µl of growth medium alone as a negative control. All incubations were carried out in the presence of 0.02% sodium azide and on wet ice. After a 30-min incubation, cells were washed three times in growth medium containing 0.02% sodium azide. Cells were resuspended in 100 µl of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins, diluted 1:40 in growth medium, and incubated for 30 min in the dark. After washing three times in growth medium containing 0.02% sodium azide, cells were resuspended in 300 µl of phosphate-buffered saline, and 10,000 cells were analyzed by a Becton Dickinson FACScan. For FACS analysis of PNA binding, cells were incubated with or without sialidase (V. cholerae), 0.06 units/ml, and analyzed for the binding of fluorescein isothiocyanate-conjugated PNA as described previously (7).

Northern Analysis of RNA—Total RNA was extracted from cell lines or breast tumors using TRIZOL as per the manufacturer’s instructions. Placental poly(A)^+ RNA was obtained from CLONTECH. RNA was run on 1% agarose, 2.2 M formaldehyde-diethyl pyrocarbonate gel in running buffer (0.02 M MOPS, 8 mM sodium acetate, 1 mM EDTA, pH 7.0, in diethyl pyrocarbonate water). After denaturing, the RNA was transferred and immobilized onto Hybond-N membrane and incubated with α-32P-labeled probe (labeled using MegaPrime kit with [α-32P]dCTP)
and hybridized overnight at 65 °C. Filters were then washed for 1 h in buffer A (0.08 M sodium phosphate, 2 mM EDTA, 5% bovine serum albumin, 10% SDS) at 65 °C, then twice in buffer B (0.16 M sodium phosphate, 4 mM EDTA, 4% SDS) for 1 h at 65 °C and then exposed to a Kodak phosphor screen overnight and visualized using a STORM scanner system.

Cell Lysis—Cells grown to around 80% confluence in a T75 flask were washed twice in 10 ml of ice-cold phosphate-buffered saline and lysed in 0.4 ml of radioimmune precipitation buffer (20 mM sodium phosphate, pH 7.2, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 109 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 10 μg/ml aprotinin). Cells were scraped off, the flask was washed with an additional 0.2 ml of radioimmune precipitation buffer, and the lysates were spun at 14,000 × g for 10 min at 4 °C. Supernatant was used for Western blotting or MUC1 immunoprecipitation.

Analysis of Hexosamine Content of MUC1—Changes in the hexosamines in the side chains of MUC1 were analyzed by high performance anion exchange chromatography (HPAEC) after metabolically labeling the glycoprotein with [3H]glucosamine hydrochloride and precipitating with the CT-1 antibody as previously described (4). Briefly, cells in 75-cm² flasks at 70–80% confluence were incubated for 2 h with medium containing reduced glucose (0.45 g/liter) before changing to low glucose medium (3 ml) containing 100 μCi/ml [3H]glucosamine hydrochloride. After overnight incubation, cells were lysed, and 500 μl of the lysate was pre-cleared with pre-immune rabbit serum and protein A-Sepharose beads. The radioactively labeled MUC1 was immunoprecipitated with 10 μl of CT-1 polyclonal antibody (30), which was directed to the cytoplasmic tail and precipitated all glycoforms of the mucin. Protein A-Sepharose was then added, and the complex was removed by centrifugation and washed thoroughly. The immunoprecipitate was eluted in 2% SDS and hydrolyzed in 2 N trifluoroacetic acid at 100 °C for 3 h and then analyzed by HPAEC on a CarboPak PA1 column by isocratic elution with 0.01 M NaOH.

Western Blotting—300 μg of cell lysate were incubated with or without 1.0 unit of C. perfringens sialidase in 50 mM sodium acetate buffer, pH 5.0, in a 37 °C water bath overnight. 50 μg of sialyated was separated on a 12% SDS-polyacrylamide gel and then blotted onto nitrocellulose in Towbin buffer using a Bio-Rad semi-dry electrophoresis. The blot was blocked overnight at 4 °C in 1× phosphate-buffered saline, pH 7.4, 0.2% Tween 20, 1.0% bovine serum albumin (PTB solution) and then incubated with biotinylated PNA (5 μg/ml in 20 ml PTB solution) for 1 h at room temperature with gentle agitation. After 3 washes in PTB solution, the blot was incubated for 1 h at room temperature with streptavidin-horseradish peroxidase (1/2000 dilution in 20 ml of PTB solution). After 3 additional washes, the bound lectin-streptavidin-horseradish peroxidase was detected using enhanced chemiluminescence (ECL) and light-sensitive film.

RESULTS
Expression of βGlcNAc T Activity in Normal and Malignant Breast Tissue and Epithelial Cell Lines—Genes coding for three enzymes able to synthesize core 2 (C2GnT1, C2GnT2, and C2GnT3) from core 1 have been isolated (20–22). Expression of these enzymes in the T47D breast cancer cell line and in the nonmalignant MTSV1–7 cells was examined by Northern analysis. Fig. 2 shows that none of these enzymes were expressed in T47D cells, whereas C2GnT1 and C2GnT3, but not C2GnT2, from core 1 have been isolated (20–22). Expression of these enzymes in the T47D breast cancer cell line and in the nonmalignant MTSV1–7 cells was examined by Northern analysis. Fig. 2 shows that none of these enzymes were expressed in T47D cells, whereas C2GnT1 and C2GnT3, but not C2GnT2, were expressed in MTSV1–7 cells (Fig. 2B, lane 1). When RNA from normal breast tissue was examined, only C2GnT2 could be detected, mRNA for the other two enzymes being undetectable (Fig. 3A). A positive control for the C2GnT2 mRNA is shown in lane 3 of Fig. 2B, containing RNA from the pancreatic carcinoma cell line HPAF (Fig. 2B, lane 3).
In situ hybridization analysis of primary breast cancers has shown that C2GnT1 levels vary widely among breast carcinomas, whereas levels of ST3Gal-I mRNA are consistently increased (19). At the time of this analysis, cDNAs coding for the C2GnT2 and C2GnT3 enzymes were not available. To document expression of all three enzymes in malignancy, we performed Northern analysis on RNA from 20 primary breast cancers. All tumors were classified as invasive ductal carcinomas Grade III, and all stained positively with the SM3 antibody. The analysis showed that C2GnT2 was not expressed in any of the tumors. On the other hand, C2GnT1 was found to be expressed at reasonable levels in 10 of the 20 cancers, the others showing weak or undetectable expression. Clearly detectable levels of C2GnT3 mRNA were seen in only one of the 20 tumors, with one other giving a weak signal. Fig. 3A shows Northern blots of four of the tumors with the three relevant probes. In Fig. 3B, lane 1 shows an example of the bands seen in 1 of the 10 tumors expressing reasonable levels of C2GnT1, whereas the other tumors (lanes 1–3) show very low expression. Fig. 3B, III, lane 1 shows the band detected in the only tumor showing a clearly detectable level of C2GnT3. This enzyme appeared to be completely absent from 18 of the 20 tumors, with one other showing a very weak band.

Thus, the key enzyme capable of synthesizing the core 2 structure in normal and malignant breast epithelial cells appears to be C2GnT1. Whereas expression of C2GnT3 is a rare event in breast cancer, the MTSV1–7 cell line was found to express this enzyme. Although retaining many features of the normal luminal epithelial cell, this line was derived by immortalizing cultured milk epithelial cells with SV40 T antigen, which may have modified gene expression. Since the T47D cell line does not express any of the core 2-synthesizing enzymes but has elevated levels of ST3Gal-I (24), it represents an appropriate cell for following the effect of overexpressing C2GnT1 on O-glycan structure.

Localization of C2GnT1 within the Golgi Apparatus—For the C2GnT1 transferase to compete with the ST3Gal-I enzyme for the core 1 substrate in breast epithelial cells, there must be some overlap in their positions in the Golgi pathway. We have previously shown that although the ST3Gal-I transferase is located mainly in the medial/trans compartments of the Golgi apparatus (14), a detectable level of expression can also be seen in the cis-Golgi. The nonmalignant mammary epithelial cell line MTSV1–7 transfected with the Myc-tagged ST3Gal-I cDNA was used for the detailed mapping of this enzyme because the structure of the Golgi apparatus is well defined in these cells. Moreover, the use of tagged constructs to localize glycosyltransferases in transfected cells has been shown to give similar results for Golgi localization to that seen with direct detection of endogenous enzymes (13). To determine whether C2GnT1 localization overlapped with ST3Gal-I, C2GnT1 cDNA was tagged with 9E10 Myc epitopes and transduced into MTSV1–7. To orient the Golgi apparatus, an antibody to β1–4 galactosyltransferase (GalT1) operative in N-linked glycosylation and that has been previously mapped to the trans cisternae and the TGN (35) was used in double-labeling experiments (data not shown). The distribution of C2GnT1 across the Golgi apparatus was determined by counting the gold particles across the Golgi stack using a method described in Whitehouse et al. (14). This method provides a more detailed analysis of the distribution of C2GnT1 across the Golgi stack rather than dividing the Golgi into each cisternae, as the stack often consist

| Cell line | β6GlcNAc transferase activity | Ratio of GalNac/GlcNAc in O-glycans attached to MUC1 produced by the cell line |
|-----------|-------------------------------|----------------------------------------------------------------------------------|
| T47D      | 0.0                           | 3.4                                                                              |
| T47D-Neo  | 0.0                           | 4.6                                                                              |
| T47D-E2B  | 2.1                           | 0.4                                                                              |
| T47D-E2J  | 8.0                           | 0.4                                                                              |
| MTSV1–7   | 2.5                           | 0.45                                                                             |

**C2GnT1, ST3Gal-I Activities Control MUC1 O-Glycan Structure**

** TABLE I **

Changes in β6GlcNAc transferase activity and hexosamine ratios in MUC1 O-glycans in C2GnT1 transfectants

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**Fig. 5. Increase in MUC1 core 2-based glycans in T47D C2GnT1 transfectants.** HPAEC analysis of radio-labeled hexosamines released after acid hydrolysis of MUC1 immunoprecipitates from T47D (a), T47D-Neo (b), T47D-E2B (expressing moderate levels of C2GnT1) (c), T47D-E2J (expressing high levels of C2GnT1) (d). Peak 1 contains GalNac, and peak 2 contains GlcNac. Open boxes, MUC1 immunoprecipitated with CT-1; closed diamonds, MUC1 immunoprecipitated with preimmune serum. Panels c and d show the results of the same hexosamine analysis of two T47D C2GnT1 transfectants, demonstrating that the MUC1 glycans expressed by these cells now contain a high GlcNAc/GalNAc ratio, as summarized in Table I.
of more than three cisternae and, therefore cannot be simplified into cis, medial, and trans. Fig. 4 shows the distribution of C2GnT1 in comparison with ST3Gal-I. The peak of C2GnT1 localization appears in the early part of the Golgi, probably corresponding to the cis. There is, however, some expression in the medial compartment and low but detectable levels in the trans-Golgi. Thus, as can be seen from Fig. 4, although on the whole C2GnT1 is localized earlier than ST3Gal-1, there is a degree of overlap between the two enzymes that could allow for competition for the core 1 substrate.

Expression of C2GnT1 in T47D Cells Modifies the Structure of the MUC1-O-glycans—To directly examine the competition between ST3Gal-I and C2GnT1, the cDNA coding for the human C2GnT1 enzyme was transfected into T47D cells (which do not normally express this enzyme), and several clones were isolated. Two cell clones were expanded for analysis, T47D-E2B and T47D-E2J. T47D-E2B was selected because it had a higher content of GlcNAc, whereas T47D-E2J was selected because it had a lower content of GlcNAc. These clones were then examined for changes in the structure of MUC1. The results are shown in Figs. 5 and 6.

The reactivity of MUC1 from T47D E2J with two other epitopes is shown in Table II. The addition of the extended O-glycans in T47D cells expressing C2GnT1 is shown in Fig. 7.

Table II: Increase in core 1 sialyltransferase activity, ST3(O), relative to the activity of enzymes synthesizing core 2 from core 1.

| Cell line | Ratio of ST3Gal-I to ST2/GNAc transferase activities | Relative SM3 staining |
|-----------|-----------------------------------------------------|-----------------------|
| T47D      | Infinity                                            | 1.00                  |
| E2B       | 0.07                                                | 0.23                  |
| E2B-ST1   | 0.20                                                | 0.20                  |
| E2B-ST2   | 0.33                                                | 0.10                  |
| E2B-ST4   | 1.67                                                | 0.91                  |

*ST3Gal(O) refers to the activity of cell extracts adding sialic acid to core 1. β6GlcNAc transferase activity refers to the activity in cell extracts adding GlcNAc to core 1 on the GalNAc moiety.
antibodies (HMFG1 and HMFG2) that recognize core protein epitopes in the tandem repeat (10) was also examined. The reactivity of the antibody HMFG2 has been shown to be reduced but not abolished when the O-glycans added to MUC1 are core 2-based (7). On the other hand, the antibody HMFG1 reacts well with MUC1 carrying core 2-based O-glycans (7). Fig. 6 shows that, as expected, the reactivity of HMFG2 with MUC1 produced by the E2J cell line is reduced, whereas HMFG1 reactivity is largely unaffected. Fig. 6 shows the staining for the high expressing clone T47D-E2J, but a 4-fold reduction in the binding of SM3 and HMFG2 is also seen with clone E2B (see below, Fig. 8). Binding of HMFG1, however, remains constant in all the cell lines, indicating that the level of MUC1 expression is similar.

Increasing the Level of the ST3Gal-I Transferase in T47D Cells Expressing C2GnT1 Induces Re-expression of the SM3 and HMFG2 Epitopes—The data of Figs. 5 and 6 demonstrate that the C2GnT1 enzyme can override the glycosylation pattern of T47D cells, leading to chain extension and masking of the SM3 epitope and a reduction in the expression of the HMFG2 epitope. This effect might be predictable from the fact that the C2GnT1 enzyme (when present) has earlier access to the core 1 substrate and, therefore, makes it unavailable to the ST3Gal-I. On the other hand, we have observed that some breast cancers and breast cancer cell lines (e.g., MCF-7) express the C2GnT1 enzyme, but the SM3 epitope is not completely masked (14, 19). Analysis by in situ hybridization, however, showed that most of the breast cancers express increased levels of the ST3Gal-I enzyme. Indeed the level of expression of ST3Gal-I appears to correlate with tumor grade (19). Moreover, the increase in expression of the mRNA coding for ST3Gal-I has now been shown to be accompanied by an increase in the level of protein using immunohistochemical analysis with a recently developed monoclonal antibody to this enzyme.3

To test whether increased expression of ST3Gal-I can compete effectively with C2GnT1 for the core 1 substrate in breast cancer cells, cDNA coding for ST3Gal-I (tagged with the 9E10 Myc epitope) was introduced into the core 2-expressing cell lines T47D-E2B and T47D-E2J. The transfected cells were then analyzed for ST3(O) and β6GlcNAc transferase activities, for hexosamine content of O-glycans attached to MUC1, and for expression of the SM3 epitope.

Table II shows the ratio of activities of ST3(O) transferase and β6GlcNAc transferase for several ST3Gal-I-transfected clones derived from the E2B cell line together with the level of staining with SM3. The core 1 sialyltransferase activity in cell extracts could include other ST3(O) enzymes besides ST3Gal-I. Similarly, the core 2-synthesizing activity could formally include other enzymes catalyzing this reaction. However, because the respective activities increase with the transfection of ST3Gal-I and C2GnT1, it is highly probable that the activities being measured are ST3Gal-I and C2GnT1. Moreover, the clone T47D-E2B-ST4 showing the highest level of expression of ST3Gal-I also showed a high level of expression of mRNA coding for this enzyme (data not shown). That the ST3Gal-I transferase is functional in vitro in fully sialylating core 1 structures is shown by the complete loss of reactivity of the transfectants with peanut lectin (shown for T47D-E2B-ST4 in Fig. 7).

It can be seen from Table II that re-expression of the SM3 epitope correlates with the highest expression of ST3(O) activity in clone T47D-E2B-ST4. The detailed FACS analysis of antibody reactivity is shown in Fig. 8 for T47D-E2B-ST4 compared with the E2B and parental T47D cell lines. Clearly, whereas expression of the MUC1 epitopes recognized by SM3 and HMFG2 are significantly decreased in the E2B cells expressing moderate levels of C2GnT1, their expression is increased in ST4 cells, where the ratio of ST3Gal-I activity to C2GnT1 activity is high. In contrast to the results with the E2B cell line, none of the ST3Gal-I transfectants of the high C2GnT1 line, E2J, showed re-expression of the SM3 epitope by FACS analysis (data not shown).

The appearance of the SM3 epitope was accompanied by changes in the composition of the O-glycans attached to MUC1. Thus, analysis of the hexosamines in O-glycans attached to MUC1 from T47D-E2B-ST4 cells demonstrated a decrease in the ratio of GlcNAc to GalNAc (compared with T47D-E2B) as indicated in Fig. 9. Since in T47D-E2B cells the only pathway leading to chain branching/extension from core 1 is via core 2, thus incorporating GlcNAc, the results demonstrate unequivocal control of core 1 branching by ST3Gal-I.

3 U. Mandel, J. M. Burchell, J. Taylor-Papadimitriou, and H. Clausen, manuscript in preparation.

FIG. 8. ST3Gal-I transduction of the C2GnT1-expressing E2B cells leads to recovery of SM3 and HMFG2 staining. FACS analysis of the effect of ST3Gal-I expression in T47D-E2B cells on MUC1-specific antibody reactivity. A, T47D; B, T47D-E2B; C, T47D-E2B-ST4. Cells incubated with no primary antibody (dashed line), HMFG-1 (bold face line), HMFG-2 (dotted line), SM3 (light face line). Binding was detected by fluorescein isothiocyanate-conjugated rabbit anti-mouse secondary antibody. Note that there was no change in the expression of the HMFG1 epitope.
FIG. 9. ST3Gal-I transfection of T47D-E2B cells leads to an increase in the proportion of GalNAc in O-glycans attached to MUC1. Hexosamine analysis of MUC1 O-glycans produced by T47D C2GnT1/ST3Gal-I transfectants. HPAEC analysis of radiolabeled hexosamines released after acid hydrolysis of MUC1 immunoprecipitates from T47D parental cell line (a), T47D-E2B-Puro (expressing core 2 and the selectable marker) (b), T47D-E2B-ST4 (c). Peak 1 contains GlcNAc; peak 2 contains GalNAc. Open square, CT-1; closed diamonds, preimmune serum. Panel c shows the result of the hexosamine analysis of a T47D C2GnT1/ST3Gal I transfectant, which shows that the MUC1 glycans expressed by these cells now contain a lower GlcNAc/GalNAc ratio relative to the parental E2B as summarized in Table II.

DISCUSSION

The O-glycans added in mucin-type O-glycosylation are constructed by adding sugars individually and sequentially as the protein/glycoprotein passes through the Golgi apparatus. This means that, providing the relevant glycosyltransferases overlap in the Golgi, enzymes that use the same substrate but add different sugars can compete for the common substrate. Two enzymes, C2GnT1 and ST3Gal-I, both of which can use core 1 as a substrate, appear to be important players in defining the final structure of the O-glycans added to MUC1 in breast cancer, since they control chain branching/extension and chain termination, respectively. Although it should be noted that C2GnT3 mRNA was detected in a small number of breast carcinomas (1/20), nevertheless C2GnT1 appears to be the dominant and, hence, influential, core 2 synthesizing glycosyltransferase in both normal and malignant tissue from the mammary gland. Our previous data showed that ST3Gal-I is localized mainly in the medial/trans-Golgi, with detectable levels in the cis-Golgi (14). We now show that in mammary epithelial cells, C2GnT1 is located mainly in the cis-Golgi but with some expression in the medial cisterna and low but detectable levels in the trans-Golgi (Fig. 4). This is in agreement with other investigators who have found, using confocal microscopy, C2GnT1 to be mainly in the cis-medial-Golgi of Chinese hamster ovary cells (38).

To determine whether the level of overlap is sufficient to allow competition between the two enzymes, we have developed transfecteds of a breast cancer cell line T47D that show different ratios of expression of C2GnT1 and ST3Gal-I. To demonstrate competition between the enzymes in vivo, changes in O-glycan composition were studied by determining the relative levels of the hexosamines GalNAc and GlcNAc in MUC1 O-glycans. It should be noted that we have previously shown a decrease in the ratio of GlcNAc to GalNAc in MUC1 O-glycans in MTSV1–7 cells transfected with ST3Gal-I (14). However, this cell line shows activity not only of two core 2-synthesizing enzymes but also of the β3GlcNAc transferase, which adds GlcNAc to the Gal moiety in core 1 (24). Thus the ST3Gal-I enzyme could theoretically compete with this activity or with C2GnT3. T47D cells, however, do not express either of these enzymes (Ref. 24 and this paper); therefore the reduction in the GlcNAc to GalNAc ratio seen in the ST3Gal-I-transfected T47D E2B cells can be unequivocally attributed to competition between the ST3Gal-I and C2GnT1 transferases. Thus, even though the evidence for localization suggests that the peak localization of C2GnT1 is positioned earlier than the peak localization of the ST3Gal-I enzyme in the Golgi pathway, when ST3Gal-I activity is increased, this termination pathway dominates over the C2GnT1-branching pathway.

Our interest in the competition between the two enzymes analyzed here stemmed from the observation that ST3Gal-I activity was found to be consistently increased, whereas the level of C2GnT1 activity was not always reduced in breast cancers. Moreover, there was a strong correlation between the levels of expression of ST3Gal-I and the SM3 epitope (19). In this report, we show that SM3 and HMFG2 reactivity to MUC1 is dependent on the relative activities of both C2GnT1 and ST3Gal-I. The results are consistent with the idea that in breast cancers, where the levels of ST3Gal-I are increased, competition with C2GnT1 is effective, resulting in a reduced content of GlcNAc in the O-glycans attached to the cancer mucin and exposure of the SM3 epitope. It is of interest to note that even in the rare tumors expressing the C2GnT3 mRNA, the SM3 epitope was detectable, suggesting that the ST3Gal-I enzyme can also compete effectively with this core 2-synthesizing enzyme. Competition in vivo between ST3Gal-I and the
β6GlcNAc transferase expressed in T cells, which could be the C2GnT3 enzyme (21), has also been reported (39). These studies used mice defective in ST3Gal-I to demonstrate that the structure of the O-glycans on molecules such as leukosialin found on peripheral CD8+ T cells has a dramatic effect on the survival and differentiation of these cells. Whether the increased expression of ST3Gal-I, which we have identified in breast cancers, affects function, possibly by affecting cell interactions, is unclear. Certainly core 1 carrying a sialic acid in α2,3 linkage is a major ligand for sialoadhesin, and MUC1 carrying such glycans interacts well with this sialic acid binding protein found on macrophages, which can be found as infiltrates in a proportion of breast cancers (37).

The effect of increasing C2GnT1 activity on MUC1 glycosylation has been studied in a pancreatic carcinoma cell line where again the SM3 epitope was lost (36). The loss of the SM3 glycosylation has been studied in a pancreatic carcinoma cell line infiltrates in a proportion of breast cancers (37).

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