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**SPECIFIC ACCEPTORS FOR TRANSFERASES TO SACCHARIDES AND
METHOD FOR OBTAINING AND USING SAME**

Cross Reference to Related applications

This Application claims priority from United States Provisional Application Serial number 60/727,359, incorporated herein by reference.

Statement regarding Federally Sponsored Research or Development

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Background of the Invention

This invention relates to acceptors for transferases, more particularly glycosyltransferases and sulfotransferases and the screening and use of both known and novel acceptors (substrates) for the detection and quantification of specific transferases.

An important area of cancer research is to search for biomarkers for a very early detection of the disease, thus avoiding the cancer spread and metastasis. Prostate specific antigen (PSA), the biomarker of prostate cancer [Chu, 1997; Brawer, 1999; Milford *et al.*, 2001] performs poorly when used to differentiate prostate cancer from benign prostatic hyperplasia [Bonn, 2002]. Pancreatic cancer is another leading cause of cancer related deaths in Western countries [Magnani *et al.*, 1983; Mangray & King, 1998; Lillemoe *et al.*, 2000]. Monoclonal antibodies for CA19-9, a mucin-associated sialyl Lewis^a, along with other serum tumor markers have been proposed for diagnosis and follow up of these diseases [Magnani *et al.*, 1983]. CA 125 is used world wide as a marker for detection and follow up of ovarian cancer with some limitations [Bast *et al.*, 2002]. At present, biomarkers unique for cancers of breast, colon, stomach, lung and other organs are practically non-existent.

While considerable advances have been made in genomic and proteomic approaches to cancer research, few reports describe cancer glycomics. As an example, genechips have been used to measure the expression of cellular glycosyltransferases

mRNA in human colon tissue. However, mRNA expression may not reflect enzyme levels, which in turn may not predict glycosylation patterns.

Changes in glycosylation are nevertheless commonly observed in human carcinomas [Dennis *et al.*, 1999; Brockhausen, 1999] and may contribute to the malignant phenotype downstream of certain oncogenic events. Carbohydrate antigens associated with cancer can be divided into three major classes: (i) Glycosphingolipids of ganglio and globo series. (ii) Lacto series type 1 (Gal β 1,3GlcNAc) and type 2 (Gal β 1,4GlcNAc) chains carrying sialyl Le^a and sialyl Le^x determinants that can occur in both tumor-associated glycolipids and glycoproteins. (iii) Tumor-associated glycoprotein antigens may be either N- or O-Glycans. Recent studies emphasize the importance of understanding the structure of carbohydrates present in tumor tissue to meet at least two clinical needs. In one aspect, antibodies against unique carbohydrate in tumor-associated antigens, especially those secreted into blood, may provide new and better biomarkers. Secondly, glycans uniquely expressed on cancer cells provide targets for novel cancer therapy. The levels of glycosyltransferase activities are altered in cancer cells and hence target structures can be selected on an enzymatic basis considering the specificity of the particular enzymes involved in the assembly of these complex carbohydrate antigens. Detailed structural determination of carbohydrate chains of already known tumor associated antigens has shown the existence of both N- and O-glycan chains in CA125 [Wong *et al.*, 2003], a glycosylation difference between prostate cancer cell PSA and normal PSA [Okada *et al.*, 2001; Peracaula *et al.*, 2003a; Prakash & Robbins, 2000; Ohyama *et al.*, 2004], and a difference in N-glycan chains of human pancreatic ribonuclease isolated from healthy pancreas vs. pancreatic cancer [Peracaula *et al.*, 2003b]. It has become apparent from these structural analyses of glycans that major changes are at the outer end of carbohydrate chains in cancer-associated antigens. This has prompted the use of lectins to detect the outer end glycosylation changes of tumor-associated glycoconjugates. The changes in the activities of glycosyltransferases reflect the structures of glycans expressed by cancer cells. Thus a knowledge of glycosyltransferase activities in cancer cells can reveal valuable information about the structures likely to be expressed by cancer cells and allow the identification and selection of better carbohydrate epitopes for

immunohistochemical detection of cancer. Tumor associated mucins from breast, pancreas and colon carcinoma are known to contain mucin core 2 branched structures [Fukuda, 1996; Hollingsworth & Swanson, 2004]. The target epitopes that can occur as part of core 2 O-linked glycoproteins associated with cancers can be identified by measuring the activities of various glycosyltransferases on defined acceptors. Mucin core 2 structure Gal β 1, 3(GlcNAc β 1, 6) GalNAc α -Ser/Thr serves as a carrier of selectin ligands [Li *et al.*, 1996]. The malignant potential of a tumor cell is yet to be defined in terms of the relative amounts of sialyl Lewis^x and sialyl Lewis^a, which serve as the selectin ligands, to that of sialyl Tn and Tn epitopes expressed by the same tumor cell [Komminoth *et al.*, 1991; Piller *et al.*, 1991]. As cancer-associated specific changes in the cellular repertoire of glycosyltransferases define the final glycosylation profiles of glycoconjugates [Müller & Hanisch, 2002], the present study was undertaken to examine the patterns of various glycosyltransferase activities in several cancer cells and then verifying these patterns in two tumor tissues.

The structural variability of glycans is dictated by tissue-specific regulation of glycosyltransferase genes, the availability of sugar nucleotides and competition between enzymes for acceptor intermediates during glycan elongation. The studies of Müller & Hanisch [2002] on recombinant MUC1 probe expressed in four breast cancer cell lines indicate that epigenetic parameters like the rate of Golgi passage and the topology of the protein substrate within the Golgi compartment are less important with respect to the final glycosylation profiles than the cellular repertoire of glycosyltransferases. Several glycosyltransferases and Gal3-O-Sulfotransferases are capable of elongating mucin core 2 tetrasaccharide as well as the Globo backbone unit. Such action of these enzymes could lead to a complexity of cancer associated terminal glycan structures, as illustrated in **Fig. 1**. Müller & Hanisch [2002] expressed a MUC1 fusion protein MFP6 in the breast cancer cell lines ZR-75-1, MDA-MB-231, MCF-7 and T47D and studied the O-glycans of the fusion proteins after releasing by hydrazinolysis and then labeling with 2-amino benzamide.

Sialic acids play an important role in a variety of biological processes, like cell-cell communication, cell-substrate interaction, adhesion, maintenance of serum glycoproteins in the circulation, and protein targeting. Cell surface sialic acid occurs

in a variety of structures, which change in a regulated manner during development, differentiation, and oncogenic transformation. Very little is known about the regulation of cell surface sialyl oligosaccharides, which are involved in cellular processes. Sialic acids occur at the terminal positions of the carbohydrate groups of glycoproteins and glycolipids. The transfer of sialic acid from CMP-Sia to these positions is catalyzed post-translationally by a family of glycosyltransferases called sialyltransferases.

To date 19 different human sialyltransferase genes have been identified that catalyze the transfer of sialic acid from CMP-Sia into an $\alpha 2,3$ -, $\alpha 2,6$ - or $\alpha 2,8$ -linkage to the terminal position of carbohydrates on glycoproteins and glycolipids. Analysis of the protein sequences of sialyltransferases reveals, as for most glycosyltransferases, a topological feature characteristic for Type 2 transmembrane proteins. All sialyltransferases cloned to date contain two conserved sialylmotifs in the catalytic part of the enzyme. A large sialylmotif of approximately 48 amino acids is termed the "L (large)-sialylmotif" which participates in the binding of the donor substrate CMP-Neu5Ac and a smaller sequence of about 23 amino acids towards the C-terminal is termed the "S (short)-sialylmotif" which participates in the binding of both the donor and the acceptor. Also, a very short sequence, the "VS-motif", further down towards the C-terminal and comprising only 3-5 amino acids has been identified as a conserved sequence of importance for the enzymatic activity.

Distinct cell-surface carbohydrates are expressed in tissue- and cell-specific manners during development and in adulthood. Aberrations in cell-surface carbohydrates often are associated with malignant transformation and other pathological condition. Among various cell-type-specific carbohydrates, sialyl Lewis x, NeuNAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ (Fuc $\alpha 1 \rightarrow 3$)GlcNAc $\beta \rightarrow$ R, is expressed on neutrophils, monocytes and certain T-lymphocytes and plays a key role in the recruitment of leukocytes. E- and P-selectin expressed on activated endothelial cells capture these leukocytes through binding to sialyl Lewis x, allowing them to roll, which leads to extravasation of leukocytes at inflammatory sites. Lymphocyte circulation is directed by interaction between L-selectin on lymphocytes and the sulfated form of sialyl Lewis x present on L-selectin receptors that are restricted to high endothelial venules.

Such an initial interaction leads to extravasation of lymphocytes from the intravascular compartment to the lymphatic compartment.

The amounts of sialyl Lewis x and sialyl Lewis a, NeuNAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 4)GlcNAc $\rightarrow$ R, which also has been shown to bind to E-selectin are increased significantly in tumor cells such as carcinoma and leukemia. In breast and colonic carcinoma patients, the presence of sialyl Lewis x and sialyl Lewis a is correlated with poor prognosis. These results strongly suggest that blood-borne tumor cells may use a carbohydrate-selectin (or a selectin-related molecule) interaction when tumor cells adhere to the endothelia at metastatic sites.

Tumor dissemination may thus be inhibited by use of sialyl Lewis x oligosaccharides as antagonists. Lewis x is recognized by CD94 on NK cells. Apparently, D94 binds more efficiently to sialyl Lewis x densely attached to carrier glycans than to sialyl Lewis x sparsely attached to carrier glycans. By contrast, sparsely or moderately attached sialyl Lewis x facilitates tumor metastasis. Thus a difference in sialyl Lewis x expression leads to entirely different biological consequences.

Cancer-associated specific changes in the cellular repertoire of glycosyltransferases apparently thus define the final glycosylation profiles of glycoconjugates. The structural analyses of glycans indicate that major changes are at the outer end of carbohydrate chains in cancer-associated antigens. The changes in the activities of glycosyltransferase reflect the altered structures of glycans expressed by cancer cells. Thus a knowledge of glycosyltransferase activities in cancer cells can reveal valuable information about the structures likely to be expressed by cancer cells and allow the identification and selection of better carbohydrate epitopes for development of monoclonal antibodies which can prove to be better biomarkers for cancer. For gaining this knowledge, it is mandatory that specific and sensitive acceptors should become available for measuring the level of various glycosyltransferase activities in cancer sera, cancer cells and tumor tissues. The above important aspect has prompted the development of well-defined acceptors for measuring sialyltransferase and glycosyltransferase activities in presence of other sialyl and glycosyltransferase activities in accordance with the present invention.

Brief Description of the Several Views of the Drawings

Fig. 1A) A composite picture of Biogel P₂ Column-elution profiles for [¹⁴C] sialylated products arising from the action of cloned α 2,3(O)ST on mucin core 2 acceptors Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (○—○—○), 3-O-SulfoGal β 1,4GlcNAc β 1,6 (Gal β 1,3)GalNAc α -O-Bn (●—●—●) and 3-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (●---●---●). CMP[¹⁴C]NeuAc emerged from the Biogel P₂ Column at 56-64 mL with a peak at 60 mL.

Fig. 1B) shows thin layer chromatography [Solvent System: 1-Propanol/NH₄OH/H₂O v/v (12/2/5)] followed by autoradiography for [¹⁴C] sialyl products isolated from the following acceptors as shown in Fig. 1A: 3-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (lane 1), Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (lane 2) and 3-O-SulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (lane 3).

Fig. 2 shows proposed biosynthetic pathways for mucin core 2 heptasaccharide with sialyl Le^x (PSGL-1), core 2 hexasaccharide containing GalNAc Le^x (potential selectin ligand), potential Siglec ligands containing 6'-sialyl, 6-sulfo LacNAc, and core 2 oligosaccharide with 6-Sulfo sialyl Le^x (GlyCAM-1). Key: □ = α -GalNAc; ■ = GlcNAc; ● = Gal; ◆ = NeuAc; □ = Fuc and S = Sulfo group.

Fig. 3 shows sialyltransferase activity at varying concentration of mucin core 2 based tetrasaccharide acceptors for cloned α 2,6(N)ST (panel A), α 2,3(N)ST (panel B), α 2,3(O)ST (panel C) and LNCaP α 2,3(O)ST (panel D). The acceptor used in A and B are Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (●—●—●) and 4-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (○—○—○). In panel C and D the acceptors are Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (●—●—●) and Gal β 1,4GlcNAc β 1,6(4-O-MeGal β 1,3)GalNAc α -O-Bn (○—○—○). Dotted lines in panel C denote enzymatic reaction data when donor CMP-NeuAc was used at the higher concentration in the reaction mixture.

Fig. 4 shows thin layer chromatography of non-radioactive acceptors lane 1. 3-O-MeGal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn, lane 2. Fuc α 1,2Gal β 1, 3(GlcNAc β 1,6)GalNAc α -O-Bn, lane 3. 3-O-SulfoGal β 1,3 (GlcNAc β 1,6)GalNAc α -O-Bn and lane 4. Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al in two solvent systems 1-propanol/NH₄OH/H₂O (12/2/5) in Panel A (developed once) and CHCl₃/CH₃OH/H₂O (5/4/1) in Panel C (developed twice). Thin layer chromatography of [9 ³H] sialyl compounds obtained by the action of cloned α 2,3(O)ST on the above acceptors (1. to 4. in the same sequence). The solvent in Panel B was 1-propanol /NH₄OH/H₂O (12/2/5) (developed once) and in Panel D it was CHCl₃/CH₃OH/H₂O (5/4/1) (developed twice).

Fig. 5 shows WGA-agarose chromatography of [9-³H] sialylated product obtained following the action of cloned α 2,3(O)ST on: A. 3-O-SulfoGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn, B. 3-O-MeGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn, C. Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al, D. Fuc α 1,2Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn, E. A mixture of the product of B above incubated with and without β -N-acetylhexosaminidase, F. Product of C above incubated with β -N-acetylhexosaminidase, G. Product of D above incubated with β -N-acetylhexosaminidase. In all runs, elution was performed with 0.5M GlcNAc after the 10th fraction.

Fig. 6 shows cloned α 2,3(O)ST activity at varying concentrations of acceptors containing modified Galactosyl residue linked β 1,3 to α -GalNAc. A. Acceptor concentration versus enzyme activity for D-Fuc β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn (●—●—●), 4-O-MeGal β 1,3GalNAc α -O-Bn (○—○—○), 4-FGal β 1,3GalNAc α -O-Bn (▲—▲—▲) and D-Fuc β 1,3GalNAc α -O-Bn (□—□—□). B. Lineweaver-Burke plot for data in panel A. C. Acceptor concentration versus enzyme activity for Gal β 1,3GalNAc β 1,3Gal α -O-Me (□—□—□), 3-O-SulfoGal β 1,3GalNAc β 1,3Gal α -O-Me (▲—▲—▲), D-Fuc β 1,3GalNAc β 1,3Gal α -O-Me (○—○—○) and 3-O-SulfoD-Fuc β 1,3GalNAc β 1,3Gal α -O-Me (●—●—●). D. Lineweaver-Burke plot for data in panel C. E. Acceptor concentration versus enzyme activity for

Fuc α 1,2Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn (CMP-NeuAc:15 μ M) ($\circ$ — $\circ$ — $\circ$),
 Fuc α 1,2Gal β 1,3 (GlcNAc β 1,6)GalNAc α -O-Bn (CMP-NeuAc:150 μ M) ($\square$ — $\square$ — $\square$),
 3-O-MeGal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn (CMP-NeuAc:0.3 μ M) ($\square$ — $\square$ — $\square$)
 and 3-O-MeGal β 1,3(6-O-Me)GalNAc α -O-Bn (CMP-NeuAc:0.3 μ M) ($\blacksquare$ — $\blacksquare$ — $\blacksquare$)F.
 Lineweaver-Burke plot for data in panel E.

Fig. 7 shows at A fragmentation of compound 6; at B fragmentation of compound 23; at C fragmentation of compound 7 and at D fragmentation of compound 18 in Table VI under the same CID condition of ESI-MS. Fragments are annotated according to the scheme proposed by Domon and Costello (42).

Fig. 8 shows MS² spectra of double charged molecular species [M-2H]²⁻ at m/z 604 derived from A. compound 6; B. compound 23; C. compound 7 and D. compound 18 in Table VI.

Fig. 9 shows MS³ spectra of A. Y'₂ ion at m/z 917 derived from compound 6; B. Y₃ ion or Y'₂ ion at m/z 917 derived from compound 23 or compound 7 and C. Y₃ ion at m/z 913 derived from compound 18 in Table VI.

Fig. 10 shows various potential competition for glycosyl and sulfotransferases acting on mucin core 2 and Globo backbone.

Brief Description of the Invention

The present invention enables an association of signature carbohydrate structures with individual cancers.

Well-defined acceptors thus provide tools to examine enzyme activities that extend results available using other experimental methods. The unique acceptors, of the present invention, for these enzymes can be used in presence of another enzyme that transfers sugar at other position of the acceptor. In accordance with the present invention, it has thus been found that modified analogs of acceptors with fluoro or methyl groups can be better and specific acceptors for transferase enzymes. For example, we have observed that MeO-2Gal β 1→3GlcNAc β 1-O-Benzyl is a novel

acceptor for STIV as this compound is not prone to α 2,3 sialyltransferase STI,II and α 2,6-sialyltransferase activities.

The invention thus includes a method for determining substrates specific for a transferase enzyme selected from the group consisting of glycosyltransferases and sulfotransferases. The method includes the steps of:

- a) selecting a substrate to be tested for specificity for sulfotransferases and for glycosyltransferases, from tri, tetra or penta saccharide compounds containing at least one $\rightarrow$ GlcNAc β 1 saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow$ 3GalNAc α -OR, $\rightarrow$ 3GlcNAc β -OR or $\rightarrow$ 3Gal α -O-R where R is alkyl, aryl, azido or allyl all of 1 through 8 carbon atoms where R may be substituted with halo, -OH, lower alkyl, -SO₃, or -NO₂ ;
- b) combining the substrate separately with each glycosyltransferase or sulfotransferase in a series, for transfer of a particular glycosyl or sulfo moiety, to a saccharide, in conjunction with a donor compound for the particular moiety, where the moiety is radio labeled, in a buffered aqueous solution of about pH of about 6 to about 7, to form incubation mixtures;
- c) incubating the incubation mixtures at a temperature of from about 18 to about 40 degrees Celcius for from about 30 minutes to about four hours;
- d) testing the resulting incubation mixtures of each separate glycosyltransferase or sulfotransferase for reaction product comprising a chemical combination of substrate and particular moiety to determine effectiveness of each separate glycosyltransferase or sulfotransferase in transferring the particular moiety to the substrate; and.
- e) comparing the effectiveness of each glycosyltransferase or sulfotransferase in transferring the particular moiety to determine whether a particular glycosyltransferase or sulfotransferase acts to transfer the particular moiety to the substrate when the other glycosyltransferases or sulfotransferases do not do so, thus determining whether the substrate acts specifically with a particular glycosyltransferase or sulfotransferase.

The invention also includes a method for the specific detection of a specific transferase for a specific moiety selected from sulfotransferases and glycosyltransferases including the steps of:

- a) selecting a substrate specific for the transferase from tri, tetra or penta saccharide compounds containing at least one pre-terminal saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow 3\text{GalNAc}\alpha\text{-OR}$, $\rightarrow 3\text{Gal}\alpha\text{-O-R}$ or $\rightarrow 3\text{GlcNAc}\beta\text{-OR}$ where R contains 1 to 12 carbon atoms and is alkyl, aryl, azido or allyl and R may be substituted with halo, -OH, lower alkyl, -SO₃ or -NO₂;
- b) combining the substrate with a sample to be tested for the specific transferase in conjunction with a donor compound for the particular moiety, in a buffered aqueous solution of about pH 6 to about pH 7, to form an incubation mixture;
- c) incubating the incubation mixture at a temperature of from about 18 to about 40 degrees Celcius for from about 30 minutes to about four hours; and
- d) testing the resulting incubation mixture for reaction product comprising a chemical combination of substrate and particular moiety to determine the presence of the particular transferase in the sample.

Steps a) through d) are commonly repeated with different concentrations of substrate to determine the minimum concentration of substrate required to maximize quantity of obtained combination of substrate and particular moiety and the determined minimum concentration is correlated with quantity of the specific transferase in the sample.

The invention also includes the newly discovered substrates $\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 6(\text{MeO-4Gal}\beta 1\rightarrow 4)\text{GalNAc}\alpha \rightarrow \text{O-R}$; $\text{MeO-Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 6(\text{Gal}\beta 1\rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$; $\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 6(\text{F-4Gal}\beta 1\rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$; and $\text{F-4Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 6(\text{Gal}\beta 1\rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$ where R contains 1 to 12 carbon atoms and is alkyl, aryl, azido, or allyl, that may be substituted with halo, -OH, -SO₃, or -NO₂.

Detailed Description of the Invention

In accordance with the invention it has been found that many of the substrates used in the invention terminate in $\rightarrow 3\text{GalNAc}\alpha\text{-OR}$ and many of the substrates used in the invention also contain at least one of Gal β 1, DFuc β 1 or an additional $\rightarrow\text{GlcNAc}\beta$ 1 where the Gal β 1, DFuc β 1 or additional $\rightarrow\text{GlcNAc}\beta$ 1 may be substituted with MeO-, EtO-, Allyl-O, or N₃-. The transferase is commonly selected from

sialyltransferases, fucosyltransferases, N-acetylglucosaminyl transferases, N-acetylgalactosaminyl transferases, and galactosyltransferases.

The incubation temperature in the method is usually from about 35 to about 40°C and is most commonly about 37°C.

The substrate may be a novel oligosaccharide compound containing 3 to 5 linked monosaccharides containing at least one $\rightarrow$ GlcNAc β 1 saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow$ 3GalNAc α -OR where R is alkyl, aryl or allyl all of 1 through 8 carbon atoms where R may be substituted with halo, -OH, -SO₃, or -NO₂ and further containing at least one of Gal β 1, DFuc β 1 or an additional $\rightarrow$ GlcNAc β 1 where the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 may be substituted with MeO-, EtO-, Allyl-O, or N₃-.

More specifically the novel compound may be selected from the group consisting of Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-4Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; MeO-4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(F-4Gal β 1 $\rightarrow$ 3) GalNAc α $\rightarrow$ O-R; F-4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; MeO-3Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6) GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6) GalNAc α $\rightarrow$ O-R; D-Fuc β 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 3Gal α -O-methyl; and GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(GalNAc β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R.

The substrate specific for the transferase is usually a tri, tetra or penta saccharide compound containing at least one pre-terminal saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow$ 3GalNAc α -OR, $\rightarrow$ 3Gal α -O-R or $\rightarrow$ 3GlcNAc β -OR where R contains 1 to 12 carbon atoms and is alkyl, aryl or allyl and R may be substituted with halo, -OH, lower alkyl, -SO₃, azido or -NO₂. R is commonly aryl that may be substituted with -OH, halo, or lower alkyl. R is usually, allyl, benzyl, methyl, azido or nitrophenyl and most commonly benzyl.

The substrate usually contains at least one GalNAc β 1 saccharide and most commonly terminates in $\rightarrow$ 3GalNAc α -OR. The substrate also commonly contains at least one of Gal β 1, DFuc β 1 or an additional $\rightarrow$ GlcNAc β 1 where the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 may be substituted with MeO-, EtO-, Allyl-O, or N₃-.

The substrate, is most commonly unsubstituted, with the exception of saccharide linkages, NAc groups and terminal groups. With the exception of

saccharide linkages, the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 are usually unsubstituted.

Following are specific examples of specific substrates, their corresponding specific transferases and appropriate donor compound for providing the transferred moiety.

1. The specific substrate is Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-3Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase IV (ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
2. The specific substrate is MeO-Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
3. The substrate is Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(F-3Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase IV (ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
4. The substrate is F-3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
5. The substrate is Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-4Gal β 1 $\rightarrow$ 4)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,4 N-acetylglucosaminyl transferase II (α 1,4-GNTII) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
6. The substrate is (MeO-4Gal β 1 $\rightarrow$ 4)GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,4 N-acetylglucosaminyl transferase I (α 1,4-GNTI) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
7. The substrate is Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(F-4Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,4 N-acetylglucosaminyl transferase II (α 1,4-GNTII) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
8. The substrate is F-4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1 $\rightarrow$ 4 N-acetylglucosaminyl transferase I (α 1,4-GNTI) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
9. The substrate is NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-3Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is the sulfotransferase,

galactose 6 sulfotransferase I (Gal-6-SulfoTI) and the donor compound is 3'phosphoadenosine 5'phosphosulfate (PAPS).

10. The substrate is $\text{MeO-3Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{NeuAc}\alpha 2 \rightarrow 3)\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is the sulfotransferase, galactose 6 sulfotransferase II (Gal-6-SulfoTII) and the donor compound is 3'phosphoadenosine 5'phosphosulfate (PAPS).
11. The substrate is $\text{Gal}\beta 1 \rightarrow 4\text{MeO-3GlcNAc}\beta 1 \rightarrow 6(\text{GalNAc}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,2$ L-fucosyltransferase I ($\alpha 1,2$ -L-FT I) and the donor compound is guanosine diphosphate fucose (GDP-fucose).
12. The substrate is $\text{GalNAc}\beta 1 \rightarrow 4(\text{MeO-3GlcNAc})\beta 1 \rightarrow 6(\text{Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,2$ L-fucosyltransferase II ($\alpha 1,2$ -L-FT II) and the donor compound is guanosine diphosphate fucose (GDP-fucose).
13. The substrate is $\text{GalNAc}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{GalNAc}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,3$ L-fucosyltransferase ($\alpha 1,3$ -L-FT) and the donor compound is guanosine diphosphate fucose (GDP-fucose).
14. The substrate is $\text{Gal}\beta 1 \rightarrow 3(\text{GlcNAc}\beta 1 \rightarrow 6)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\beta 1 \rightarrow 3/4$ N-acetyl galactosaminyl transferase ($\beta 1 \rightarrow 3/4$ GalNAc-T) and the donor compound is uridine diphosphate galactosamine (UDP-GalNAc).
15. The substrate is $\text{MeO-3Gal}\beta 1 \rightarrow 3(\text{GlcNAc}\beta 1 \rightarrow 6)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is an αX N-acetyl glucosaminyl transferase ($\alpha X\text{GlcNAc-T}$) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
16. The substrate is $2\text{-O-MeGal}\beta 1 \rightarrow 4\text{GlcNAc}\beta\text{-O-benzyl}$, the specific transferase is sialyl transferase IV (Gal3ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
17. The substrate is $4\text{-O-MeGal}\beta 1 \rightarrow 4\text{GlcNAc}\beta\text{-O-benzyl}$, the specific transferase is sialyl transferase IV (Gal3ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
18. The substrate is $\text{D-Fuc}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha\text{-O-methyl}$, the specific transferase is sialyl transferase I/II (Gal3ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

19. The substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

20. The substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

21. The substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

22. The substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

Sialic acids are key determinants in many carbohydrates involved in biological recognition. As a further aspect of the present invention, We studied the acceptor specificities of three cloned sialyltransferases (STs) α 2,3(N)ST, α 2,3(O)ST and α 2,6(N)ST, and another α 2,3(O)ST present in prostate cancer cell LNCaP towards mucin core-2 tetrasaccharide [Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn] and Globo [Gal β 1,3GalNAc β 1,3Gal α -O-Me] structures containing sialyl-, fucosyl-, sulfo-, methyl- or fluoro- substituents by identifying the products by electrospray ionization tandem mass spectral analysis and other biochemical methods. The Globo precursor was an efficient acceptor for both α 2,3(N)ST and α 2,3(O)ST whereas only α 2,3(O)ST used its deoxy analog [D-Fuc β 1,3GalNAc β 1,3-Gal α -O-Me]; 2-O-MeGal β 1,3GlcNAc and 4-OMeGal β 1,4GlcNAc were specific acceptors for α 2,3(N)ST. Other major findings in this study were: **i)** α 2,3 sialylation of β 1,3Gal in mucin core 2 can proceed even after α 1,3fucosylation of β 1,6-linked LacNAc; **ii)** sialylation of β 1,3 Gal must precede the sialylation of β 1,4Gal for favorable biosynthesis of mucin core 2 compounds; **iii)** α 2,3 sialylation of 6-O-SulfoLacNAc moiety in mucin core 2 (eg. GlyCAM-1) is facilitated when β 1,3Gal has already been α 2,3 sialylated; **iv)** α 2,6(N)ST was absolutely specific for the β 1,4 Gal in mucin core 2. Either α 1,3 fucosylation or 6-O-Sulfation of the GlcNAc moiety reduced the

activity. Sialylation of $\beta 1,3\text{Gal}$ in addition to 6-O-sulfation of GlcNAc moiety abolished the activity; **v)** prior $\alpha 2,3$ -sialylation or 3-O-sulfation of $\beta 1,3\text{Gal}$ would not affect $\alpha 2,6$ -sialylation of $\text{Gal}\beta 1,4\text{GlcNAc}$ of mucin core 2; **vi)** 3- or 4-Fluoro substituent in $\beta 1,4\text{Gal}$ resulted in poor acceptors for the cloned $\alpha 2,6(\text{N})\text{ST}$ and $\alpha 2,3(\text{N})\text{ST}$ whereas 4-Fluoro or 4-OMe $\text{Gal}\beta 1,3\text{GalNAc}\alpha$ was a good acceptor for cloned $\alpha 2,3(\text{O})\text{ST}$; **vii)** 4-O-methylation of $\beta 1,4\text{Gal}$ abolished the acceptor ability towards $\alpha 2,6(\text{N})\text{ST}$ but increased the acceptor efficiency considerably towards $\alpha 2,3(\text{N})\text{ST}$; **viii)** just like LNCaP $\alpha 1,2$ -FT and Gal-3-O-sulfotransferase T2, the cloned $\alpha 2,3(\text{N})\text{ST}$ which modifies terminal Gal in $\text{Gal}\beta 1,4\text{GlcNAc}$ also efficiently utilizes the terminal $\beta 1,3\text{Gal}$ in the Globo backbone. Utilization of C-3 block compounds such as 3-O-sulfo- $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-OMe}$ as acceptors by cloned $\alpha 2,3(\text{O})\text{ST}$ and analyses of the resulting products by lectin chromatography and mass spectrometry indicate that $\alpha 2,3(\text{O})\text{ST}$ is capable of attaching NeuAc to another position in C-3 substituted $\beta 1,3\text{Gal}$.

Sialyl groups of cell surface and secreted glycoconjugates (1,2) can act as binding targets for viruses, bacteria, parasites and toxins (3,4) and also can recognize mammalian selectin and Siglec lectins (3). Sialyltransferases (STs) attach sialic acid to galactose via $\alpha 2,3$ and $\alpha 2,6$ glycosidic bonds, and to GalNAc via $\alpha 2,6$ linkages and also form polysialic acid via $\alpha 2,8$ -linkage.

Siglecs in contrast to the majority of immunoglobulin superfamily members, which recognize protein ligands, bind to specific sialylated glycans (5-7). The carbohydrate recognition motif of Siglec-2, Siglec-5 and Siglec-7 have been identified as $\text{NeuAc}\alpha 2,6\text{Gal}\beta 1,4\text{Glc}$ (8-10). A role for $\alpha 2,6$ linked sialic acid as a modulator of immune cell interaction in B-cells is evident from the finding that B-cell activation with polyclonal anti-IgM and interferon- γ , leads to specific loss of $\alpha 2,6$ linked sialic acid, and this creates access to costimulatory molecules for the cell surface (11).

Selectins are another family of sialic acid recognizing adhesion molecules, containing amino terminal C-type lectin domains (12-15). They recognize sialyl Lewis-x or sialyl Lewis-a motifs (16-18). Our studies confirmed the importance of $\alpha 2,3$ linked sialic acid and $\alpha 1,3/4$ linked fucose for selectin recognition of ligands,

and they demonstrate the role of the mucin core 2 structure in enhancing L- and P-selectin binding to carbohydrates (19). We have also demonstrated the contribution of the NeuAc α 2,3Gal β 1,3GalNAc α - sequence of mucin core 2 structure in modulating L- and P-selectin binding function (20). Finally, we also showed a unique carbohydrate sequence lacking sialic acid namely GalNAc β 1,4(Fuc α 1,3)GlcNAc β -(GalNAc Lewis x) that could act as a ligand for E-selectin (20). As compared to NeuAc α 2,3Gal β 1,4(Fuc α 1,3) GlcNAc β -O-Me (sialyl Lewis-x), we found GalNAc β 1,4(Fuc α 1,3)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3) GalNAc α -O-Me to be a 5-6 fold better inhibitor of L- and P-Selectin binding.

Previously, we used various modified disaccharides as acceptors for studying the specificities of a purified porcine liver α 2,3ST acting on Gal β 1,3GalNAc α - and a cloned α 2,3ST utilizing Gal β 1,3/4GlcNAc β - (21). In view of the importance of sialic acid residues in various biological recognition phenomena, we decided to study in detail the acceptor substrate specificities of three clonal sialyltransferases and also another sialyltransferase that is strictly specific for Gal β 1,3GalNAc α - sequence. A variety of mucin core 2 and Globo based synthetic compounds and related simple structures were utilized as acceptors in order to gain insight into the sialylation sequence in the biosynthesis of complex carbohydrate ligands. The study also reveals a novel action of cloned α 2,3(O)sialyltransferase in catalyzing the attachment of sialic acid to another position of Galactose in Gal β 1,3GalNAc moiety containing a C-3 substituent.

The prostate carcinoma cell line, LNCaP, was grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (penicillin, streptomycin and amphotericin B) under conditions recommended by American Type Culture Collection (Manassas, VA). The cells were homogenized with 0.1M Tris-Maleate pH6.3 containing 2% Triton X-100 using a Dounce all glass hand-operated homogenizer. The homogenate was centrifuged at 16,000g for 1 h at 4°C. Protein concentration in supernatant was measured using the BCA assay (Pierce Biotech, Inc., Rockford, IL), with BSA as the standard. The supernatant was adjusted to 5mg

protein/ml by adding the necessary amount of extraction buffer and then stored frozen at -20°C until use. 10 μl aliquot of this extract was used in assays run in duplicate.

Cloned Sialyltransferases:

Rat recombinant $\alpha 2,3(\text{O})\text{ST}$, $\alpha 2,3(\text{N})\text{ST}$ and $\alpha 2,6(\text{N})\text{ST}$ were purchased from Calbiochem and stored at either -20°C or -70°C as recommended by the supplier. Suitable aliquots were diluted with 1.0ml of 0.1M NaCacodylate buffer pH6.0 containing 2% Triton CF-54 and 2% BSA and used in the experiments; $\alpha 2,3(\text{O})\text{ST}$ and $\alpha 2,6(\text{N})\text{ST}$ as diluted above were found to retain full activity for at least three months when stored frozen at -20°C . $\alpha 2,3(\text{N})\text{ST}$ was diluted just before use in the experiment. 10 μl aliquots of the diluted enzymes were used in the assays run in duplicate.

Synthetic acceptors:

The synthesis of several compounds that are used as acceptors in the present study have been published (20,22-24). The synthesis of acceptors containing the Globo H precursor structure, namely, $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Al}$; $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; $\text{D-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; $3\text{-O-SulfoGal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and $3\text{-O-Sulfo-D-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and mucin core 2 tetrasaccharides containing 4-O-Me group and complex structural units will be published elsewhere. Mass spectrometry analysis of many of these compounds is presented in this manuscript. The synthesis of mucin core 2 tetrasaccharides containing 3-F group has been reported (25).

Macromolecular and natural acceptors:

Acrylamide copolymer of $\text{Gal}\beta 1,3\text{GalNAc}\alpha\text{-O-Al}$ synthesized by the procedure of Horejsi *et al.* (26) and Fetuin triantennary asialo glycopeptide were available from earlier studies of this laboratory (21, 27-29).

Assay of sialyltransferases:

The incubation mixtures run in duplicate contained 0.1M NaCacodylate buffer pH6.0, the acceptor (typically at 7.5mM, or as indicated in some experiments), CMP-[9- ^3H] NeuAc (typically 0.2 μCi ; 20 $\mu\text{Ci/nmol}$ or as indicated in some experiments) and the enzyme in a total volume of 20 μl . The control incubation mixtures contained everything except the acceptor. Incubation was carried out for 2h at 37°C . The

enzymatic transfer of [9-³H] NeuAc to a typical acceptor was linear for 2h and less than 30% CMP-[9-³H] NeuAc was utilized. Chromatography, using either Dowex-1-Formate, Sep-Pak C18 or Biogel P2, was applied to separate radioactive product from un-reacted [9-³H] NeuAc as described below. The values for the duplicate runs did not vary by more than 5%.

The radioactive products from neutral allyl and methyl glycosides as well as non-glycosides were measured by fractionation on Dowex-1-Formate (Bio-Rad:AG-1X8; 200-400 mesh; format form) as follows: The incubation mixture was diluted with 1.0ml water and passed through AG-1-formate column (1.0ml bed volume in a Pasteur pipet which had been washed with 5ml of 2M Formic acid followed by 10ml water). The column was washed twice with 1.0ml water after the entry of the sample and then eluted with 3.0ml of 0.1M NaCl. The radioactivity present in water and 0.1M NaCl eluates were measured separately using 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL) and a Beckman LS6500 scintillation counter. The CPM values were corrected by subtracting the blank CPM. Any radioactivity present in the water eluate (as noticed in the case of some methylglycosides) was added to the corresponding CPM value of 0.1M NaCl eluate. The radioactive products from sulfated and/or sialylated methylglycosides were also measured by the above procedure, only the elution of the AG-1-Formate column was continued further with 3.0ml of 0.2M NaCl for achieving a complete elution of the radioactive product, a correction being made as before by subtracting the corresponding blank values.

The radioactive products from benzylglycosides and monosialylated benzylglycosides were measured by hydrophobic chromatography on Sep-Pak C18 cartridge (Waters, Milford, MA) and eluting the product with 3.0ml methanol (30). The radioactivity was determined by liquid scintillation as above.

Radioactive sialylation products from sulfated benzylglycosides were quantitated by fractionation of the reaction mixture on a BioGel P2 column, since these products were not elutable from AG-1-Formate column even by 0.2M NaCl, and they did not bind to Sep-Pak C18. For such work, a Biogel P2 column (Fine Mesh; 1.0x116.0cm) was used with 0.1M pyridine acetate pH5.4 as the eluent at room

temperature. The effluent fractions were monitored for radioactivity and the first peak containing radioactivity was collected, lyophilized to dryness, dissolved in 200 μ l of water and stored frozen at -20°C for thin layer chromatography (TLC) and other experimentation.

Thin layer chromatography:

TLC was carried out on Silica gel GHLF (250 μ m, scored 20X20cm; Analtech, Newark DE). The solvent systems 1-propanol/NH₄OH/H₂O (v/v 12/2/5) and CHCl₃/CH₃OH/H₂O (v/v5/4/1) were used. The acceptor compounds were located on the plates by spraying with sulfuric acid in ethanol and heating at 100°C. The radioactive products were located by scraping 0.5cm width segments of silica gel and soaking in 2.0ml water in vials followed by liquid scintillation counting. Fluorography of the TLC plates was done at -70°C using Bio-Max MS Film (Eastman Kodak) after spraying the TLC plates with Enhance (Dupont).

WGA-, PNA- and RCA-I-agarose affinity chromatography:

A column of 5ml bed volume of lectin-agarose (Vector Lab, Burlingame, CA) was employed using 10mM Hepes pH7.5 containing 0.1mM CaCl₂, 0.01mM MnCl₂ and 0.1% NaN₃ as the running buffer. Fractions of 1.0ml were collected. The bound material was then eluted with 0.5M GlcNAc after the 10th fraction in the case of WGA-agarose, or with 0.2M Galactose after the 12th fraction for the RCA-I-agarose and PNA-agarose columns in the same buffer as recommended by the manufacturer. Fractionation was done at room temperature.

Mass spectral analysis of enzymatically sialylated compounds:

Reaction mixtures contained 0.3 μ mol acceptor, 0.2 μ mol CMP-NeuAc, 1.0 μ Ci CMP [9-³H] NeuAc, 30 μ g BSA, 0.1M Na Cacodylate pH 6.0 and 20milliunit of the cloned sialyltransferase in a total volume of 100 μ l and incubated for 18h at 37°C. After incubation, the reaction mixture was diluted with 1.0ml water and then subjected to column chromatography on a Biogel P₂ column (1.0x116.0 cm) as described above. The first peak containing the radioactive product was collected, lyophilized to dryness, dissolved in 200 μ l water and stored frozen at -20°C until mass spectral analysis.

MSⁿ experiments (31) were carried out with an Esquire-LC, Bruker-HP ion trap (Bremen, Germany). Samples at 10pM/μL in MeOH were infused into the electrospray source via a 50μm id fused silica capillary using a syringe pump at a flow rate of 5μl/min. Nitrogen was used as the nebulizing gas (at 5-6 psi) and also as the drying gas (5-7 l/min at 200°C). The potentials of the spray needle, capillary exit, and skimmer were set to ± 4,000, 90-150, and 25-50 V, respectively. Helium was the buffer/collision gas. For each spectrum 100-500 scans were averaged. The fragmentation amplitude was varied between 0.8 and 1.5 depending on the experiment design. The fragmentation amplitude was 1.15 for MS² and 1.20 for MS³. The fragmentation time was 40 ms. Typically, the low mass cutoff was set at slightly less than 1/3rd the precursor m/z value.

All monosialyated or monosulfated oligosaccharides were found to lose protons easily to yield [M-H]⁻ ion in negative mode. The oligosaccharides bearing both sialyl and sulfate groups were detected as double charged ions [M-2H]²⁻ in negative mode. The monosialyated or monosulfated oligosaccharides yielded singly charged doubly sodiated ions [M-H+2Na]⁺ in positive mode. The oligosaccharides bearing both sialyl and sulfate group were detected as doubly charged quadruply sodiated ions [M-2H+4Na]²⁺ in positive mode. In contrast to the positive mode, the negative mode could easily avoid the interference of ions from impurity and this yielded higher signal intensity of the product ions. However, the sodiated adduct ions detected in the positive mode provided additional information of the molecular weight, and they confirmed the results got from negative polarity electrospray mass spectra.

Sialyltransferase assay

Sialyltransferase activity was measured using methods which we have documented elsewhere (21). In addition to these published protocols, in the current manuscript, the BioGel P2 column was used to separate [9-³H] labeled benzyl glycosides that were both sulfated and sialylated from unreacted CMP-[9-³H]NeuAc (Fig. 1). In this figure, sample Biogel P2 elution profiles are shown for three radioactive products along with corresponding autoradiographs of these products following TLC.

Modified disaccharides and GloboH based acceptors can distinguish between the cloned enzymes (Table I, part A)

The cloned $\alpha 2,3(\text{N})\text{ST}$ and $\alpha 2,6(\text{N})\text{ST}$ displayed preference for Gal $\beta 1,4\text{GlcNAc}\beta\text{-O-Al}$ rather than Gal $\beta 1,3\text{GalNAc}\alpha\text{-O-Al}$, though $\alpha 2,3(\text{N})\text{ST}$ showed 24.1% activity on the latter compound; $\alpha 2,3(\text{O})\text{ST}$ and LNCaP STs, on the other hand, showed higher specificity for Gal $\beta 1,3\text{GalNAc}\alpha\text{-O-Al}$ compared to Gal $\beta 1,4\text{GlcNAc}\beta\text{-O-Al}$ and also acted efficiently on Gal $\beta 1,3(\text{GlcNAc}\beta 1,6)\text{GalNAc}\alpha\text{-O-Al}$.

We examined various modifications of the above disaccharides to determine if unique acceptors could be identified. We observed that $\alpha 2,6(\text{N})\text{ST}$ utilized Gal $\beta 1,4\text{GlcNAc}\beta\text{-O-Al}$ and 2-O-MeGal $\beta 1,4\text{GlcNAc}$ as acceptors (100.0% and 90.1% active), but had very low activity towards 3-O-MeGal $\beta 1,4\text{GlcNAc}$ (8.7% active), Gal $\beta 1,3\text{GlcNAc}\beta\text{-O-Al}$ (3.0%), 2-O-MeGal $\beta 1,3\text{GlcNAc}$ (3.7%) and 2-O-MeGal $\beta 1,3\text{GlcNAc}\beta\text{-O-Bn}$ (0%). $\alpha 2,3(\text{O})\text{ST}$ exhibited either very low or negligible activity with acceptors 2-O-MeGal $\beta 1,3\text{GlcNAc}$ (2.9%), 2-O-MeGal $\beta 1,4\text{GlcNAc}$ (0.2%) and 3-O-MeGal $\beta 1,4\text{GlcNAc}$ (3.6%). $\alpha 2,3(\text{N})\text{ST}$ was the only enzyme that acted efficiently to sialylate 2-O-MeGal $\beta 1,3\text{GlcNAc}$ (115.3%), suggesting that this may be a unique acceptor for this enzyme. It also acted efficiently on Gal $\beta 1,3\text{GlcNAc}\beta\text{-O-Al}$ (123.1%) and 2-O-Me-Gal $\beta 1,4\text{GlcNAc}$ (60.0%).

The GloboH precursor Gal $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and its deoxy analog D-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{-Gal-O-Me}$ could be used to distinguish between the enzymes. $\alpha 2,3(\text{O})\text{ST}$ utilized both the GloboH precursor (120.8% active) and its deoxy analog

(115.6%). $\alpha 2,3(N)$ ST on the other hand acted efficiently on the Globo H precursor (106.8% active) but not its deoxy analog (3.1% active) indicating that the C-6 hydroxyl group of the $\beta 1,3$ -linked Gal in Globo H precursor is absolutely required for this enzyme activity. In contrast, $\alpha 2,6(N)$ ST was inactive with both the GloboH based acceptor (1.1%) and its deoxy analog (2.5%). The sialyltransferase from LNCaP utilized these two acceptors at lower activities of 34.3% and 9.6% respectively. Key results regarding the specificity for GloboH precursors were verified using mass spectroscopy in Table VI. Overall, these results demonstrate that 2-O-MeGal $\beta 1,3$ GlcNAc is a unique acceptor for $\alpha 2,3(N)$ ST. All enzymes can also be distinguished by their action on the Globo H precursor and its deoxy analog.

Specificity of sialyltransferases for core-2 structure

The STs acted on the core-2 tetrasaccharide and 3-O-methyl analogs of this molecule in a manner that was largely expected based on our above studies with disaccharides (Table I, part B). $\alpha 2,3(N)$ ST and $\alpha 2,6(N)$ ST showed specificity towards the Gal $\beta 1,4$ GlcNAc β side chain in the tetrasaccharide while the sialyltransferases from LNCaP preferred to act on the Gal $\beta 1,3$ GalNAc α moiety. While $\alpha 2,3(N)$ ST was 24.1% active towards Gal $\beta 1,3$ GalNAc α -O-Al it displayed somewhat lower activity (6.1% activity) towards 3-O-MeGal $\beta 1,4$ GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Bn. Surprisingly, $\alpha 2,3(O)$ ST displayed activity towards both methyl analogs of the tetrasaccharide suggesting that it can have multiple sites of action including, but not limited to, the 3-position of Gal in Gal $\beta 1,3$ GalNAc α . This finding is elaborated upon later in Results.

Defining specific acceptors to distinguish between sialyltransferases from LNCaP and $\alpha 2,3(O)$ ST

The sialyltransferase from LNCaP exhibited strict specificity for Gal $\beta 1,3$ GalNAc α while $\alpha 2,3(O)$ ST displayed broader specificity. This is evident upon comparison of the two methylated tetrasaccharides, 3-O-MeGal $\beta 1,4$ GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Bn and Gal $\beta 1,4$ GlcNAc $\beta 1,6$ (3-O-MeGal $\beta 1,3$)GalNAc α -O-Bn. While the cloned enzyme was 100.0% and 49.6% active respectively towards these acceptors, the LNCaP enzyme was 100.9% and 3.0% active

respectively. The strict specificity of LNCaP enzyme towards Gal β 1,3GalNAc α is also evident from its negligible activity towards Gal β 1,3GlcNAc β 1,3Gal β -O-Me (1.4%) as compared to the cloned enzyme (37.2%). Further, these two enzymes differ in their action since, while the cloned enzyme utilized both Gal β 1,3GalNAc α -O-Al and the T-hapten in the mucin core 2 structure as acceptors to the same extent (112.0% and 100.0%, respectively.), the LNCaP enzyme was only 1/3 efficient towards Gal β 1,3GalNAc α -O-Al (34.4% active). The cloned enzyme and the LNCaP enzyme differed in using Gal β 1,3GlcNAc β -O-Al (42.1% and 3.7% active respectively). Finally, the addition of Gal to the β 1,6-linked GlcNAc of mucin core 2 structure increased the activity of LNCaP enzyme (from 60.4% to 100.0%) in contrast to the behavior of the cloned enzyme (from 120.5% to 100.0%).

In order to define a simple specific acceptor for assaying cloned α 2,3(O)ST, in presence of other α 2,3(O)STs like those resembling LNCaP ST, we examined the action of both enzymes on a series of disaccharide analogs, namely, 3-O-MeGal β 1,3GalNAc α -O-Bn; 4-O-MeGal β 1,3GalNAc α -O-Bn; 4-FGal β 1,3GalNAc α -O-Bn; Gal β 1,3(6-OMe)GalNAc α -O-Bn and 3-O-MeGal β 1,3(6-O-Me)GalNAc α -O-Bn along with Gal β 1,3GalNAc α -O-Bn (Table II). Mass spectroscopy analysis was performed with some of the enzymatic products as discussed in Table VI. These studies were conducted at two different acceptor concentrations. We observed that the cloned enzyme exhibited 70.4% and 55.9% activities towards 3-O-MeGal β 1,3GalNAc α -O-Bn and 3-O-MeGal β 1,3(6-O-Me)GalNAc α -O-Bn respectively as compared to Gal β 1,3GalNAc α -O-Bn at 0.75 mM, which is the saturation point for the maximum activity. LNCaP α 2,3(O)ST exhibited negligible activities towards these acceptors (2.6% and 1.1% respectively). Hence, 3-O-MeGal β 1,3GalNAc α -O-Bn and 3-O-MeGal β 1,3(6-O-Me)GalNAc α -O-Bn could be used as specific acceptors for the cloned α 2,3(O)ST enzyme. In fact, the latter compound may be absolutely specific for this enzyme since α 2,6sialyltransferase acting on α -GalNAc cannot act on this acceptor.

Biosynthesis of mucin core-2 based glycans

Enzyme specificity data of the present study (Table I, Part B) indicates the possible sequence in sialylation in the biosynthetic pathways of glycans such as PSGL-1, GalNAc Lewis-x, GlyCAM-1 and potential Siglec ligands (Fig. 2). However, one must be aware of the possibilities *in vivo* that a) the recombinant enzymes may not fully acquire the conformation of the native enzymes; b) the concentration of the substrates in the cell may dictate the action of enzymes in the competing pathways involved in multiple glycoconjugate synthesis; c) the conformation of the active site of these enzymes is sensitive to temperature and pH and d) alternative, albeit minor pathways for the same glycan elongation may exist in the cell.

A. PSGL-1 and GalNAc-Lewis x

Singly fucosylated mucin core-2 based glycans with terminal sialyl Lewis x structure and a tyrosine-sulfated peptide display high affinity binding for P- and L-selectin (32). Several investigator have also demonstrated that fucosylation can be the last step in the biosynthesis of glycans borne on PSGL-1 (33-37). Our enzymatic studies provide additional insight into the biosynthesis of such mucins.

First, our results suggest that $\alpha 2,3$ sialylation of Gal residue can proceed even after $\alpha 1,3$ fucosylation of GlcNAc residue. In support of this proposition, we observed that Lewis x and (GalNAc) Lewis x determinants on the core-2 mucin did not affect the activity of clonal $\alpha 2,3$ (O)ST. [Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me: 133.4% GalNAc $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me: 134.9%]. Sialyltransferase from LNCaP was also active towards the GalNAc Lewis-x containing mucin core 2 acceptor, albeit at a lower level (21.1% activity); Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me and Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$) GalNAc α -O-Bn served as acceptors for $\alpha 2,3$ (N)ST to a significant, albeit low level (19.0% and 27.1% active respectively).

Second, regarding the sequence of sialylation, our results suggest that sialylation of $\beta 1,3$ -linked Gal must precede the sialylation of $\beta 1,4$ linked Gal for favorable biosynthesis of mucin core 2 backbone compounds. This proposition is supported by the following lines of evidence: i) Sialylation of $\beta 1,3$ -linked Gal in

mucin core 2 enhanced the $\alpha 2,3$ sialylation of $\beta 1,4$ linked Gal via $\alpha 2,3(N)$ ST as evident from a comparison of the activity of this enzyme towards Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α - and Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$)GalNAc α - (19.0% and 27.1%, respectively), and upon comparison of Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α - with Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$)GalNAc α - (28.1% and 50.8%, respectively; ii) When the LacNAc moiety of mucin core 2 was $\alpha 2,3$ sialylated, as evident from using the acceptor NeuAc $\alpha 2,3$ Gal $\beta 1,4$ GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me, the $\alpha 2,3$ sialylation of Gal $\beta 1,3$ GalNAc α -moiety was greatly reduced (Cloned $\alpha 2,3(O)$ ST enzyme 35.2% and LNCaP enzyme 5.1%).

Taken together, the above results suggest that Leukosialin would form from mucin core 2 tetrasaccharide by the sequential action of $\alpha 2,3(O)$ ST and $\alpha 2,3(N)$ ST. PSGL-1 glycans would form from further action of FTVII. PSGL-1 can also arise from mucin core 2 tetrasaccharide by the sequential action of FTIV (or possibly FTIII, FTV or FTVI) followed by $\alpha 2,3(O)$ ST and $\alpha 2,3(N)$ ST (Fig. 2). GalNAc-Lewis x type structures on the core-2 tetrasaccharide can likewise be formed by the sequential action of $\beta 1,4$ GalNAc transferase, $\alpha 1,3$ fucosyltransferase and $\alpha 2,3(O)$ ST (Fig. 2).

B. GlyCAM-1

From previous studies, it is evident that GlcNAc:6-O-sulfotransferase does not act on the core-2 tetrasaccharide since it requires terminal GlcNAc for its action (38-40). This suggests that 6-sulfation precedes $\beta 1,4$ Galactosyltransferase activity in core-2 mucins. Building upon this observation, in the current work, we examined the action of sialyltransferases on an array of 6-O-sulfated structures to determine the potential biosynthetic pathway that leads to formation of sulfated core-2 structures. First, we observed that $\alpha 2,3(N)$ ST acted more efficiently on the sialylated sulfated mucin core 2 compound Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$)GalNAc α -O-Me (50.8% active) compared to Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me (28.1% active). Also, this 3'Sialyl,6-O-Sulfo Lewis x determinant dramatically decreased the activity of clonal $\alpha 2,3(O)$ ST down to 7.4%. These observations support the proposition that, like PSGL-1, $\alpha 2,3$ sialylation of 6-O-SulfoLacNAc moiety in

mucin core 2 is facilitated when the β 1,3-linked Gal moiety has already been α 2,3 sialylated. In other words, α 2,3(O)ST action likely precedes α 2,3(N)ST action in the case of GlyCAM-1 biosynthesis.

We observed that human FTVII enzyme, obtained from extracts of COS-7 cells that were transfected with FTVII cDNA, were capable of efficiently transferring [14 C]Fuc to the acceptor NeuAc α 2,3Gal β 1,4(6-O-Sulfo)GlcNAc β 1,3Gal β 1,4(6-O-Sulfo)GlcNAc β -O-Me (Chandrasekaran, Xia, Neelamegham and Matta, unpublished results). Further the fucosylated acceptor Gal β 1,4(6-O-Sulfo)(Fuc α 1,3)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α -O-Me had very low activity (1.7% active) towards α 2,3(N)ST suggesting that fucosylation must occur after sialylation. Based on the above, we suggest that the biosynthesis of GlyCAM-1 starting from GlcNAc β 1,6(Gal β 1,3)GalNAc α -, involves the sequential action of GlcNAc:6-O-Sulfo-T, β 1,4Gal-T, α 2,3(O)ST, α 2,3(N)ST and finally FTVII (Fig. 2). The sialylation steps in this reaction scheme were validated by analyzing reaction products using mass spectroscopy (Table VI).

While our data above suggests that sulfation must be one of the first steps in the biosynthetic pathway, we also note that 6-O-Sulfation of β 1,6-linked GlcNAc in mucin core 2 reduces the activity of α 2,3(O)STs. In the regard, Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Me was 27.2% active towards clonal α 2,3(O)ST and 14.8% active towards sialyltransferase from LNCaP. Similarly, cloned α 2,3(O)ST was 49.4% and 69.4% active with acceptors 6-O-SulfoGlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn and Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn respectively. Not only 6-O-sulfation, but 3-O-sulfation of LacNAc moiety also decreased the enzyme activity of α 2,3(O)ST to act on the core-2 mucin since 3-O-sulfo Gal β 1,4 GlcNAc β 1,6 (Gal β 1,3)GalNAc α -O-Bn was only 70.3% active. Our previous study indicated that sialylated core-2 acceptor, namely NeuAc α 2,3Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn, can act as an acceptor for GlcNAc:6-O-sulfotransferase (40). Thus, as an alternate scheme (not shown in Fig. 2), it may be possible that α 2,3(O)ST action may precede 6-O-sulfation of GlcNAc in the GlyCAM-1 biosynthetic pathway.

C. Siglec ligands

The present study reports the formation of α 2,6sialylated and 6-O-sulfated mucin core-2 based compounds which may serve as potential ligands of Siglecs. In our experiments (Table I, part B), we observed that α 2,6(N)ST was absolutely specific for the β 1,4 linked Gal moiety in mucin core 2. Either α 1,3 fucosylation or 6-O-Sulfation of the GlcNAc moiety reduced the activity (31.0% and 30.6%, respectively). Sialylation of β 1,3-linked Gal in mucin core 2 in addition to 6-O-sulfation of GlcNAc moiety reduced the activity drastically: Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α -O-Me was active at 0.9% and Gal β 1,4(6-O-Sulfo)(Fuc α 1,3)GlcNAc β 1,6 (NeuAc α 2,3Gal β 1,3)GalNAc α -O-Me was 0% active. This suggests that action of α 2,3(O)ST cannot be followed by α 2,6(N)ST action in the case of 6-O-sulfated core-2 mucin (concept illustrated in Fig. 2). Gal β 1,4(Fuc α 1,3)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3) GalNAc α -O-Bn, on the other hand, served as a reasonable acceptor for α 2,6(N)ST (31.0%), suggesting that prior α 2,3-sialylation of β 1,3-linked Gal would not affect α 2,6-sialylation of β 1,4-linked Gal in non-sulfated mucin core 2 tetrasaccharide. Also, 3-O-sulfation of β 1,3-linked Gal in mucin core 2 did not affect the activity of α 2,6(N)ST (127.4%). Based on these results, the complex Siglec ligand, namely, NeuAc α 2,6Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α - could arise from Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6 (Gal β 1,3)GalNAc α - by the sequential action of α 2,6(N)ST and α 2,3(O)ST. The formation of a more complex ligand, namely, NeuAc α 2,6Gal β 1,4(Fuc α 1,3)(6-O-Sulfo)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α - seems to be impossible since NeuAc α 2,6Gal β 1,4GlcNAc is inactive as an acceptor for α 1,3-L-fucosyltransferases (41).

Mucin Core 2 based compounds as acceptors for sialyltransferases

We examined the action of the sialyltransferases on modified core-2 structures where key hydroxyl groups were replaced with either O-methyl or fluoro substituents (Table III). We observed that the cloned α 2,3(O)ST utilized all acceptors including Gal β 1,4GlcNAc β 1,6(3-FGal β 1,3)GalNAc α -O-Bn, Gal β 1,4 GlcNAc β 1,6(3-O-MeGal β 1,3)GalNAc α -O-Bn and Gal β 1,4 GlcNAc β 1,6(4-O-MeGal β 1,3)GalNAc α -O-Bn exhibiting 77.2%, 49.6% and 101.3% activity respectively. On the other hand, LNCaP α 2,3(O)ST acted only on Gal β 1,4GlcNAc β 1,6(4-O-MeGal β 1,3)GalNAc α -O-

Bn (112.0%), and both 3-O-Methyl and 3-Fluoro substitution of the β 1,3 linked Gal abrogated enzyme action. The cloned α 2,6(N)ST showed very low activity towards 4-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn (12.6%) whereas α 2,3(N)ST exhibited 99.8% activity towards this acceptor. On the contrary, 3-O-Methyl, 3-Fluoro and 4-Fluoro substitution of Gal in the β 1,4 linked Gal resulted in the formation of poor acceptors for both enzymes. The results indicate that besides transferring sialic acid to the 3-position of β 1,3-linked Gal, α 2,3(O)ST also induces sialylation of the acceptor at another position: either at the C-3 hydroxyl group of β 1,4 linked Gal or at another -OH group on either β 1,4 or β 1,3 linked Gal.

We examined in detail, the effect of 4-O-methylation of Gal moiety on the efficiency of mucin core 2 acceptors towards sialyltransferases (Fig. 3). When the activity of α 2,6(N)ST was measured at varying concentrations of Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn and 4-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn separately, it was found (Fig. 3A) that the latter compound was almost inactive at the maximum concentration (0.75mM) tested whereas the former compound showed considerable activity even at the lowest concentration (0.15mM) tested. When the activity of α 2,3(N)ST was measured with the above two acceptors in the same manner (Fig. 3B), the latter compound was four fold active at the lowest concentration (0.05mM) tested and two-fold active at the maximum concentration (0.25mM) tested as compared to the activities of former compound at these concentrations. Thus 4-O-methylation of β 1,4-linked Gal abolished the acceptor ability towards α 2,6(N)ST but increased the acceptor efficiency considerably towards α 2,3(N)ST.

When the activity of cloned α 2,3(O)ST was measured at varying concentration of Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn and Gal β 1,4GlcNAc β 1,6(4-O-MeGal β 1,3)GalNAc α -O-Bn separately (Fig. 3C), the former compound was found as a slightly better acceptor at the standard incubation conditions. At a higher concentration of the donor CMP-NeuAc in the reaction mixture, the efficiency difference between these two acceptors was highly pronounced. On the contrary, LNCaP α 2,3(O)ST (Fig. 3D) utilized the latter compound more efficiently as an

acceptor, reaching the maximum activity at 0.15mM whereas the former compound exhibited maximum activity at 0.60mM. Thus 4-O-methylation of β 1,3-linked Gal resulted in entirely different influence on the activities of cloned α 2,3(O)ST and LNCaP α 2,3(O)ST.

Probing the location of sialylation by cloned α 2,3(O)ST on the acceptor GlcNAc β 1,6(3-O-MeGal β 1,3) GalNAc α -O-Bn

With the objective of determining if sialylation by cloned α 2,3(O)ST takes place on the β 1,4-linked Gal attached to GlcNAc, we examined the enzymatic products from the acceptors 3-O-MeGal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn, Fuc α 1,2Gal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn and 3-O-Sulfo Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn. The [$9\text{-}^3\text{H}$] sialylated products from the acceptors 3-O-MeGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn and Fuc α 1,2Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn were isolated by Sep-Pak C18 method. The [$9\text{-}^3\text{H}$] sialylated products from the acceptors, Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al and 3-O-SulfoGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn were isolated by Biogel P₂ Column chromatography. These [$9\text{-}^3\text{H}$] sialyl compounds were subjected to TLC using two different solvent systems (Fig. 4). The location of the radioactive compounds on TLC plates was determined by scraping $\frac{1}{2}$ cm width segments followed by liquid scintillation counting. Such studies indicated that only a single product was being formed. It was found that the enzyme exhibited 24.7%, 45.4% and 54.7% activities respectively towards these acceptors when its activity towards reference molecule Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al was set to 100% (Table IV).

The sialylated products from all these acceptors except 3-O-SulfoGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn exhibited binding to WGA-agarose via GlcNAc (Fig. 5A, B, C and D). This indicates that 3-O-Sulfo group on β 1,3-linked Gal in mucin core 2 trisaccharide abolishes the binding of WGA to β 1,6 linked GlcNAc of mucin core 2. After digestion of these radioactive products with β -N-acetylhexosaminidase (Jack bean), the resulting radioactive compounds did not bind to WGA-agarose due to the absence of GlcNAc (Fig. 5E, F and G). Overall, this set of

experiments suggest that sialylation by $\alpha 2,3(\text{O})\text{ST}$ can take place on the $\beta 1,3$ -linked Gal at a position other than the 3-hydroxyl group.

Characterization of sialylated product arising from Gal $\beta 1,4\text{GlcNAc}\beta 1,6(3\text{-O-MeGal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ by the action of cloned $\alpha 2,3(\text{O})\text{ST}$

We isolated the products by Sep-Pak C18 method and then subjected it to RCA-I-agarose chromatography (data not shown). As anticipated [^3H]NeuAc $\alpha 2,3\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(3\text{-O-MeGal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ arising from the action of $\alpha 2,3(\text{N})\text{ST}$ did not bind to this column. The sialylated compounds formed from Gal $\beta 1,4\text{GlcNAc}\beta 1,6(3\text{-O-MeGal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ and Gal $\beta 1,4\text{GlcNAc}\beta 1,6(\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ by the action of cloned $\alpha 2,3(\text{O})\text{ST}$, however, exhibited weak binding to this column. The results indicated very strongly that sialylation by $\alpha 2,3(\text{O})\text{ST}$ did not take place on the $\beta 1,4$ linked Gal moiety, and this is in agreement with the data reported above using the WGA-agarose chromatography.

Evidence for sialylation of other than C-3 or C-6 hydroxyl group in the $\beta 1,3$ -linked Gal moiety

The action of $\alpha 2,3(\text{O})\text{ST}$ on a series of compounds over varying acceptor concentrations was examined in order to better define its specificity. In some of the molecules Gal was replaced by D-Fuc since both monosaccharides are identical, except that D-Fuc (6-deoxy Gal) lacks a hydroxyl group at the C-6 position. The compound D-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ as shown above served as good acceptor for the cloned $\alpha 2,3(\text{O})\text{ST}$ indicating that the C-6 OH group of $\beta 1,3$ -linked Gal may not be the target of sialylation. In order to gain evidence in support of this contention, we also examined D-Fuc $\beta 1,3\text{GalNAc}\alpha\text{-O-Bn}$; D-Fuc $\beta 1,3(\text{GlcNAc}\beta 1,6)\text{GalNAc}\alpha\text{-O-Bn}$; D-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; Gal $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and 3-O-SulfoD-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ as acceptors for the cloned $\alpha 2,3(\text{O})\text{ST}$ (Fig. 6A and Fig. 6C). Both D-Fuc $\beta 1,3\text{GalNAc}\alpha\text{-O-Bn}$ and D-Fuc $\beta 1,3(\text{GlcNAc}\beta 1,6)\text{GalNAc}\alpha\text{-O-Bn}$ at 0.75mM concentration were found to be 100% efficient as acceptors as compared to 3-O-MeGal $\beta 1,4\text{GlcNAc}\beta 1,6(\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$. Further, [^3H] sialyl compound

arising from the former did not bind to WGA-agarose column whereas that from the latter did bind to this column (data not shown), indicating that sialylation occurs on the D-Fucosyl moiety only.

When the activity of the cloned $\alpha 2,3(\text{O})\text{ST}$ was measured by varying the concentration of Globo H analogs, we found (Fig. 6C) that $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and $\text{D-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ were efficient acceptors, whereas both $3\text{-O-SulfoGal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and $3\text{-O-SulfoD-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ also served as acceptors but to a less extent. Detailed mass spectroscopy analysis was performed with $3\text{-O-SulfoD-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ (compound S4 in Table VI) and its reaction product (compound 14) fragmentation analysis using MS^n analysis (Supplemental Data). This verified the attachment of sialic acid to this molecule, which is devoid of free hydroxyl at the C-3 and C-6 positions. $\text{D-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ was about 50% and 100% efficient as compared to $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ at 0.15mM and 0.75mM respectively, indicating that C-6 hydroxyl group is required for the maximum enzyme affinity towards the acceptors. Further, almost the same acceptor efficiency of $3\text{-O-SulfoGal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and $3\text{-O-SulfoD-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ (Fig. 6C; Table V) would suggest that C-6 hydroxyl group of Gal moiety is unlikely the site of sialylation in the acceptor $3\text{-O-SulfoGal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$. Sialylation likely takes place at the C-2 or C-4 positions at $\beta 1,3$ attached Gal.

The unique catalytic activities of the cloned $\alpha 2,3(\text{O})\text{ST}$ on the modified terminal $\beta 1,3\text{Galactosyl}$ residues

A measurement of the clonal $\alpha 2,3(\text{O})\text{ST}$ activity at varying concentration of various acceptors followed by a determination of K_m and V_{max} values by Lineweaver-Burke plot (see Fig. 6 and Table V) indicated that the Globo backbone compounds $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; $3\text{-O-SulfoGal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; $\text{D-Fuc}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$; the T-hapten compounds $4\text{-O-MeGal}\beta 1,3\text{GalNAc}\alpha\text{-O-Bn}$; $4\text{-FGal}\beta 1,3\text{GalNAc}\alpha\text{-O-Bn}$ and $\text{D-Fuc}\beta 1,3\text{GalNAc}\alpha\text{-O-Bn}$ and the mucin core 2 compounds $\text{D-Fuc}\beta 1,3(\text{GlcNAc}\beta 1,6)\text{GalNAc}\alpha\text{-O-Bn}$ and $\text{Fuca}1,2\text{Gal}\beta 1,3(\text{GlcNAc}\beta 1,6)\text{GalNAc}\alpha\text{-O-Bn}$ exhibited K_m values (mM) in the range

from 0.12 up to 2.00 and Vmax values (pmol/h) in the range from 197 to 1873. The results would indicate that the synthesis of these unique sialylated compounds is feasible by utilizing this enzyme.

ESI-MS/MSⁿ analysis of the enzymatically sialylated compounds

To confirm our finding that $\alpha 2,3(\text{O})\text{ST}$ can catalyze novel sialylation, we performed MSⁿ spectra analysis with selected enzymatically synthesized products under collision-induced dissociation (CID). Four isomeric pentasaccharides (compounds 6, 7, 18 and 23) with different sialyl linkages (sial $\alpha 2,3$, sial $\alpha 2,2/4$ and sial $\alpha 2,6$) and a sulfate residue (at 3-position of either galactose terminal in mucin core-2 tetrasaccharide) (Fig. 7) were detected as double charged ion $[\text{M}-2\text{H}]^{2-}$ at m/z 604 in negative mode and doubly charged quadruply sodiated ions $[\text{M}-2\text{H}+4\text{Na}]^{2+}$ at m/z 650 in positive mode (Table VI). MS² spectra of molecular species at m/z 604 derived from the four isomers are shown in Figure 8. The fragmentations identified in Figures 8 and 9 were shown in Figure 7 by using established Domon-Costello nomenclature (42). The glycosidic cleavage between sialyl group and branched mucin core-2 yielded B ions (B'₁ ion or B₁ ion) representing sialyl group and Y ions (Y'₂ ion or Y₃ ion) representing the sulfated branched mucin core-2 tetrasaccharides. As seen, the ratios of B ion to Y ions are significantly different among the different linkages (see Fig. 8). The same CID patterns are seen in Figure 8A and 8B indicating that compound 6 and 23 shared a common linkage, which is distinct from compound 7 and 18. Based on the enzymatic studies this is likely an $\alpha 2,3$ linkage. The Y₃ ion from compound 18 is at m/z 913 which is significantly different from the corresponding ions at m/z 917 from compound 6 and 23. This difference in molecular weight could be attributed to the formation of a lactone bond between two primary hydroxyl groups of Gal and GlcNAc in the Gal $\beta 1,4$ GlcNAc linkage of the branched mucin core 2 tetrasaccharide based on the mechanism reported by Ulrik et. al. (43) The spectrum of compound 7 (Fig. 8C) is distinct from the spectra of compounds 6, 18 and 23 indicating a different sialyl linkage and thus likely representing either $\alpha 2,2$ or $\alpha 2,4$ linkage of sialic acid.

MS³ spectra of the Y ions representing sulfated branched mucin core 2 structures are shown in Fig. 9. The B₂ ion at m/z 444 shown in Figure 9A confirms that the sulfate group of 6 is on the $\beta 1,4$ linked Gal of the mucin core-2

tetrasaccharide. Similarly, the Y_1 ions at m/z of 552 in Figures 9B and 9C indicate that the sulfate group of compound 23, 7 and 18 are on the Gal of Gal β 1,3GalNAc in the tetrasaccharide. The formation of a lactone bond was also confirmed by the Y_2 ion at m/z 753 and B_4 ion at m/z 807 of compound 18 shown in Figure 9C, which is different from the corresponding ion at m/z 755 and m/z 809 in Figure 9A and 9B.

The acceptor specificity of three cloned rat sialyltransferases and a human sialyltransferase from prostate cancer cell line LNCaP has been examined. By defining the specificity of these enzymes for an array of carbohydrate acceptors based on the core-2 and globo structures we: i) Define unique acceptors that can be used to characterize each of these enzymes in a complex mixture of glycosyltransferases, ii) Describe potential biochemical pathways that can lead to the synthesis of the ligands for selectins and Siglecs, and iii) Propose that the C-2 or C-4 position of β 1,3 linked Gal in the core-2 mucin may be a site for the action of cloned α 2,3(O)ST, provided the C-3 position has a substituent other than sialic acid.

Some pertinent observations on rat recombinant and LNCaP sialyltransferases

Others investigators have performed studies with the sialyltransferases examined in the current manuscript. Chemo-enzymatic studies of Takano *et. al.* (44) identified only one product, namely Gal β 1,4GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α -O-R, when the tetrasaccharide acceptor was incubated at 37°C with the rat recombinant α 2,3(O)ST. On the other hand, they identified the above monosialyl compound (8%) and disialyl compound (15%) in addition to the typical product, namely, NeuAc α 2,3Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-R (57%) when the tetrasaccharide acceptor was incubated at 37°C with rat recombinant α 2,3(N)ST at pH 7.4 for 24 h. Sengupta *et. al.* (45) made similar observation with the rat recombinant α 2,3(N)ST when the tetrasaccharide acceptor was incubated at pH 7.4 at 37°C for 18 h. In order to compare our findings with these investigators, we also extended the reaction times in our experiments from 2h to 20h under our standard experimental conditions (pH 6.0 at 37°C) for the acceptor Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn and utilized all three rat recombinant enzymes α 2,3(N)ST, α 2,6(N)ST and α 2,3(O)ST. The [9-³H] sialyl products arising from the tetrasaccharide acceptor were isolated using the Sep-Pak C18 cartridge. PNA-agarose affinity chromatography of the radioactive products showed that 92%, 93% and 0% of the radioactivity respectively were specifically bound to the column, indicating the identity of the bound products as [9-³H]NeuAc α 2,3Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn and [9-³H] NeuAc α 2,6Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn in case of

$\alpha 2,3(\text{N})\text{ST}$ and $\alpha 2,6(\text{N})\text{ST}$, and the unbound product as $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(9\text{-}^3\text{HNeuAc}\alpha 2,3\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ in case of $\alpha 2,3(\text{O})\text{ST}$. Triton X-100 solubilized extract of LNCaP cells contained >95.0% $\alpha 2,3(\text{O})\text{ST}$ activity as evident from the activity of the following acceptors: 3-O-Me $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ (100.0%); $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(3\text{-O-MeGal}\beta,13)\text{GalNAc}$ (3.0%) and $\text{Gal}\beta 1,4\text{GlcNAc}\beta\text{-O-Al}$ (0.5%). Overall, our results are in agreement with other data in literature, and some differences observed may be attributed to variation in incubation conditions between the studies.

Unique activities of clonal $\alpha 2,3(\text{O})\text{ST}$ on a different position other than C-3 of $\beta 1,3\text{-linked Gal}$

The clonal $\alpha 2,3(\text{O})\text{ST}$ was active with 3-O-Me $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ (100.0%) and $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(3\text{-O-MeGal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ (49.6%) but was almost inactive towards $\text{Gal}\beta 1,4\text{GlcNAc}\beta\text{-O-Al}$ (4.7%). When $\text{Gal}\beta 1,4\text{GlcNAc}\beta 1,6(\text{Gal}\beta 1,3)\text{GalNAc}\alpha\text{-O-Bn}$ was used as an acceptor, 100% of the isolated [$9\text{-}^3\text{H}$] sialyl product did not bind to PNA-agarose column, indicating that sialylation took place only on the $\beta 1,3\text{-linked Gal}$ moiety. This suggests two possibilities. One, that sialylation might occur on $\beta 1,4\text{-linked Gal}$ when there is a C-3 block on $\beta 1,3\text{-linked Gal}$ moiety. Two, that sialylation may take place on some other position in $\beta 1,3\text{-linked Gal}$ moiety. In order to explore these possibilities, we used mucin core 2 trisaccharide containing different substituents on $\beta 1,3\text{-linked Gal}$ moiety as acceptor for the clonal $\alpha 2,3(\text{O})\text{ST}$. Using tandem mass spectral analysis, WGA-agarose affinity chromatography on [$9\text{-}^3\text{H}$] sialylated products before and after $\beta\text{-N-acetyl hexosaminidase}$ treatment and also employing RCA-agarose affinity chromatography on [$9\text{-}^3\text{H}$] sialyl products from mucin core 2 tetrasaccharide acceptors, we demonstrate unequivocally that sialylation took place on a different position of 3-O-substituted $\beta 1,3\text{-linked Gal}$. Since both 3-O-Sulfo D-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ and 3-O-Sulfo $\text{Gal}\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$ served as acceptors with almost same efficiency for the clonal $\alpha 2,3(\text{O})\text{ST}$, the C-6 position in C-3 blocked $\beta 1,3\text{-linked Gal}$ is unlikely to be the site for sialylation. It is possible that one of the remaining hydroxyl groups (C-2 or C-4) is the site of action of

this enzyme. However, Kobata and his group (46) reported the occurrence of NeuAc α 2,4Gal terminal N-glycan chains in Cold-insoluble globulin isolated from bovine plasma. Further, 3-SulfoGal β 1,3GalNAc β 1,3Gal α 1,4Gal β →Glc β ceramide has been reported to occur in rat kidney (47). In fact, the sequence 3-Sulfo-Gal β 1,3GalNAc β is known to occur in several glycolipids (48).

Prediction of potential inhibitors of Siglec binding

Our biochemical investigations suggest new molecules that may act as efficient inhibitors of Siglec binding to its ligand. In this regard, recently, Blixt *et al.* (49) assessed the sialoside specificity of the Siglec family using a novel multivalent platform comprising biotinylated sialosides bound to a streptavidin-alkaline phosphatase conjugate. Human Siglecs 2,3,7,8,9 and 10 exhibited significant binding to the sialoside-SAAP probe H containing NeuAc α 2,6Gal β 1,4GlcNAc. They observed that there was little correlation between the IC₅₀ values and the ability of the corresponding SAAP probe to bind to immobilized Siglec chimeras. They also found in the binding assay that the length of the spacer arm can influence the interaction of a sialoside sequence with a given Siglec. In spite of these pitfalls, these authors came up with a remarkable finding that NeuAc α 2,6(Gal β 1,3)GalNAc α -O-Thr, NeuAc α 2,3Gal β 1,3GalNAc α -O-Thr and NeuAc α 2,3Gal β 1,3 (NeuAc α 2,6)GalNAc α -O-Thr are 100, 600 and 3,000 fold more potent than NeuAc α 2,6GalNAc α -O-Thr in inhibiting Siglec 4 (MAG) binding. Based on our studies, it now appears the following mucin core-2 based compounds may also be potential inhibitors for Siglec 4 and perhaps other Siglec binding also: NeuAc α 2,3Gal β 1,3(GlcNAc β 1,6)GalNAc α -; NeuAc α 2,3Gal β 1,3(NeuAc α 2,3Gal β 1,4GlcNAc β 1,6) GalNAc α -; NeuAc α 2,3Gal β 1,3(NeuAc α 2,6Gal β 1,4GlcNAc β 1,6) GalNAc α -; NeuAc α , α 2,3Gal β 1,3(6-O-Sulfo)GalNAc α -; NeuAc α 2,3Gal β 1,3(3-O-SulfoGal β 1,4GlcNAc β 1,6)GalNAc α -; NeuAc α 2,3Gal β 1,3[Gal β 1,4(6-O-Sulfo)GlcNAc β 1,6]GalNAc α - and NeuAc α 2,3Gal β 1,3(6-O-SulfoGal β 1,4GlcNAc β 1,6) GalNAc α . In fact, blixt *et. al.* (50) have recently reported that NeuAc α 2,6Gal β 1,4(6-O-Sulfo)GlcNAc β - is far better than the corresponding non-sulfated structure in binding to human CD22.

Enzymes that act at the C-3 position of Gal in LacNAc also act efficiently on the Globo acceptor

Earlier, we found that prostate cancer cell LNCaP α 1,2-L-FT exhibited 4 fold greater activity towards β 1,4-linked Gal as compared to its activity with β 1,3-linked Gal in mucin core 2 tetrasaccharide (51); the same enzyme also utilized the Globo backbone structures Gal β 1,3GalNAc β 1,3Gal α -O-Me and D-Fuc β 1,3GalNAc β 1,3Gal α -O-Me very efficiently. Subsequently, we observed that Gal:3-O-Sulfotransferase, Gal3ST-2, which exhibits specificity for LacNAc Type 2 structure also acts efficiently on the Globo structures (52). Extending these observations, in the present study, we show that α 2,3(N)ST acted with equal efficiency on LacNAc and the Globo structure Gal β 1,3GalNAc β 1,3Gal α -O-Me. Thus, we discovered a physiological correlation that different enzymes modifying terminal β 1,4-linked Gal also efficiently utilize the terminal β 1,3-linked Gal in the Globo backbone.

Rationale for the difference in the acceptor specificity of α 2,3(O)ST from liver and prostate

All cloned mammalian sialyltransferases contain L- S- and VS- sialyl motifs (53-55). The L- sialyl motif was shown to bind to the donor CMP-NeuAc (56) while the S- motif participated in the binding of both donor and acceptor glycans (59). The exact role for VS- motif in the catalytic process had not been identified. Kitazume-Kawaguchi *et al* (60) suggested that His could be a catalytic residue for all sialyltransferases.

Three genes were identified to encode for human sialyltransferases ST-4a, b and c, which catalyze α 2,3 sialylation of Gal β 1,3GalNAc α -. Each enzyme followed a distinct pattern of tissue specific expression (59). The level of ST-4a mRNA was relatively similar in most tissues examined except for brain and skeletal muscle which show little and strong expression, respectively (59). ST-4b was identified only in liver and, surprisingly, was not detectable in HepG₂; ST-4c was most strongly expressed in heart, placenta, testis and ovary. Prostate tissue also predominantly contain ST-4c (57). In the context of our study, it is possible that the human counterpart for rat liver recombinant α 2,3(O)ST is ST-4b, and based on the tissue of origin the

sialyltransferases from prostate cells LNCaP may be ST-4c. It remains to be seen whether such a difference in the expression of ST-4 transcripts between liver and prostate can account for the difference in the intricate substrate specificities of enzymes from these sources.

Enzymatic studies with synthetic acceptors can complement conventional lectin and antibody studies

A differential expression of mRNAs for $\alpha 2,3$ and $\alpha 2,6$ sialyltransferases has been noted in rat and human tissues (60, 61). However, the expression of mRNA does not always correlate with actual enzyme levels, which in turn, cannot always predict glycosylation. Martin *et. al.* (62) examined the terminal glycans in tissues of normal mice and mice deficient in ST6Gal I or ST3Gal I, by using plant lectins as histochemical probes. They found cell type-specific expression of different linkages of terminal sialic acids in a variety of mouse tissues and identified multiple changes in cell type-specific glycan structures in various tissues of deficient mice. The present study was able to provide valuable information on the intricate substrate specificities of sialyltransferases. Such information will be helpful for meaningful interpretation of the histology data obtained using lectin and antibody probes on the tissues of normal and knockout mice. Studies of glycosyltransferase activity using an array of carbohydrate acceptors can also complement genomic and proteomic data that is becoming available from various high-throughput assays.

In order to test the hypothesis of predicting glycan signatures from the pattern of glycosyltransferase activities, we first examined the levels of activities of various enzymes in the four cell lines ZR-75-1, T47D, MDA-MB-231 and MCF-7, followed by other cell lines as reported in **Table II**. The levels of the carbohydrate structures likely to arise from these enzyme activities are presented in **Table III**. Müller & Hanisch [2002] found that NeuAc $\alpha 2,3$ Gal $\beta 1,3$ GalNAc α - units constituted the major structure of MFP6 proteins expressed in T47D (68.6%), MDA-MB-231 (65.5%) and ZR-75-1 (86.1%). The present invention also shows that $\alpha 2,3$ -Sia-TI or TII was the most dominant enzyme as compared to $\alpha 2,3$ -sia-TIV in all these cell lines. They also observed in MCF-7 that sialylated carbohydrate chains comprise only 5.2% of the O-glycan chains.

As found in accordance with the present invention, except for ZR-75-1, which exhibits high sialyltransferase activities, T47D, MDA-MB-231 and MCF-7 expresses almost the same level of α 2,3-sialyl-TI or TII. MDA-MB231 and ZR75-1 cell extracts contain α 2,3-Sialyl-T IV enzyme that constructs the NeuAc2,3Gal β 1,4GlcNAc and Müller & Hanisch [2002] report this sequence in the oligosaccharide chains of O-glycans of cell lines MDA-MB231 and ZR75-1 but not in MCF-7 cell line.

The target compounds are specifically designed to measure a particular sialyltransferase activity in presence of other sialyltransferase and glycosyltransferase activities and hence will be very useful in measuring these activities accurately in biological samples.

As a further extension of the present invention it has been found that structural studies, using specific substrates of the invention, on carbohydrate chains released from cancer associated glycoprotein antigens reveal major changes at the outer ends of the oligosaccharide chains in cancer. In accordance with the present invention, various cancer cell lines have been examined for the levels of fucosyl-, β -galactosyl-, β -N-acetylgalactosaminyl-, sialyl- and sulfotransferase activities which generate the outer ends of the oligosaccharide chains in glycans for identifying the signature glycan structures expressed by cancer cells. We have identified glycosyltransferases activities at the levels that would give rise to non-reducing carbohydrate ends of O-glycans as reported by others in breast cancer cell lines T47D, ZR75-1, MCF-7 and MDA-MB-231. Two distinct Gal: 3-O-sulfotransferases, one being specific for T-hapten Gal β 1 $\rightarrow$ 3GalNAc α - and other exhibiting vast preference for the Gal β 1 $\rightarrow$ 4GlcNAc terminal unit in O-glycans are expressed respectively by breast and colon cancer cell lines. We extended our study to ovarian cancer cells SW626 & PA-1 and hepatic cancer cells HepG₂. Our studies show that α 1,2-L-fucosyl-T, α 2,3(N) sialyl-T, and 3-O-Sulfo-T capable of acting on mucin core 2 tetrasaccharide Gal β 1,4GlcNAc β 1,6(3-O-MeGal β 1,3)GalNAc α - can act on Globo H antigen backbone Gal β 1,3GalNAc β 1,3Gal α -. Thus, our study is able to identify signature carbohydrate moieties belonging to certain cancer-associated glycolipids. Briefly, the present study finds i) 3'-Sulfo-T-hapten as a measure of the tumorigenic potential and 3'-sialyl T-

hapten & Lewis^a as prevalent structures in breast cancer cells; ii) 3'-Sulfo Lewis^x, 3-O-Sulfo Globo unit and 3-Fucosyl Chitobiose core as unique structures associated with colon cancer cells; iii) favorable synthesis of polylactosamine chain and T-hapten in ovarian cancer cells as they contain negligible sialyltransferase activities and iv) 6'-sialyl LacNAc unit and 3'-sialyl T-hapten as the prevalent structure in hepatic cancer cell glycans. Thus, it is evident from the present study that different cancer cells express unique glycan epitopes, thus providing novel targets for cancer diagnosis and treatment.

The structural variability of glycans is dictated by tissue-specific regulation of glycosyltransferase genes, the availability of sugar nucleotides and competition between enzymes for acceptor intermediates during glycan elongation. The studies of Müller & Hanisch [2002] on recombinant MUC1 probe expressed in four breast cancer cell lines indicate that epigenetic parameters like the rate of Golgi passage and the topology of the protein substrate within the Golgi compartment are less important with respect to the final glycosylation profiles than the cellular repertoire of glycosyltransferases. Several glycosyltransferases and Gal3-O-Sulfotransferases are capable of elongating mucin core 2 tetrasaccharide as well as the Globo backbone unit. Such action of these enzymes could lead to a complexity of cancer associated terminal glycan structures, as illustrated in **Fig. 10**.

Müller & Hanisch [2002] expressed a MUC1 fusion protein MFP6 in the breast cancer cell lines ZR-75-1, MDA-MB-231, MCF-7 and T47D and studied the O-glycans of the fusion proteins after releasing by hydrazinolysis and then labeling with 2-amino benzamide. In order to test the hypothesis of predicting glycan signatures from the pattern of glycosyltransferase activities, we first examined the levels of activities of various enzymes in these four cell lines followed by other cell lines as reported in **Table VII**. The levels of the carbohydrate structures likely to arise from these enzyme activities are presented in **Table VIII**. Müller & Hanisch [2002] found that NeuAc α 2,3Gal β 1,3GalNAc α - units constituted the major structure of MFP6 proteins expressed in T47D (68.6%), MDA-MB-231 (65.5%) and ZR-75-1 (86.1%). Our present study also showed that α 2,3-Sia-TI or TII was the most dominant enzyme

as compared to $\alpha 2,3$ -sialyl-TIV in all these cell lines. They also observed in MCF-7 that sialylated carbohydrate chains comprise only 5.2% of the O-glycan chains. The present study finds that except for ZR-75-1, which exhibits high sialyltransferase activities, T47D, MDA-MB-231 and MCF-7 expressed almost the same level of $\alpha 2,3$ -sialyl-TI or TII. MDA-MB231 and ZR75-1 cell extracts contain $\alpha 2,3$ -Sialyl-T IV enzyme that constructs the NeuAc $\alpha 2,3$ Gal $\beta 1,4$ GlcNAc and Müller & Hanisch [2002] report this sequence in the oligosaccharide chains of O-glycans of cell lines MDA-MB231 and ZR75-1 but not in MCF-7 cell line. Our enzymatic studies have demonstrated that MCF-7 does not contain $\alpha 2,3$ -Sialyl-T IV but expresses $\alpha 1,2$ -L-FT and $\alpha 1,3$ -L-FT capable of generating the Fuc $\alpha 1,2$ Gal and Fuc $\alpha 1,3$ GlcNAc linkages, respectively. Thus, presence of these fucosyltransferases suggested the existence of Fuc $\alpha 1,2$ Gal $\beta 1,3$ GalNAc and Gal $\beta 1,4$ (Fuc $\alpha 1,3$)GlcNAc, the structures that were reported to be part of O-glycans in MCF-7 [Müller & Hanisch, 2002]. The lack of $\alpha 2,3$ -sialyl-T IV activity suggests the absence of sialyl Lewis^x structure in MCF-7, which agrees with another report [Kumamoto *et al.* 1998]. However, Lewis^x structure moiety linked at C-6 position of GalNAc is reported to be part of O-glycans in MCF-7. Overall the expression profiles of $\alpha(2,3)$ -sialyl-Ts and fucosyltransferases in our studies agree with the structures reported by Müller & Hanisch [2002] for these breast cancer cell lines.

Since sulfotransferases and sialyltransferases can compete for the same site of acceptors, it became important to study sulfotransferases. Among the breast cell lines, DU4475 is unique as it has negligible sialyltransferase activities but high level of 3-O-sulfotransferase specific for Gal $\beta 1,4$ GlcNAc. MCF-7 and ZR-75-1 also can express this enzyme at a low level. The presence of 1,3-FT would generate sulfo Lewis^x in these cell lines. MCF-7 contains both $\alpha 1,2$ -L-FT and $\alpha 1,3$ -L-FT activities which can generate Lewis^y moiety but it is not reported in O-glycans [Müller & Hanisch, 2002]. Gal3-O-Sulfo-T seems to prevent the action of $\alpha 1,2$ -L-FT on Gal $\beta 1,4$ GlcNAc. The cell lines MDA-435/LCC6, MDA-435/LCC6^{MDR1} and even DU4475 have sulfotransferase activity which incorporates sulfate at C-3 position of galactose in T antigen to give 3'-Sulfo-T-hapten. We were the first to report that a core 2 structure

distinguishes the sulfotransferase activities in breast and colon cell lines [Chandrasekaran *et al.*, 1997; Chandrasekaran *et al.*, 1999]. Two distinct types of Gal: 3-O-sulfotransferases were revealed, one being specific for the Gal β 1,3GalNAc α - moiety is expressed by breast cancer cell lines and the other showing preference for the Gal β 1,4GlcNAc branch in mucin core 2 is present in colon cancer cell lines and some breast cell lines [Chandrasekaran *et al.*, 1999]. In this context, the availability of modified analogs of core 2 tetrasaccharide has proven to be important in determining the uniqueness of these enzymes. The specificity of sulfotransferase in colon cancer cell lines and DU4475 breast cell line predicts the presence of 3-O-Sulfo Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -. On the other hand, Gal3Sulfo T-4 cloned by Seko *et al.* [2001] is present in breast cancer cell lines, synthesizing Gal β 1,4GlcNAc β 1,6(3-O-SulfoGal β 1,3)GalNAc α sequence. These core 2 structures would serve as acceptors for α (2,3)-sialyltransferase and α (1,3)L-fucosyltransferase to generate more complex carbohydrate structures. The levels of glycosyltransferase and sulfotransferase activities in colon and as a comparison, that of ovarian cancer cell lines are reported in **Table IX**. A comparison of the levels of the predicted carbohydrate structures arising from these enzyme activities is presented in **Table VIII**. Three colon cell lines we have tested had very low α 1,2-FT activity but a high level of both α 1,3- and α 1,4-FT activities, the α 1,4-FT activity being highest in Colo205. They all expressed FTVI activity, the level being highest in LS180. Colo205 showed a very weak expression of 3-O-Sulfotransferase activity towards Gal β 1,4GlcNAc β . On the contrary high levels of this enzyme activity are present in LS180 and SW1116. Since the levels of both α 2,3-sia-TIV and α 1,2-FT activities appear to be low, in all cases, this would suggest that Gal β 1,4GlcNAc β arm in core 2 tetrasaccharide is prone to the action of Gal:3-O-sulfotransferase to give 3-O-sulfoGal β 1,4GlcNAc β . The dominance of 3'Sulfo Lewis^x determinant in LS180 and SW1116 can be expected as these cells contain a high level of α 1,3-L-fucosyltransferase activity. The enzyme α 2,3-sia-TI or TII activity is dominated in LS180 and SW1116. The occurrence of 3'Sulfo Lewis^x determinant has also been demonstrated in mucin glycoprotein expressed by LS174T-HM7, a highly metastatic subline of colon carcinoma LS174T [Capon *et al.*, 1997]. The expression of di-O-

sulfated mucin core 2 tetrasaccharide seems to be favorable with DU4475 since it lacks all sialyltransferase activities. Our present findings demonstrate the power of well-defined acceptors for studying these enzymes. Brown *et al.* [2003] analyzed mRNA from LS180 cells using Glyco-V1 Genechip micorarrays and detected only α 2,3-Sia-TIV. On the contrary, the present study identified α 2,3-Sia-TI or TII as the overwhelming sialyltransferase activity not only in LS180, but also in other colon cell lines Colo205 and SW1116. In agreement with our present data on fucosyltransferase activities, they also detected FTIII and FTVI in LS180 cells. But the presence of FTVII in colon cancer cells could not be ruled out, when considering that Fetuin triant sialylglycopeptide acted as a good acceptor for the FTs of colon cancer cell lines. Glycosyltransferase expression in human colonic tissues was examined by Kemmner *et al.* [2003] using oligonucleotide microarrays. Their restricted analysis of 39 glycosyltransferases present on the Genechip U95A indicated that sialyltransferases α 2,6-Sia-TI, α 2,3-Sia-TIV, fucosyltransferases FTIII, FTVI and FTVIII(α 1,6-L-FT) and also GalNAcT-1(polypeptide:GalNAc-T) and β 1,4GalT-2 may be responsible for the aberrant biosynthesis of carbohydrates in colonic carcinogenesis and metastasis. Their findings seem to fit with the present data on the pattern of most of the glycosyltransferase activities obtained for colon cancer cell lines.

In accordance with the invention, we also investigated the specificity of α (2, 3) Sia-T, Gal-3-O-Sulfo-T and α 1,2-Fuc-T using core 2 branched structure and Gal β 1,3GalNAc β 1,3Gal α - sequence which occurs in glycolipids as globo H precursor. The Globo H antigen was originally characterized by Hakomori and co-workers in a human breast cancer cell line [Bremer *et al.*, 1984]. Immunostaining using murine MoAb MBr1 demonstrated this antigen in other human tissues including prostate cancer and small cell lung carcinoma. We reported that α 1,2-Fuc-T in LNCaP cells acts more efficiently (4 fold) on the mucin core 2 Gal β 1,4GlcNAc β unit as well as on the Globo H precursor trisaccharide as compared to its activity towards Gal β 1,3GalNAc β -arm in mucin core-2 [Chandrasekaran *et al.*, 2002]. In another study, we examined the specificity of three cloned Gal 3 sulfotransferases [Chandrasekaran *et al.*, 2004]. Gal 3SulfoT-2 which is known to incorporate sulfate at C-3 position of galactose in Gal β 1,4GlcNAc β behaves similar to α 1,2-Fuc-T

transferase and $\alpha 2,3$ -Sia-TIV towards these substrates; it acted with the same efficiency on both the Globo trisaccharide and core-2 tetrasaccharide. Gal-3-sulfoT4 can also utilize the globo H precursor to the same extent as Gal $\beta 1,3$ GalNAc α -. $\alpha 2,3$ -Sia-TI or TII generates NeuAc $\alpha 2,3$ Gal $\beta 1,3$ GalNAc- in mucin and also acts on Globo H precursor to give NeuAc $\alpha 2,3$ Gal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α $\rightarrow$ sequence. $\alpha 2,3$ -sia-TII has been reported to be involved in the biosynthesis of NeuAc $\alpha 2,3$ Gal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α ,3Gal $\beta 1,4$ Glc β Cer [Saito *et al.*, 2003; Maccioni *et al.*, 1999]. There is some controversy on the specificity of $\alpha 2,3$ -Sia-TIV [Kono *et al.*, 1997]. Some groups report that this enzyme is involved in the synthesis of NeuAc $\alpha 2,3$ Gal $\beta 1,3$ GalNAc β in glycolipids, while other groups suggest that it can generate NeuAc $\alpha 2,3$ Gal $\beta 1,4$ GlcNAc β followed by fucosylation to give SLe^x [Kitagawa, 1994; Sasaki *et al.*, 1993]. Our studies demonstrate clearly the specificity of $\alpha 2,3$ -Sia-TIV towards mucin core 2 Gal $\beta 1,4$ GlcNAc unit as well as Gal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α - sequence.

Our study indicates three types of sulfated carbohydrate epitopes 3-O-SulfoGal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -, 3-O-SulfoGal $\beta 1,3$ GalNAc α - and 3-O-SulfoGal $\beta 1,4$ GlcNAc β as the signatures of cancer cells. The 3-O-Sulfo-T activity towards Gal $\beta 1,4$ GlcNAc β - in DU4475 is 18-fold that of MCF-7 and ZR-75-1, which are the only other two breast cancer cell lines expressing this activity. These results would indicate the signature carbohydrate structure expressed by DU4475 as 3'-Sulfo Lewis^x. It is interesting to note that the tumorigenic breast cell lines MDA-MB-435/LCC6 and MDA-MB-435/LCC6^{MDR1} exhibit about 9- and 4-fold Gal:3-O-Sulfotransferase activity specific for Gal $\beta 1,3$ GalNAc α - unit as compared to its level in the non-tumorigenic parent cell line MDA-MB-435S. Further, the former cell lines contain 4-5-fold GlcNAc: $\beta 1,4$ Gal-T activity and less sialyltransferase activities as compared to the latter. This, it appears that an increase in Gal:3-O-Sulfotransferase with a concomitant decrease in Gal:3-O-Sialyltransferase acting on Gal $\beta 1,3$ GalNAc α - has a relationship to the tumorigenic potential of breast cancer cell lines. The absence of FT activities in MDA-MB-435 series would suggest that 3-O-

SulfoGal β 1,3GalNAc α - as well as 3-O-SulfoGal β 1,4GlcNAc β could be signature structures of tumorigenic breast cancer cells.

For comparison purposes, the enzyme activity levels of two ovarian cancer cell lines SW626 and PA-1 (**Table IV**) were examined and the predicted carbohydrate structures are presented in **Table III**. These cell lines contain only a small amount of sialyltransferase and glycan sulfotransferase activities and considerably a low level of α 1,3-L-Fucosyltransferase activity as compared to the colon cancer cell lines, indicating that the biosynthesis of polylactosamine chain is quite favored in these cell lines. The hepatic cancer cell line HepG2 appears to be capable of synthesizing α 2, 6-sialylated N-glycans and α 2, 3-sialyl T-hapten. It is interesting to note that, except for the occurrence of a significant level of α 1,6-L-FT activity, other FT activities are either negligible or absent in HepG2 thus indicating the existence of a reciprocal relationship in the expression of α 2,6(N)Sialyltransferase and α 1,2 and α 1,3/4-L-Fucosyltransferases. From the level of α 1,6-L-FT activity it is apparent that, as compared to T47D cell line, the other breast cell lines BT20, MCF-7 and MDA-MB-435 series, respectively, express about 2-, 3- and 4-fold and the rest, namely MDA-MB-231, DU4475 AND ZR-75-1 express about 5-fold α 1,6-Fucosylated N-glycans. The colon cancer cells Colo205, LS180 and SW1116 contained almost the same level of α 1,6-FT activity indicating the presence of same amount of α 1,6-Fucosylated N-glycans. Both sialo and asialo Fetuin triantennary glycopeptides served as good acceptors for the fucosyltransferases of the colon cell lines, indicating the facile synthesis of sialyl Lewis^x and sialyl Lewis^a. Ancrod was also utilized as an acceptor that would further indicate the formation of sialyl Lewis^a. Hence, Colo205, LS180 and SW1116 could express sialyl Lewis^a, sialyl Lewis^x and sialyl T-hapten.

We compared a breast tumor with colon tumor for glycosyltransferase activities (see **Table V**). The colon tumor in comparison to the breast tumor had 18-fold α 1,3-FT activity. The α 1,4-FT activity in breast tumor was comparatively negligible; but there was a significant level of α 1,2-FT activity. These FT activities, thus, mirrored the O-glycan chains from MUC1 fusion protein of MCF-7 reported by Muller and Hanisch. In contrast to the presence of Gal β 1,4GlcNAc utilizing Gal:3-O-Sulfotransferase in MCF-7, ZR-75-1 and DU4475, the other Sulfo-T specific for

Gal β 1,3GalNAc was found in this breast tumor. We identified the latter enzyme in MDA-MB-435 series and DU4475 and in a number of breast tumor specimens in an earlier study [Chandrasekaran *et al.*, 1999].

It is interesting to note that the tumorigenic cell lines MDA-435/LCC6 and MDA-435/LCC6^{MDR1} as compared to the non-tumorigenic parent cell line MDA-MB-435S express 4-5 fold GlcNAc: β 1,3/4Gal-T activity; DU4475 expressing negligible amount of sialyltransferase activities, as well as a low level of GlcNAc: β 1,3/4Gal-T activity as compared to other cell lines, contains a high level of GlcNAc: β 1,3/4GalNAc-T activity as well as α -GalNAc: β 1,3Gal-T activity. In this context, it makes sense in comparing this data to the situation in breast tumor. As compared to the colon tumor specimen, the breast tumor specimen showed a high level of GlcNAc: β 1,3/4GalNAc-T activity and a low level of α 2,3(O)ST and α -GalNAc: β 1,3Gal-T activities.

Our analysis of glycosyltransferase activities in a colon tumor tissue (see Table IV) indicated a pattern of fucosyltransferase activities akin to that of colon cancer cells being present in this tissue. From the levels of α 1,2-FT activity in tumor specimens as measured with four different acceptors (see Table III), it became evident that Globo-based acceptors, namely Gal β 1,3GalNAc: β 1,3Gal α -O-Me and D-Fuc β 1,3GalNAc: β 1,3Gal α -O-Me are better acceptors than the Core 1 acceptor Gal β 1,3GalNAc α -O-Al and Gal β -O-Bn for tumor α 1,2-FT. Further, the dominance of Gal:3-O-Sulfotransferase activity specific for Gal β 1,4GlcNAc over α 2,3 (N) sialyltransferase activity in colon cancer cell lines and colon tumor tissue would suggest that 3-Sulfo Lewis^x terminal carbohydrate would be a signature carbohydrate structure associated with colon cancer.

Further, it is quite interesting to note that while the breast tumor tissue akin to breast cancer cell lines was almost devoid of FTVI activity, the colon tumor tissue contained a significant level of this activity thus resembling the colon cancer cell lines. It appears that N-glycans with 3-Fucosyl Chitobiose core could be a signature carbohydrate structure associated with colon cancer.

The present invention has well documented that $\alpha 2,3$ (O) ST activity is the most predominant sialyltransferase activity ranging 70-90% of the total sialylating activity in breast and colon cancer cell lines as well as in the two tumor tissues that were examined. We have also found (Chandrasekaran *et al.* submitted for publication) that cloned $\alpha 2,3$ (O) ST (ST3 Gal II) can sialylate very efficiently not only T-hapten (Gal $\beta 1,3$ GalNAc α -) and Globo backbone (Gal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -) but also LacNac type 1 (Gal $\beta 1,3$ GlcNAc β -). We have shown previously [Chandrasekaran *et al.* 1997; Chandrasekaran *et al.*] that Gal:3-O-Sulfotransferases of human breast and colon tumor tissues, which act on Gal $\beta 1,3$ GalNAc α - and Gal $\beta 1,4$ GlcNAc β - respectively, act poorly on Gal $\beta 1,3$ GlcNAc β -. Thus, it appears that the formation of 3'- sialyl Lewis^a and 3'- sulfo Lewis x could be the favorable events in colon cancer. Further the predominancy of $\alpha 2,3$ (O) ST activity in breast and colon cancer cells precludes the possibility of mucin core 1 chain (Gal $\beta 1,3$ GalNAc α -O-Ser/Thr) elongation in these cells. But such as possibility seems to exist in ovarian cancer cells, as they express almost negligible amount of sialyltransferase activities.

A knowledge of glycoconjugate biosynthesis and the existing structures of glycans is helpful in interpreting the glycosyltransferase activity patterns. For example, $\alpha 2,6$ -Sia-TI prefers LacNAc type II glycan branches located at the $\alpha 1-3$ linked mannose branch of N-glycans [Joziassse *et al.*, 1987]. A lack of core 2 $\beta 1,6$ GlcNAc-T activity can lead to O-glycans having extended core 1 [Ellies *et al.*, 1998]. Le^a, Le^b and sialyl Le^a are known to be located at C-3 branch from GalNAc α . A monoclonal antibody specific for 3-O-sulfated Lewis a tetrasaccharide is capable of binding to sulfomucin, a high molecular weight glycoprotein in colon mucosa, suggesting the existence of 3-O-SulfoGal $\beta 1,3$ (Fuc $\alpha,4$)GlcNAc $\beta 1,3$, Gal $\beta 1,3$ as part of sulfomucin [Yamachika *et al.*, 1997]. A decrease of this epitope in colon cancer is reported whereas the expression of sulfo Lewis^x is not altered [Yamachika *et al.*, 1997]. Thus, in accordance with the invention, activities and specificity of the chain terminating enzymes such as fucosyl-, N-acetylgalactosaminyl-, sialyl- and sulfotransferases from cancer cells and tissues along with a knowledge of oligosaccharide biosynthesis would help to determine and select the carbohydrate epitopes present at the outer ends of known as well as unidentified cancer-associated antigens for immunological targeting.

The colon carcinoma cell line, Colo205, the hepatic carcinoma cell line, HepG₂, the breast carcinoma cell lines, BT20 and MCF-7, and the ovarian teratocarcinoma cell line, PA-1, were grown in minimal essential medium; the colon carcinoma cell line, LS180, and the breast carcinoma cell line, DU4475, were grown in RPCI 1640; the colon carcinoma cell line, SW1116, the ovarian carcinoma cell line, SW626, the breast carcinoma cell lines, MDA-MB-231, MDA-MB-435S, MDA-435/LCC6 and MDA-435/LCC6^{MDRI} were grown in Leibovitz's L-15 medium. All media were supplemented with 10% fetal bovine serum and the antibiotics, penicillin, streptomycin and amphotericin B in 250mL T-flasks under conditions as recommended by American Type Culture Collection, except for DU4475, which was grown as a suspension. MDA-435/LCC6 and MDA-435/LCC6^{MDRI} were kindly provided by Dr. Ralph Bernacki of this Institute. The cells were homogenized with 0.1M Tris-Maleate pH 7.2 containing 2% Triton X-100 using a Dounce all glass hand-operated homogenizer. The homogenate was centrifuged at 16,000g for 1h at 4°C. Protein was measured on the supernatants by the BCA micro method (Pierce Chemical Co.) with BSA as the standard. The supernatants were adjusted to 5mg protein per mL by adding the necessary amount of extraction buffer and then stored frozen at -20°C until use [Chandrasekaran *et al.*, 2003]. Aliquots (10µL) of the extracts were used in assays run in duplicate.

Colon tumor tissue (CC9338) and breast tumor tissue (BC9400) were obtained during surgical procedures at Roswell Park Cancer Institute and stored frozen within 1h at -70°C. The tissues were homogenized at 4°C with 4 volumes of 0.1M Tris Maleate pH 7.2 0.1% NaN₃ using kinematica. After adjusting the concentration of TritonX-100 to 2%, these homogenates were mixed in the cold room for 1h using Speci-Mix (Thermolyne) and then centrifuged at 20,000g for 1h at 4°C. The clear fat-free supernatant was stored frozen at -20°C until use. Aliquots of 10µL from this extract were used in assays run in duplicates. Glycosyltransferase activity in cell lysate was determined by mixing the lysates with acceptor and radiolabeled donor (monosaccharide) under the reaction conditions detailed below, followed by separation of un-reacted donor from the radioactive product using anionic or

hydrophobic chromatography. In all cases, the radioactive content of isolated products was determined by using 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL) and a Beckman LS6500 scintillation counter. Controls for each assay contained the reaction mixture with everything except the acceptor. Radioactivity of product was subtracted from that of control to obtain the results presented in the Tables. All assays were run in duplicate. Results from duplicate runs did not vary by more than 5%.

The acceptors used for measuring the glycosyltransferase and sulfotransferase activities are given in **Table I**. The following are the conditions for individual enzymatic assays. Reaction temperature in all cases was 37°C. α 2,3- and α 2,6 sialyltransferase (ST) assay reactions proceeded for 2h in a mixture containing 100mM sodium cacodylate buffer (pH 6.0), 7.5mM acceptor, CMP-[9-³H]NeuAc (typically 0.2 μ Ci) and 10 μ l cell extract in a total volume of 20 μ l [Chandrasekaran *et al.*, 1995]. β GlcNAc: β 1,4Gal-T and α GalNAc: β 1,3Gal-T assay mixtures in duplicate contained 0.1M Hepes-NaOH pH 7.0, 7mM ATP, 20mM Mn acetate, 1mM UDP-Gal, UDP [¹⁴C]Gal (0.05 μ Ci; 327mCi/mmol; Amersham), 0.5mM acceptor (unless otherwise stated) and the enzyme in a total volume of 20 μ L. It was incubated for 4h [Chandrasekaran *et al.*, 2001]. β GlcNAc: β 1,4GalNAc-T assay mixtures in duplicate contained 0.1M Hepes-NaOH pH 7.0, 7mM ATP, 20mM Mn acetate. UDP [³H] GalNAc (0.20 μ Ci; 7.8Ci/mmol; New England Nuclear Corp.) 7.5mM acceptor (unless otherwise stated) and the enzyme in a total volume of 20 μ L and incubated for 4h [Chandrasekaran *et al.*, 2001].

α 1,2, α 1,6-, α 1,3- and α 1,4-fucosyltransferase (FT) assay reactions were carried out for 2h in a reaction mixture containing 50mM Hepes buffer (pH 7.5), 5mM MnCl₂, 7mM ATP, 3mM NaN₃, 3mM synthetic acceptor or 40 μ g of fetuin-based acceptor, 0.05 μ Ci GDP-[¹⁴C]Fuc (290mCi/mmol) and 10 μ l cell extract in a total volume of 20 μ l [Chandrasekaran *et al.*, 1996]. Sulfotransferase (Sulfo T) assay reactions took 2h and required a mixture containing 100mM Tris-maleate (pH 7.2), 5mM Mg Acetate, 5mM ATP, 10mM NaF, 10mM BAL, 7.5mM acceptor, 0.5 μ Ci of

[³⁵S]PAPS (specific activity 2.4Ci/mmol) and 10μl of cell extract in a total volume of 30μl [Chandrasekaran *et al.*, 2004].

Dowex-1-Cl or Sep-Pak C18 cartridges were used to isolate radiolabeled product from the reaction mixture. For GalT, GalNAc-T and FT assays, the incubation mixture was diluted with 1ml water and passed through a 1ml Dowex-1-Cl column [Chandrasekaran *et al.*, 1996; Chandrasekaran *et al.*, 2001]. The column was washed twice with 1ml of water. The breakthrough and the water wash contained the [¹⁴C]-galactosylated or [¹⁴C]-fucosylated products formed with neutral acceptors. 3ml of 0.1M NaCl was used to obtain [¹⁴C]-fucosylated products from sialylated acceptors after water elution. For sialyltransferase assays, the radioactive products from benzylglycosides were separated by hydrophobic chromatography on Sep-Pak C18 cartridge (Waters, Milford, MA), and elution of the product was done with 3ml methanol [Palcic *et al.*, 1988]. For sulfotransferase assays, elution of the [³⁵S]-sulfated compound from Dowex-1-Cl column was achieved by 3ml of 0.2M NaCl [Chandrasekaran *et al.*, 2004].

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Table I

Acceptor-substrate specificity analysis of the cloned sialyltransferases and LNCaP cell $\alpha 2,3(\text{O})\text{ST}$

Acceptor (7.5mM)	Sialyltransferase activity (%)			
	Cloned $\alpha 2,3(\text{N})\text{ST}$	Cloned $\alpha 2,6(\text{N})\text{S}_\text{T}$	Cloned $\alpha 2,3(\text{O})\text{ST}$	LNCaP $\alpha 2,3(\text{O})\text{S}_\text{T}$
PART A:				
Gal β 1,4GlcNAc β -O-Al	100.0 ^a	100.0 ^b	4.7	0.5
Gal β 1,3GalNAc α -O-Al	24.1	0.2	112.0	34.4
Gal β 1,4GlcNAc β 1,3Gal β 1,4GlcNAc β -O-Bn			8.7	
Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al			120.5 ¹	60.4
Gal β 1,3GlcNAc β 1,3Gal β -O-Me			37.2	1.4
2-O-MeGal β 1,4GlcNAc	60.0	90.1	0.2	
3-O-MeGal β 1,4GlcNAc	1.4	8.7	3.6	
Gal β 1,3GlcNAc β -O-Al	123.1	3.0	42.1	3.7
2-O-MeGal β 1,3GlcNAc	115.3	3.7	2.9	
2-O-MeGal β 1,3GlcNAc β -O-Bn		0		
Gal β 1,3GalNAc β 1,3Gal α -O-Me	106.8 ²⁰	1.1	120.8 ²	34.3
D-Fuc β 1,3GalNAc β 1,3Gal α -O-Me	3.1	2.5	115.6 ³	9.6
Fetuin triantennary asialo gp (100 μ g)	66.1			
Gal β 1,3GalNAc α -O-Al/AA-CP (100 μ g)	1.7			
PART B:				
Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn	100.7	93.3	94.4 ⁴	
3-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn	6.1	4.4	100.0 ^c	100.0 ^d
Gal β 1,4GlcNAc β 1,6(3-O-MeGal β 1,3)GalNAc α -O-Bn	95.3	97.7	49.6	3.0
Gal β 1,4(Fuca1,3)GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Me	19.0	7.1	133.4	
Gal β 1,4(Fuca1,3)GlcNAc β 1,6(NeuAc α 2,3Gal β 1,3)GalNAc α -O-Bn	27.1	31.0		
NeuAc α 2,3Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Me			35.2	5.1
GalNAc β 1,4(Fuca1,3)GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Me			134.9	21.1

Acceptor (7.5mM)	Cloned $\alpha 2,3(N)ST$	Cloned $\alpha 2,6(N)S$ T	Cloned $\alpha 2,3(O)ST$	LNCaP $\alpha 2,3(O)S$ T
6-O-SulfoGlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Bn				
Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Bn			49.4 ^{e,5}	
Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me	28.1 ²¹	30.6 ₁₇	69.4 ^e	
Gal $\beta 1,4$ (6-O-Sulfo)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$)GalNAc α -O-Me	50.8 ²²	0.9		
Gal $\beta 1,4$ (6-O-Sulfo)(Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (NeuAc $\alpha 2,3$ Gal $\beta 1,3$)GalNAc α -O-Me	1.7	0		
NeuAc $\alpha 2,3$ Gal $\beta 1,4$ (6-O-Sulfo)(Fuc $\alpha 1,3$)GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Me			7.4	
3-O-SulfoGal $\beta 1,4$ GlcNAc $\beta 1,6$ (Gal $\beta 1,3$)GalNAc α -O-Bn			70.3 ^{e,6}	
Gal $\beta 1,4$ GlcNAc $\beta 1,6$ (3-O-SulfoGal $\beta 1,3$)GalNAc α -O-Bn		127.4 ^{e,18}	33.1 ^{e,7}	

100% represents ^a: 29520 CPM; ^b: 43500 CPM; ^c: 40560 CPM and ^d: 30930 CPM

^e: These values were obtained by quantitating the [¹⁴C] sialylated products from fractionation of the reaction mixture on a Biogel P2 column 1-7, 17-18, 20-22 : Mass spectroscopy analysis of these reaction products was performed in Table VI and supplemental data

Table II

Unique sialylation by cloned $\alpha 2,3(\text{O})\text{ST}$ results in identification of specific acceptors that can measure $\alpha 2,3(\text{O})\text{ST}$ activity in the presence of related enzymes

Acceptor	Sialyltransferase Activity (%)			
	Acceptor (0.75mM)		Acceptor (7.5mM)	
	Cloned $\alpha 2,3(\text{O})\text{ST}$	LNCaP $\alpha 2,3(\text{O})\text{ST}$	Cloned $\alpha 2,3(\text{O})\text{ST}$	LNCaP $\alpha 2,3(\text{O})\text{ST}$
Gal β 1,3GalNAc α -O-Bn	100.0 ⁸ (42550 CPM)	100.0 (30490 CPM)	100.0 ⁸ (43600 CPM)	100.0 (30600 CPM)
3-O-MeGal β 1,3GalNAc α -O-Bn	70.4	2.6	101.8	15.5
4-O-MeGal β 1,3GalNAc α -O-Bn	104.6 ⁹	141.5	105.0 ⁹	188.7
4-F-Gal β 1,3GalNAc α -O-Bn	104.6 ¹⁰	44.8	102.3 ¹⁰	105.1
Gal β 1,3(6-O-Me)GalNAc α -O-Bn	107.6 ¹¹	119.2	107.3 ¹¹	189.4
3-O-MeGal β 1,3(6-O-Me)GalNAc α -O-Bn	55.9	1.1	ND	ND

ND: Not determined

⁸⁻¹¹: Mass spectroscopy analysis of these reaction products was performed in Table VI and supplemental data

Table III

Sialyltransferase Activities towards Mucin Core 2 based Compounds

Mucin Core 2 Compound [7.5mM]	Cloned Sialyltransferase Activities			
	α 2,6(N)ST %	α 2,3(N) ST %	α 2,3(O) ST %	LNCaP α 2,3(O)ST %
3-O- MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α - O-Bn	6.4	4.5	100.0 ^c	100.0 ^d
Gal β 1,4GlcNAc β 1,6(3-O- MeGal β 1,3)GalNAc α -O-Bn	100.0 ^a	100.0 ^b	49.6	3.0
4-O- MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α - O-Bn	12.6	99.8	103.0	97.1
Gal β 1,4GlcNAc β 1,6(4-O- MeGal β 1,3)GalNAc α -O-Bn	101.3	100.6	101.3	112.0
3-FGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α - O-Bn	11.3	7.2	102.9 ¹²	91.5
Gal β 1,4GlcNAc β 1,6(3-FGal β 1,3)GalNAc α - O-Bn	77.7 ¹⁹	96.9 ²⁵	77.2	1.7
4-FGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α - O-Bn	2.7	7.9	102.1	ND

ND: Not determined

100% represents ^a: 30440CPM; ^b: 38140 CPM; ^c: 39200 CPM and ^d: 30100 CPM^{12, 19, 25}: Mass spectroscopy analysis of these reaction products was performed in Table VI and supplemental data

Table IVUnique sialylation of mucin core 2 based compounds by Cloned $\alpha 2,3(O)ST$

Compound [7.5mM]	Sialyltransferase activity (%)
Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al ^a	100.0 (29860 CPM) ¹
3-O-MeGal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn ^b	24.7
Fuc α 1,2Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn ^b	45.4
3-O-SulfoGal β 1,3(GlcNAc β 1,6) GalNAc α -O-Bn ^a	54.7

^a The [$9-^3H$] sialylated product was quantitated by BioGel P₂ Column Chromatography

^b Sep-Pak C18 fractionation procedure

¹: Mass spectroscopy analysis of this reaction product was performed in Table VI and supplemental data

Table V

Discerning the unique catalytic abilities of the cloned $\alpha 2,3(O)ST$ by utilizing an array of synthetic compounds containing modified terminal $\beta 1,3$ -Galactosyl residue as acceptors.

Compounds	^a CMP-NeuAc(150 μ M)		
	Km (mM)		Vmax(pmol/h)
a) Globo backbone:			
i) Gal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -O-Me	0.12		1293 ¹³
ii) 3-O-SulfoGal $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -O-Me		1.25	
416			
iii) D-Fuc $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -O-Me	0.56		1873
iv) 3-O-SulfoD-Fuc $\beta 1,3$ GalNAc $\beta 1,3$ Gal α -O-Me	2.00	14	624
b) T-hapten:			
i) 4-O-MeGal $\beta 1,3$ GalNAc α -O-Bn	0.13		1250
ii) 4-FGal $\beta 1,3$ GalNAc α -O-Bn	0.25		1442
iii) D-Fuc $\beta 1,3$ GalNAc α -O-Bn	0.21	15	1250
c) Mucin Core 2:			
i) D-Fuc $\beta 1,3$ (GlcNAc $\beta 1,6$)GalNAc α -O-Bn	0.28	16	1562
ii) Fuca1,2Gal $\beta 1,3$ (GlcNAc $\beta 1,6$)GalNAc α -O-Bn	1.82		197

^a The reaction mixtures (20 μ l) contained 0.2 μ Ci CMP[9-³H]NeuAc in 150 μ M CMP-NeuAc

¹³⁻¹⁶: Mass spectroscopy analysis of these reaction products was performed in Table VI and supplemental data. S4 (Table VI) is the synthetic acceptors that yields product ¹⁴.

Table VI

Mass spectral analysis of enzymatically sialylated acceptors

Compound a No.	Oligosaccharides	Yield ^c (nmol)	Calcd. MW	Found (m/z)	
				Negative mode	Positive Mode
1	Gal β 1,3(GlcNAc β 1,6)GalNAc α -O-Al $\uparrow$ α 2,3 NeuAc	146	917.3	[M-H] ⁻ : 916.0	
2	Gal β 1,3GalNAc β 1,3Gal α -O-Me $\uparrow$ α 2,3 NeuAc	138	850.3	[M-H] ⁻ : 848.9	[M- H+2Na] ⁺ : 894.7
3	D-Fuc β 1,3GalNAc β 1,3Gal α -O-Me $\uparrow$ α 2,3 NeuAc	160	835.2	[M-H] ⁻ : 833.8	[M- H+2Na] ⁺ : 879.2
4	Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn $\uparrow$ α 2,3 NeuAc	63	1129.4	[M-H] ⁻ : 1127.1	[M- H+2Na] ⁺ : 1173.5
5	6-O-SulfoGlcNAc β 1,6 (Gal β 1,3)GalNAc α -O-Al $\uparrow$ α 2,3 NeuAc	143	997.9	[M-2H] ²⁻ : 498.0	[M- 2H+4Na] ²⁺ : 544.0
6	3-O-SulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α - O-Bn $\uparrow$ α 2,3 NeuAc	145	1210.1	[M-2H] ²⁻ : 604.1	[M- 2H+4Na] ²⁺ : 650.3
7	Gal β 1,4GlcNAc β 1,6(3-O-SulfoGal β 1,3)GalNAc α - O-Bn $\uparrow$ α 2,2/4 ^b NeuAc	11	1210.1	[M-2H] ²⁻ : 604.1	[M- 2H+4Na] ²⁺ : 649.9
8	Gal β 1,3GalNAc α -O-Bn $\uparrow$ α 2,3 NeuAc	146	764.3	[M-H] ⁻ : 763.4	
9	4-O-MeGal β 1,3GalNAc α -O-Bn $\uparrow$ α 2,3 NeuAc	140	778.7	[M-H] ⁻ : 777.6	
10	4-F-Gal β 1,3GalNAc α -O-Bn $\uparrow$ α 2,3 NeuAc	124	766.2	[M-H] ⁻ : 764.9	[M- H+2Na] ⁺ : 813.4
11	Gal β 1,3(6-O-Me)GalNAc α -O-Bn $\uparrow$ α 2,3	117	778.7	[M-H] ⁻ : 777.5	[M- H+2Na] ⁺ : 823.3
12	3-F-Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn $\uparrow$ α 2,3 NeuAc	94	1131.4	[M-H] ⁻ : 1129.4	

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Compound No.	Oligosaccharides	Yield ^c (nmol)	Calcd. MW	Found (m/z)	
				Negative mode	Positive Mode
13	3-O-SulfoGalβ1,3GalNAcβ1,3Galα-O-Me ↑ α2,2/4 ^b NeuAc	13	931.2	[M-2H] ²⁻ : 463.9	
14	(3-O-Sulfo)D-Fucβ1,3GalNAcβ1,3Galα-O-Me ↑ α2,2/4 ^b NeuAc	17	915.2	[M-2H] ²⁻ : 455.2	
15	D-Fucβ1,3GalNAcα-O-Bn ↑ α2,3 NeuAc	114	748.7	[M-H] ⁻ : 747.6	
16	D-Fucβ1,3(GlcNAcβ1,6)GalNAcα-O-Bn ↑ α2,3 NeuAc	118	951.3	[M-H] ⁻ : 949.7	
17	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ α2,6 NeuAc	87	1134.0	[M-2H] ²⁻ : 566.8	[M-2H+4Na] ²⁺ : 611.9
18	Galβ1,4GlcNAc β1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn ↑ α2,6 NeuAc	121	1210.1	[M-2H] ²⁻ : 604.3	[M-2H+4Na] ⁺ : 649.6
19	Galβ1,4GlcNAc β1,6(3-F-Galβ1,3)GalNAcα-O-Bn ↑ α2,6 NeuAc	69	1131.4	[M-H] ⁻ : 1128.9	
20	Galβ1,3GalNAcβ1,3Galα-O-Me ↑ α2,3 NeuAc	175	850.3	[M-H] ⁻ : 849.0	[M-H+2Na] ⁺ : 895.0
21	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ α2,3 NeuAc	129	1134.0	[M-2H] ²⁻ : 567.2	[M-2H+4Na] ²⁺ : 611.3
22	Galβ1,4(6-O-Sulfo)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me ↑ α2,3 NeuAc	159	1423.0	[M-3H] ³⁻ : 472.2	
23	Galβ1,4GlcNAc β1,6(3-O-SulfoGalβ1,3)GalNAcα-O-Bn ↑ α2,3 NeuAc	146	1210.1	[M-2H] ²⁻ : 603.4	[M-2H+4Na] ⁺ : 649.5
24	4OMeGalβ1,4GlcNAcα-O-Bn ↑ α2,3 NeuAc	174	778.7	[M-H] ⁻ : 777.3	
25	Galβ1,4GlcNAc β1,6(3-F-Galβ1,3)GalNAcα-O-Bn ↑ α2,3 NeuAc	117	1131.4	[M-H] ⁻ : 1130.3	

S1	6-O-SulfoGal β 1,3GalNAc α -O-NP $\uparrow$ α 2,6 NeuAc	875.8	[M-2H] ²⁻ : 437.1	
S2	6-O-SulfoGal β 1,4(Fuc α 1,3)GlcNAc β -O-Me $\uparrow$ α 2,3 NeuAc	914.3	[M-2H] ²⁻ : 456.1	
S3	<i>D</i> -Fuc β 1,3GalNAc β 1,3Gal α -O-Me	543.0	[M-H] ⁻ : 542.2	[M-H+2Na] ⁺ : 565.8
S4	(3-O-Sulfo) <i>D</i> -Fuc β 1,3GalNAc β 1,3Gal α -O-Me	623.0	[M-H] ⁻ : 621.6	[M-H+2Na] ⁺ : 667.8

^a **NeuAc** denotes the sialic acid that was added to specific acceptors by the action of cloned sialyltransferases: $\alpha 2,3(\text{O})\text{ST}$ (Samples 1-16), $\alpha 2,6(\text{N})\text{ST}$ (Samples 17-19) and $\alpha 2,3(\text{N})\text{ST}$ (Samples 20-25). S1-S4 are chemically synthesized compounds

^b Sialylation may take place at either the C-2 or C-4 position of Gal in the Gal β 1,3GalNAc residue of the core-2 structure

^cYield was calculated from the percentage of radioactivity incorporated into the acceptor from 200nmol of CMP-19-³HNeuAc taken in the reaction mixture

TABLE VII:

Acceptors used for assaying the enzymes in cancer cell lines

Enzyme	Acceptor used for measuring the enzyme activity
Sialyltransferase	
α 2,3-Sia-TI or TII (ST3:Gal β 1,3GalNAc)	3-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn
α 2,3-Sia-TIV (ST3: Gal β 1,4GlcNAc)	Gal β 1,4GlcNAc β 1,6(3-O-MeGal β 1,3)GalNAc α -O-Bn
α 2,6-Sia-TI (ST6:Gal β 1,4GlcNAc)	
α 2,3-Sia-TIV	2-O-MeGal β 1,3GlcNAc β -O-Bn
Gal-/GalNAc-transferase	
β -GlcNAc: β 1,4-Gal-T	3-O-MeGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn
β -GlcNAc: β 1,4-GalNAc-T	3-O-MeGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn
α -GalNAc: β 1,3-Gal-T	4-F-GlcNAc β 1,6GalNAc α -O-Bn
Fucosyltransferase	
α 1,2-Fuc-T	Gal β -O-Bn
α 1,3-Fuc-T	2-O-MeGal β 1,4GlcNAc
α 1,4-Fuc-T	2-O-MeGal β 1,3GlcNAc
	Fetuin triantennary asialo glycopeptide
	Ancrod
Fuc-TVI	GlcNAc β 1,4GlcNAc β -O-Bn
Fuc-TVII	Fetuin triantennary sialo glycopeptide
α 1,6-Fuc-T	Fetuin triantennary asialo agalacto glycopeptide
Sulfotransferase	
Gal3-O-Sulfo-T ₂ or T ₃	Gal β 1,4GlcNAc β 1,6(3-O-MeGal β 1,3)GalNAc α -O-Bn
Gal3-O-Sulfo-T ₄	3-O-MeGal β 1,4GlcNAc β 1,6(Gal β 1,3) GalNAc α -O-Bn
GlcNAc6-O-Sulfo-T	3-O-MeGal β 1,3(GlcNAc β 1,6)GalNAc α -O-Bn

TABLE VIII

Sialyl-, Fucosyl-, β -Galactosyl-, β -N-Acetylglucosaminyl- and Glycan:3-O-Sulfo-Transferase activities in breast cancer cell lines.Activity: Incorporation of radioactive sugar or [35 S] sulfate [$\text{CPM} \times 10^{-3}$] catalyzed by 1mg protein of the cell extract

ND: Not Determined

ENZYME	MDA-MB-231	MCF-7	T47D	ZR-75-1	DU4475	BT20	MDA-MB435S	MDA-435/LCC6	MDA-MDR1
<u>Sialyltransferases:</u>									
$\alpha 2,3(\text{O})\text{ST}$	126.5	105.2	86.8	368.1	8.7	197.8	257.4	171.6	159.1
$\alpha 2,3(\text{N})\text{ST}$	10.6	9.4	4.2	12.3	2.4	18.3	26.3	18.4	17.7
$\alpha 2,6(\text{N})\text{ST}$	0	3.4	8.6	74.1	0	0	86.5	56.0	64.1
<u>Fucosyltransferases:</u>									
$\alpha 1,2\text{-L-FT}$	0.4	2.9	1.2	0.3	46.9	0.4	0	0	0.1
$\alpha 1,3\text{-L-FT}$	30.5	48.1	40.6	105.3	60.4	20.8	0	0	0
$\alpha 1,4\text{-L-FT}$	0.5	1.1	0.5	156.5	255.1	241.5	0	0	0
FTVI	0	0.4	0.3	0	1.9	0	2.5	0	0
$\alpha 1,6\text{-L-FT}$	631.2	362.0	127.3	612.4	611.4	205.6	496.6	444.9	447.8
FetTA-AsialoGP	200.1	195.1	ND	ND	221.6	105.4	96.1	99.9	103.8
FetTA-SialoGP	0	0	ND	ND	31.7	9.6	0	0	0
Ancrod	0	0	ND	ND	33.8	41.3	0	0	0

 β Gal/GalNAc-Transferases:

GlcNAc: β 1,4Gal-T	494.4(100.0)	284.9(100.0)	358.1	552.7	155.5(100.0)	260.7(100.0)	80.1(100.0)	400.1(100.0)	295.8(100.0)
GlcNAc: β 1,4GalNAc-T	152.1(30.8)	13.4(4.7)	ND	ND	103.3(66.4)	33.1(12.7)	34.7(43.3)	86.2(21.5)	65.7 (22.2)
α GalNAc: β 1,3Gal-T	28.8	23.3	30.6	36.3	47.4	35.6	11.1	45.3	43.6
<u>Glycan:Sulfotransferases</u>									
Gal β 1,3GalNAc α -:	1.2	3.4	0.3	2.1	51.8	<0.1	7.2	62.6	28.1
3-O-Sulfo-T									
Gal β 1,4GlcNAc β :	0.2	19.4	0.3	19.1	361.2	0.3	0.1	0.5	0.3
3-O-Sulfo-T	0.3	0	0	0.2	0.2	0.1	0	0.2	0.2
GlcNAc:6-O-Sulfo-T									

TABLE IX

A comparison of the levels of each potential signature carbohydrate structure among several cancer cell lines.

Carbohydrate Structural Unit	Breast Cancer Cell Lines						Colon Cancer Cell Lines			Ovarian Cancer Cell Lines		Hepatic Cancer Cell Lines	
	MDA-MB-231	MCF-7	T47D	ZR-75-1	DU4675	BT20	MDA-MB-435	MDA-435	MDA-435/Colo205	LS180	SW1116	SW626	PA-1
HepG2													
	231				435S		LCC6	LCC6MDR1					
i) 6-Fucosyl Chitobiose core	5X	3X	X	5X	2X	4X	4X	4X	4X	4X	4X	3X	
ii) 3-Fucosyl Chitobiose core	O	T	T	O	T	O	O	9X	16X	X	O	O	
iii) Lewis a	O	O	O	6X	10X	O	O	O	13X	9X	X	O	
iv) Lewis x	X ^a	2X	2X	4X	3X	X	O	O	9X	9X	3X	3X	T
v) 3'-Sialyl T-hapten	4X	3X	3X	12X	T	6X	6X	X	3X	3X	T	T	6X
vi) 3'-Sulfo Lewis x	O	X	O	X	18X	O	O	O	T	10X	X	O	ND ^c
vii) 3'-Sialyl Lewis x	T ^b	T	T	T	O	T	O	O	T	T	O	O	T
viii) 3'-Sialyl Lewis a	O	O	O	T	T	O	O	O	T	T	O	O	O
ix) T-hapten	T	T	T	T	T	T	T	O	O	O	X	X	T
x) 3'-Sulfo T-hapten	O	O	O	O	7X	O	9X	4X	T	5X	T	O	ND
xi) 3'-Sialyl LacNAc	O	O	O	O	O	O	X	X	T	T	O	O	X
xii) 6'-Sialyl LacNAc	O	O	O	X	O	O	X	X	T	O	O	O	5X
xiii) Lewis b	O	O	O	O	X	O	O	O	T	T	T	T	O
xiv) Lewis y	O	O	O	O	X	O	O	O	T	T	T	T	O

^a -x denotes the lowest concentration that was considered as 1-fold when a comparison of a particular structure was made among all the cancer cell lines studied here.

^b -T refers to trace amount

^c -ND - Not Determined

TABLE X

Sialyl-, Fucosyl-, β -Galactosyl-, β -N-Acetylglucosaminyl- and Glycan:3-O-Sulfo-Transferase activities of colon cancer, ovarian cancer, leukemia and hepatic cancer cell lines..

Activity: Incorporation of radioactive sugar or [35 S] sulfate [$\text{CPM} \times 10^{-3}$] catalyzed by 1mg protein of the cell extract.

ENZYME	COLON CANCER			OVARIAN CANCER		HEPATIC CANCER
	Colo205	LS180	SW1116	SW626	PA-1	
Sialyltransferases:						
α2,3(O)ST	34.0	86.7	95.9	9.0	5.0	185.7
α2,3(N)ST	3.5	7.4	7.5	1.3	1.3	22.6
α2,6(N)ST	4.2	0	0	0	0	362.8
Fucosyltransferases:						
α1,2-L-FT	3.8	5.5	3.4	2.7	0.9	0
α1,3-L-FT	267.7	257.8	223.7	88.7	86.7	11.8
α1,4-L-FT	319.0	210.8	89.4	24.0	0.8	0
FTVI	101.3	180.3	11.8	2.3	0.8	0
α,6-L-FT	413.6	350.9	343.9	438.9	443.5	292.0
FetTA-AsialoGP	391.7	399.7	199.0	165.4	148.9	39.6
FetTA-SialoGP	227.2	251.2	61.1	0	0	43.6
Ancrod	77.5	75.0	16.5	0	0	0
β-Gal/GalNAc-Transferases:						
GlcNAc:	297.5(100.0)	226.6(100.0)	503.4(100.0)	478.2(100.0)	187.0(100.0)	478.3(100.0)
β1,4Gal-T						
GlcNAc:	27.6(9.3)	73.0(32.2)	171.5(34.1)	142.8(29.9)	75.5(40.4)	148.1(31.0)
β1,4GalNAc-T						
α-GalNAc:	31.5	23.7	62.2	65.4	29.5	146.3
β1,3Gal-T						
Glycan:Sulfotransferases:						
Galβ1,3GalNAcα-:	1.3	33.4	17.3	2.5	0.8	ND
3-O-Sulfo-T						
Galβ1,4GlcNAcβ-:	6.4	216.0	92.5	15.7	0	ND
3-O-Sulfo-T						
GlcNAc: 6-O-Sulfo-T	0.1	0.2	0.4	0	ND	ND

TABLE XI

Sialyl-, Fucosyl-, β -Galactosyl-, β -N-Acetylgalactosaminyl- and Glycan:3-O-Sulfo-Transferase activities in three different tissues.

Activity: Incorporation of radioactive sugar or [^{35}S] sulfate [$\text{CPM} \times 10^{-3}$] catalyzed by 1mg protein of the tissue extract.

ENZYME	COLON TUMOR CC9338	BREAST TUMOR BC9400
<u>Sialyltransferases:</u>		
$\alpha 2,3(\text{O})\text{ST}$	721.4	283.0
$\alpha 2,3(\text{N})\text{ST}$	7.8	2.3
$\alpha 2,6(\text{N})\text{ST}$	148.0	11.6
<u>Fucosyltransferases:</u>		
$\alpha 1,2\text{-L-FT}$:		
i) Gal β -O-Bn	20.6	9.8
ii) Gal $\beta 1,3\text{GalNAc}\alpha\text{-O-Al}$	36.8	5.5
iii) Gal $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$	49.1	13.7
iv) D-Fuc $\beta 1,3\text{GalNAc}\beta 1,3\text{Gal}\alpha\text{-O-Me}$	89.8	16.2
$\alpha 1,3\text{-L-FT:2-O-MeGal}\beta 1,4\text{GlcNAc}$	540.9	30.5
$\alpha 1,2\text{-L-FT plus } \alpha 1,4\text{-L-FT}$:		
Gal $\beta 1,3\text{GlcNAc}\beta 1,3\text{Gal}\beta\text{-O-Me}$	578.9	16.8
$\alpha 1,4\text{-L-FT}$:		
2-O-MeGal $\beta 1,3\text{GlcNAc}$	727.5	4.6
FTVI:GlcNAc $\beta 1,4\text{GlcNAc}\beta\text{-O-Bn}$	139.1	0.3
<u>β-Gal/GalNAc-Transferases:</u>		
a) β -GlcNAc: $\beta 1,4\text{Gal-T}$	421.0(100.0%)	403.4(100.0%)
b) β -GlcNAc: $\beta 1,4\text{GalNAc-T}$	85.3(20.3%)	153.2(38.0%)
α -GalNAc: $\beta 1,3\text{Gal-T}$	102.2(24.3%)	38.6(9.6%)
<u>a and b by using Other Acceptors:</u>		
i) GlcNAc $\beta 1,6\text{Man}\alpha\text{-O-Al}$:	a. 493.4(100.0%)	443.6(100.0%)
	b. 54.5(11.0%)	99.2(22.4%)
ii) GlcNAc $\beta 1,2\text{Man}\beta 1,6\text{Glc}\beta\text{-O-Al}$:	a. 277.7	70.5
	b. 25.6	57.9
iii) GlcNAc $\beta 1,3\text{Gal}\beta\text{-O-Me}$:	a. 247.9	338.5
	b. 20.9	60.9
iv) GlcNAc $\beta 1,4\text{GlcNAc}\beta\text{-O-Bn}$:	a. 307.6	436.7
	b. 18.9	57.8
<u>Glycan:Sulfotransferases:</u>		
Gal $\beta 1,3\text{GalNAc}\alpha\text{-:3-O-Sulfo-T}$	40.1	60.2
Gal $\beta 1,4\text{GlcNAc}\beta\text{-:3-O-Sulfo-T}$	181.2	5.0
GlcNAc:6-O-Sulfo-T	52.8	69.8

Table XII. Availability of Novel Acceptors for Enzymatic Studies [[#]Synthesis of compound in progress. Bolded sugar residues denote site of glycosyltransferase action].

Compound	Acceptor	Enzyme
1	Galβ1→4GlcNAcβ1→6(MeO-3Galβ1→3)GalNAcα→OB	ST IV, Sulfo T-4
2	MeO-3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα→OB	ST I, II, Sulfo T-2,3
3	Galβ1→4GlcNAcβ1→6(F-3Galβ1→3)GalNAcα→OB	Sulfo T-4
4	F-3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα→OB	Sulfo T-3
5	Galβ1→4GlcNAcβ1→6(MeO-4Galβ1→4)GalNAcα→OB	α(1,4) GNT II
6	(MeO-4Galβ1→4)GlcNAcβ1→6(Galβ1→3)GalNAcα→OB	α(1,4) GNT I
7	Galβ1→4GlcNAcβ1→6 (F-4Galβ1→3)GalNAcα→OB	α(1,4) GNT II
8	F-4Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα→OB	α(1,4) GNT I
9	NeuAcα2→3Galβ1→4GlcNAcβ1→6(MeO-3Galβ1→3)GalNAcα→OB	Gal-6-Sulfo T I
10	MeO-3Galβ1→4GlcNAcβ1→6(NeuAcα2→3)Galβ1→3GalNAcα→OB	Gal-6-Sulfo T II
11	Galβ1→4MeO-3GlcNAcβ1→6(GalNAcβ1→3)GalNAcα→OB	α(1,2)- L-FT I
12 [#]	GalNAcβ1→4(MeO-3GlcNAc)β1→6(Galβ1→3)GalNAcα→OB	α(1,2) -L-FT II
13 [#]	SE-6Galβ1→4GlcNAcβ1→6(GalNAc1→3) GalNAcα→OB	α(1,2)-L-FT I for Galβ1→4 GlcNAc
14 [#]	SE-6GalNAc1→4GlcNAcβ1→6(SE-6 Gal1→3)GalNAcα→OB	α(1,2) L-FT II

Abbreviations used herein are as follows:

AA/CP:	Acrylamide Copolymer;
Al	Allyl
BAL	British anti-Lewisite
Bn	Benzyl
BSA:	Bovine serum albumin;
CPM	Counts per minute
FetTA	Fetuin triantannery
FT	Fucosyltransferase
GlyCAM-1:	Glycosylation-dependent cell adhesion molecule-1;
Globo backbone	Gal β 1,3GalNAc β 1,3Gal α -
GP	Glycopeptide molecule-1;
LacNAc:	Gal β 1,4GlcNAc;
LacNAc type 1	Gal β 1,3GlcNAc
LacNAc type 2	Gal β 1,4GlcNAc
Lewis ^a	Gal β 1,3(Fuc α 1,4)GlcNAc
Lewis ^b	Fuc α 1,2Gal β 1,3(Fuc α 1,4)GlcNAc
Lewis ^x	Gal β 1,4(Fuc α 1,3)GlcNAc
Lewis ^y	Fuc α 1,2Gal β 1,4(Fuc α 1,3)GlcNAc
Me	Methyl

PAPS	3'-phospho adenosine, 5'-phosphosulfate
PNA:	Peanut agglutinin;
PSGL-1:	P-selectin glycoprotein ligand-1;
RCA:	Ricinus communis agglutinin;
RM:	Reaction mixture;
Siglec:	Sialic acid-binding lectin of the immunoglobulin super family;
ST:	Sialyltransferase;
T-hapten:	Gal β 1,3GalNAc α -;
TLC:	Thin layer chromatography;
WGA:	Wheat germ agglutinin.

What is claimed is:

1. A method for determining substrates specific for a transferase enzyme selected from the group consisting of glycosyltransferases and sulfotransferases comprising:

- a) selecting a substrate to be tested for specificity for sulfotransferases and for glycosyltransferases, from tri, tetra or penta saccharide compounds containing at least one $\rightarrow$ GlcNAc β 1 saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow$ 3GalNAc α -OR, $\rightarrow$ 3GlcNAc β -OR or $\rightarrow$ 3Gal α -O-R where R is alkyl, aryl, azido or allyl all of 1 through 8 carbon atoms where R may be substituted with halo, -OH, -SO₃, or -NO₂;
- b) combining the substrate separately with each glycosyltransferase or sulfo-transferase in a series, for transfer of a particular glycosyl or sulfo moiety, to a saccharide, in conjunction with a donor compound for the particular moiety, where the moiety is radio labeled, in a buffered aqueous solution of about pH of about 6 to about 7, to form incubation mixtures;
- c) incubating the incubation mixtures at a temperature of from about 18 to about 40 degrees Celcius for from about 30 minutes to about four hours;
- d) testing the resulting incubation mixtures of each separate glycosyltransferase or sulfotransferase for reaction product comprising a chemical combination of substrate and particular moiety to determine effectiveness of each separate glycosyltransferase or sulfotransferase in transferring the particular moiety to the substrate; and.
- e) comparing the effectiveness of each glycosyltransferase or sulfotransferase in transferring the particular moiety to determine whether a particular glycosyltransferase or sulfotransferase acts to transfer the particular moiety to the substrate when the other glycosyltransferases or sulfotransferases do not do so, thus determining whether the substrate acts specifically with a particular glycosyltransferase or sulfotransferase.

2. The method of claim 1 where the substrate terminates in $\rightarrow 3\text{GalNAc}\alpha\text{-OR}$.
3. The method of claim 2 where the substrate also contains at least one of $\text{Gal}\beta 1$, $\text{DFuc}\beta 1$ or an additional $\rightarrow \text{GlcNAc}\beta 1$ where the $\text{Gal}\beta 1$, $\text{DFuc}\beta 1$ or additional $\rightarrow \text{GlcNAc}\beta 1$ may be substituted with MeO- , EtO- , Allyl-O- , or $\text{N}_3\text{-}$.
4. The method of claim 1 where the transferase is a glycosyltransferase selected from the group consisting of sialyltransferases, fucosyltransferases, N-acetylglucosaminyl transferases, N-acetylgalactosaminyl transferases, and galactosyltransferases.
5. The method of claim 1 where the incubation temperature is about 35 to about 40°C .
6. The method of claim 1 where the incubation temperature is about 37°C .
7. A method for the specific detection of a specific transferase for a specific moiety selected from the group consisting of sulfotransferases and glycosyltransferases comprising:
 - a) selecting a substrate specific for the transferase from tri, tetra or penta saccharide compounds containing at least one pre-terminal saccharide that may be substituted with MeO- , EtO- , Allyl-O- , or $\text{N}_3\text{-}$ and terminating in $\rightarrow 3\text{GalNAc}\alpha\text{-OR}$, $\rightarrow 3\text{Gal}\alpha\text{-O-R}$ or $\rightarrow 3\text{GlcNAc}\beta\text{-OR}$ where R contains 1 to 12 carbon atoms and is alkyl, aryl or allyl and R may be substituted with halo, -OH, lower alkyl, -SO_3 , azido or -NO_2 ;
 - b) combining the substrate with a sample to be tested for the specific transferase in conjunction with a donor compound for the particular moiety, in a buffered aqueous solution of about pH 6 to about pH 7, to form an incubation mixture;
 - c) incubating the incubation mixture at a temperature of from about 18 to about 40 degrees Celcius for from about 30 minutes to about four hours; and
 - d) testing the resulting incubation mixture for reaction product comprising a chemical combination of substrate and particular moiety to determine the presence of the particular transferase in the sample.

8. The method of claim 7 where the substrate contains at least one GlcNAc β 1 saccharide.
9. The method of claim 7 where the substrate terminates in $\rightarrow$ 3GalNAc α -OR.
10. The method of claim 8 where the substrate, with the exception of saccharide and terminal linkages, is unsubstituted.
11. The method of claim 7 where the substrate also contains at least one of Gal β 1, DFuc β 1 or an additional $\rightarrow$ GlcNAc β 1 where the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 may be substituted with MeO-, EtO-, Allyl-O, or N₃-.
12. The method of claim 11 where, with the exception of saccharide linkages, the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 are unsubstituted.
13. The method of claim 7 where the incubation temperature is from about 35 to about 40°C.
14. The method of claim 13 where the incubation temperature is about 37°C.
15. The method of claim 7 where steps a) through d) are repeated with different concentrations of substrate to determine the minimum concentration of substrate required to maximize quantity of obtained combination of substrate and particular moiety and the determined minimum concentration is correlated with quantity of the specific transferase in the sample.
16. The method of claim 7 where R is aryl that may be substituted with -OH, halo, or lower alkyl.
17. The method of claim 7 where R is allyl, benzyl, methyl, azido or nitrophenyl.
18. The method of claim 7 where the specific substrate is Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-3Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase IV (ST IV) and the donor compound is cytidine-5' monophosphate-Nacetylneuraminic acid (CMP-sialic acid).
19. The method of claim 7 where the specific substrate is MeO-Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-Nacetylneuraminic acid (CMP-sialic acid).

20. The method of claim 7 where the substrate is $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{F-3Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is sialyl transferase IV (ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
21. The method of claim 7 where the substrate is $\text{F-3Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).
22. The method of claim 7 where the substrate is $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{MeO-4Gal}\beta 1 \rightarrow 4)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,4$ N-acetylglucosaminyl transferase II ($\alpha 1,4$ -GNTII) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
23. The method of claim 7 where the substrate is $(\text{MeO-4Gal}\beta 1 \rightarrow 4)\text{GlcNAc}\beta 1 \rightarrow 6(\text{Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,4$ N-acetylglucosaminyl transferase I ($\alpha 1,4$ -GNTI) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
24. The method of claim 7 where the substrate is $\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{F-4Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1,4$ N-acetylglucosaminyl transferase II ($\alpha 1,4$ -GNTII) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
25. The method of claim 7 where the substrate is $\text{F-4Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is $\alpha 1 \rightarrow 4$ N-acetylglucosaminyl transferase I ($\alpha 1,4$ -GNTI) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).
26. The method of claim 7 where the substrate is $\text{NeuAc}\alpha 2 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{MeO-3Gal}\beta 1 \rightarrow 3)\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific transferase is the sulfotransferase, galactose 6 sulfotransferase I (Gal-6-SulfoTI) and the donor compound is 3'phosphoadenosine 5'phosphosulfate (PAPS).
27. The method of claim 7 where the substrate is $\text{MeO-3Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 6(\text{NeuAc}\alpha 2 \rightarrow 3)\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\alpha \rightarrow \text{O-R}$, the specific

transferase is the sulfotransferase, galactose 6 sulfotransferase II (Gal-6-SulfoTII) and the donor compound is 3'phosphoadenosine 5'phosphosulfate (PAPS).

28. The method of claim 7 where the substrate is Gal β 1 $\rightarrow$ 4MeO-3GlcNAc β 1 $\rightarrow$ 6(GalNAc β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,2 L-fucosyltransferase I (α 1,2-L-FT I) and the donor compound is guanosine diphosphate fucose (GDP-fucose).

29. The method of claim 7 where the substrate is GalNAc β 1 $\rightarrow$ 4(MeO-3GlcNAc) β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,2 L-fucosyltransferase II (α 1,2-L-FT II) and the donor compound is guanosine diphosphate fucose (GDP-fucose).

30. The method of claim 7 where the substrate is GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(GalNAc β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R, the specific transferase is α 1,3 L-fucosyltransferase (α 1,3-L-FT) and the donor compound is guanosine diphosphate fucose (GDP-fucose).

31. The method of claim 7 where the substrate is Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)GalNAc α $\rightarrow$ O-R, the specific transferase is β 1 $\rightarrow$ 3/4 N-acetyl galactosaminyl transferase (β 1 $\rightarrow$ 3/4 GalNAc-T) and the donor compound is uridine diphosphate galactosamine (UDP-GalNAc).

32. The method of claim 7 where the substrate is MeO-3Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)GalNAc α $\rightarrow$ O-R, the specific transferase is an α X N-acetyl glucosaminyl transferase (α XGlcNAc-T) and the donor compound is uridine diphosphate N-acetylglucosamine (UDP-GlcNAc).

33. The method of claim 7 where the substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase IV (Gal3ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

34. The method of claim 7 where the substrate is 4-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase IV (Gal3ST IV) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

35. The method of claim 7 where the substrate is D-Fucβ1→3GalNAcβ1→3Galα-O-methyl, the specific transferase is sialyl transferase I/II (Gal3ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

36. The method of claim 7 where the substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

37. The method of claim 7 where the substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

38. The method of claim 7 where the substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

39. The method of claim 7 where the substrate is 2-O-MeGal β 1 $\rightarrow$ 4GlcNAc β -O-benzyl, the specific transferase is sialyl transferase I/II (ST I/II) and the donor compound is cytidine-5' monophosphate-N-acetylneuraminic acid (CMP-sialic acid).

40. An oligosaccharide compound comprising 3 to 5 linked monosaccharides containing at least one $\rightarrow$ GlcNAc β 1 saccharide that may be substituted with MeO-, EtO-, Allyl-O, or N₃- and terminating in $\rightarrow$ 3GalNAc α -OR where R is alkyl, aryl or allyl all of 1 through 8 carbon atoms where R may be substituted with halo, -OH, -SO₃, or -NO₂ and further containing at least one of Gal β 1, DFuc β 1 or an additional $\rightarrow$ GlcNAc β 1 where the Gal β 1, DFuc β 1 or additional $\rightarrow$ GlcNAc β 1 may be substituted with MeO-, EtO-, Allyl-O, or N₃-.

41. The compound of claim 40 selected from the group consisting of
Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-4Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; MeO-
4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R;

Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(F-4Gal β 1 $\rightarrow$ 3) GalNAc α $\rightarrow$ O-R; F-
 4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; MeO-
 3Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6)
 GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 3(GlcNAc β 1 $\rightarrow$ 6) GalNAc α $\rightarrow$ O-R; D-
 Fuc β 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 3Gal α -O-methyl; and
 GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(GalNAc β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R.

42. The compound of claim 41 where R is benzyl.

43. A compound that acts as a specific substrate for a specific glycosyl or sulfo transferase enzyme, said substrate being selected from the group consisting of: Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(MeO-4Gal β 1 $\rightarrow$ 4)GalNAc α $\rightarrow$ O-R; MeO-Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R; Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(F-4Gal β 1 $\rightarrow$ 3) GalNAc α $\rightarrow$ O-R; and F-4Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 6(Gal β 1 $\rightarrow$ 3)GalNAc α $\rightarrow$ O-R where R contains 1 to 12 carbon atoms and is alkyl, aryl, azido, or allyl, that may be substituted with halo, -OH, -SO₃, or -NO₂.