Characterization of Two Glycolipid:α2-3Sialyltransferases, SAT-3 (CMP-NeuAc:nLcOse4Cer α2-3sialyltransferase) and SAT-4 (CMP-NeuAc:GgOse4Cer α2-3sialyltransferase), from Human Colon Carcinoma (Colo 205) Cell Line[†]

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ABSTRACT: Sialyltransferase activities, SAT-3 (CMP-NeuAc:nLcOse4Cer α2-3sialyltransferase) and SAT-4 (CMP-NeuAc:GgOse4Cer α2-3sialyltransferase), in Colo 205 cells catalyze the transfer of sialic acid to the terminal galactose of GlcNAc- and GalNAc-containing glycolipid substrates, respectively. Competition kinetic studies with nLcOse4Cer and GM1 as substrates in a sialyltransferase assay show that these two activities are catalyzed by two different catalytic entities. The two enzymes were co-solubilized with taurocholate and resolved by DEAE−Cibacron Blue−Sepharose column chromatography into two elution peaks. The column eluent with SAT-3 activity failed to transfer sialic acid to asialo α_1-acid glycoprotein, indicating that this enzyme is different from the sialyltransferase (ST3N) that synthesizes NeuAcα2-3Gal linkage in asparagine-linked oligosaccharides of glycoprotein. However, SAT-3 activity can be immunoprecipitated with a polyclonal antibody produced against a protein expressed in *Escherichia coli* as GST-fusion protein from an ECB cDNA homolog of an α2-3sialyltransferase (SAT-3 or STZ) that has been cloned from human melanoma cell and human placenta. Thus a concentration-dependent decrease in the residual SAT-3 activity relative to SAT-4 activity was observed in the supernatant after precipitation of the immune complex. Expression of SAT-3 (STZ) cDNA was also detected in Colo 205 cell by RT-PCR, followed by sequence analysis of the RT-PCR product. Characterization of the catalytic reaction products of SAT-3 and SAT-4 with thin-layer chromatography, sialidase treatment, and binding to specific antibodies indicates that both SAT-3 and SAT-4 catalyze the formation of α2-3 linkage between sialic acid and terminal galactose of glycolipid substrates.

Gangliosides[1] comprise a family of acidic glycolipids that are characterized by the presence of sialic acid. They are ubiquitous in all higher eukaryotes and are implicated to be involved in cell−cell interaction (Hakomori, 1981), cell growth and differentiation (Hakomoi, 1990), and host−pathogen interaction (Karlsson, 1989). The biosynthesis of gangliosides occurs in the Golgi apparatus starting from ceramide by sequential and coordinated addition of glucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and sialic acid (Basu, S., 1966; Schachter & Roseman, 1980; Basu, S. C., 1991; van Echten & Sandhoff, 1993). These reactions are catalyzed by specific glycosyltransferases. Each of these glycosyltransferases is specific for the terminal acceptor sugar, the anomeric linkage formed by the enzyme and in some cases the specificity extends beyond to include the structure of the glycolipid (Basu, S., et al., 1990). Sialyltransferases form a subclass of these glycosyltransferases and catalyze the transfer of sialic acid (NeuAc) from CMP-NeuAc to terminal galactose or another sialic acid. At least five different sialyltransferase activities (Basu, S., 1966; Kaufman et al., 1967; Basu, S., & Basu, M., 1982; Higashi et al., 1985; Basu, M., et al., 1987; Basu, S., et al., 1995; Freischutz et al., 1994) are known to be required for the synthesis of the sialylated glycolipids.

The present studies are concerned with the two sialyltransferase activities, CMP-NeuAc:nLcOse4Cer α2-3sialyltransferase (SAT-3) and CMP-NeuAc:GgOse4Cer α2-3sialyltransferase (SAT-4), in human colon carcinoma cells (Colo 205) catalyzing the following reactions, respectively:

$$\text{CMP}[^3\text{H}]\text{NeuAc} + \text{nLcOse4Cer} \rightarrow [^3\text{H}]\text{NeuAc}(\alpha2\text{-}3)\text{nLcOse4Cer (LM1)} + \text{CMP}$$

$$\text{CMP}[^3\text{H}]\text{NeuAc} + \text{GgOse4Cer} \rightarrow [^3\text{H}]\text{NeuAc}(\alpha2\text{-}3)\text{GgOse4Cer (GM1b)} + \text{CMP}$$

These two enzyme activities have been reported previously in embryonic chicken brain and bovine spleen (Basu, S., & Basu, M., 1982; Basu, S., et al., 1988). The SAT-3 activity is involved in the biosynthesis of sialyl-Le^x epitope (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc-) on glycolipid. Sialyl-Le^x is

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[1] Abbreviations: GgOse4Cer or Gg4 (Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer); GM1 (Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glc-cer); nLcOse4Cer or nLc4 (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer); SA$^-\alpha_1$AGP (asialo α_1-acid glycoprotein); DSS (detergent-solubilized supernatant); TDC (taurodeoxycholate); ECB (embryonic chicken brain); SAT-1 (CMP-NeuAc:LcOse2Cer α2-3sialyltransferase); SAT-2 (CMP-NeuAc:GM3 α2-3sialyltransferase).

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known as an oncofetal antigen. Using monoclonal antibodies in tissue immunostaining, Le^x and sialyl-Le^x antigens have been shown to be only weakly expressed in a limited number of adult cells in normal gastrointestinal tissue but are strongly expressed in fetal tissue as well as in adenocarcinoma derived from gastrointestinal, pulmobronchial, and breast tissues (Fukushi et al., 1984, 1985). In addition, increased expression of sialyl-Le^x determinants was suggested to contribute to metastatic behavior of carcinoma cells (Polley et al., 1991; Matsushita et al., 1991; Hakomori, 1991). The sialyl-Le^x epitope has also been found to be the ligand for selectins, the adhesion molecules involved in leukocyte migration and recruitment at the site of inflammation (Lowe et al., 1990; Phillips et al., 1990; Polley et al., 1991). Our results show that, in Colo 205 cells, this enzyme activity is catalyzed by an enzyme different from that involved in the terminal sialylation of GM1 or Gg4 in the ganglio series glycolipid biosynthesis pathway. SAT-3 in Colo 205 also appears to be different from the sialyltransferase that transfers to terminal galactose of asparagine-linked glycoprotein.

MATERIALS AND METHODS

Materials. The following materials were obtained from commercial sources: sodium taurocholate, Triton CF-54, Triton X-100, taurodeoxycholate, non-radioactive CMP-NeuAc (Sigma Chemical Co.); Zwittergent detergent 3-14 (Calbiochem); TLC plates (Brinkman Instruments); Whatman 3MM and SG-81 papers (VWR); CMP-[^{3}H]NeuAc (American Radiolabeled Chemicals, Inc.); CMP-[^{14}C]NeuAc (New England Nuclear, Inc.). Tissue culture flasks and 96-well titer plates for ELISA were obtained from Corning Glass Works. RPMI-1640 medium, penicillin, and streptomycin are obtained from Gibco Laboratories. Sterile filtered bovine serum (REHATUHIN) was obtained from Intergen Co. (Purchase, NY). *Clostridium perfringens* neuraminidase was purchased from Sigma Chemical Co. Neuraminidase from Newcastle disease virus was isolated from allantoic fluid of a virus-infected 11-day-old fertile chicken egg. Anti-LM1 was produced in our laboratory by injecting rabbit with LM1-conjugated BSA.

Preparation of the Substrates. Neolactotetraosylceramide, nLc4, the glycosphingolipid acceptor substrate for SAT-3, was prepared by controlled hydrolysis of nLc5 from bovine erythrocyte with α-galactosidase from fig (Li & Li, 1972). The glycosphingolipid acceptor substrate of SAT-4, GM1, was isolated from bovine brain, while gangliotetraosylceramide (Gg4) was prepared by controlled hydrolysis of GM1 with 0.2 N formic acid at 80 °C.

Commercially obtained human α_1-acid glycoprotein (α_1-AGP) was freed from contaminant serum albumin by passage through a Cibacron Blue agarose column. Purified α_1-AGP was subsequently desialylated by treatment with 0.1 M formic acid at 80 °C for 2 h and then dialyzed against distilled water and lyophilized.

Cell Culture. Human colon carcinoma Colo 205 cells were routinely grown and maintained in culture in a 250 mL (75 cm^2) T-flask containing RPMI-1640 medium supplemented with 10% fetal calf serum in the presence of penicillin (50 units/mL) and streptomycin (50 μg/mL). The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 (pH 7.0) at 37 °C. The medium was changed once before harvesting. When monolayer reached confluence, the cells were subcultured using phosphate-buffered saline (PBS) containing 0.1% EDTA and harvested using 20 mM HEPES buffer, pH 7.0, containing 0.32 M sucrose, 0.1% mercaptoethanol, and 1 mM EDTA.

Solubilization of SAT-3 and SAT-4 Activities. Packed cells were homogenized with 2 volumes of homogenization buffer containing 15 mM cacodylate-HCl (pH 6.8), 1 mM EDTA 0.15 M NaCl, 25 mM $MgCl_2$, 5 mM benzamidine, 0.005% soyabean-trypsin inhibitor, and 20% glycerol. Homogenate was centrifuged at 100 000*g* in 2 Beckman ultracentrifuge, at 4 °C, for 60 min. The pellet was homogenized with 2 volumes of the homogenization buffer, and taurocholate in 2:1 protein:detergent ratio was added and stirred with magnetic stirrer for 90 min at 4 °C. The mixture was then centrifuged at 100 000*g* in a Beckman ultracentrifuge, at 4 °C, for 60 min, and detergent soluble supernatant (DSS) was collected.

DEAE−Cibacron Blue Column Chromatography. A 2 mL amount of Colo 205 DSS was loaded on a 6 mL DEAE-Cibacron Blue-agarose column equilibrated with buffer (buffer A) containing 15 mM cacodyate-HCl (pH 6.8), 1 mM EDTA, 0.15 M sodium acetate, 25 mM $MgCl_2$, 20% glycerol, and 1% taurocholate. The elution of protein was continuously monitored by absorbance measurement at 280 nm, as shown in the elution profile (Figure 5). Buffer A, buffer B (containing 0.3 M sodium acetate in buffer A), and buffer C (containing 0.5 M sodium acetate in buffer A) were used successively to elute bound protein, followed by elution with a linear sodium acetate gradient with buffer C as the starting buffer and buffer D (1.25 M sodium acetate in buffer A) as the limit buffer. Fractions of 1.0 mL were collected and assayed for sialyltransferase activities with nLc4 and GM1 as acceptor substrates.

Assay of Sialyltransferase Activity. Enzymatic assays of SAT-3 and SAT-4 were carried out as described previously (Basu, S., et al., 1988), with little modification to provide the optimum conditions for SAT-3 and SAT-4 activities. The incubation mixture contained the following components in a final incubation volume of 35 μL: aceeptor glycolipid (20 nmol or at concentrations indicated in the figures) or glycoprotein (at concentrations indicated in the figures), 10 μL of 1 M cacodylate-HCl buffer (pH 6.8) containing 25 μM $MgCl_2$, 20 μL of enzyme fraction containing 100−200 μg of protein, 300 μM of CMP-[^{14}C]NeuAc in 5 μL (30 000−35 000 cpm). Detergent taurocholate (sodium salt) was added to the incubation mixture in the protein:detergent ratio of 0.5, when assays were performed with an unsolubilized enzyme fraction. Incubation was for 60−120 minutes, at 37 °C, and the reaction was terminated by the addition of 100 μL of chloroform:methanol (2:1). The whole mixture was spotted on Whatman 3 MM paper followed by descending chromatography or high-voltage electrophoresis using 0.1% sodium borate buffer. The appropriate areas of each chromatogram were cut out, and the amount of radiolabeled sialic acid transferred to glycolipid substrate was determined by a Beckman liquid scintillation counter (model LS-3133T).

Isolation of the Radiolabeled Products. [^{14}C]NeuAc-nLcOse4Cer or [^{14}C]NeuAc-GgOse4Cer was isolated from 20-fold increased incubation mixtures. The incubation mixtures were applied to a C_{18} Sep-Pak cartridge, washed with 5 mL of 0.01 M KCl and 25 mL of distilled water, and the ^{14}C-glycolipid fraction was eluted with 5 mL of methanol

followed by 10 mL of chloroform:methanol (1:1). They sialylated products were separated from unreacted substrate by DEAE−Sephadex A-50 column chromatography. The radioactive product eluted with 0.1 M sodium acetate.

Neuraminidase Treatment of Isolated Ganglioside Products. ^{14}C-labeled products of SAT-3 and SAT-4 (3000 cpm each) were incubated with 0.05 units of *C. perfingens* neuraminidase or 20 μL of Newcastle disease disease virus lysate at 37 °C for 12 h, in 20 μL of 0.2 M sodium acetate buffer (pH 5.5), 25 μg of taurodeoxycholate, and 5 μL of 0.1 M $CaCl_2$. The incubation mixtures were spotted on Whatman 3MM paper, and high-voltage electrophoresis was performed. The appropriate areas of each chromatogram were cut out, and the amount of [^{14}C]NeuAc recovered after neuraminidase treatment was quantitated in a liquid scintillation counter.

Binding of Anti-LM1 and Anti-SSEA-4 to SAT-3 and SAT-4 Products, LM1 and GM1a, Respectively. Glycolipid dissolved in methanol was spotted in the wells of polystyrene titer plates (Corning) and allowed to dry. Washing buffer (50 μL; containing 4.5 mM barbital, 4.5 mM sodium barbital, 0.15 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% ovalbumin, and 0.1% gelatin) was added to each well and incubated for 60 min, at 37 °C. An aliqout of 50 μL of anti-LM1 or MC-813-70 anti SSEA-4 (IgG3) (Kannagi et al., 1983), diluted 5-fold with dilution buffer (same as washing buffer, except containing 1.0% ovalbumin instead of 0.1%), was added and incubated for 60 min, at 37 °C. Each well was then washed with 250 μL of washing buffer, three times. An aliquot of 50 μL of a second antibody, HRP conjugated goat anti-mouse or anti-rabbit polyvalent IgG, diluted (1:100) in dilution buffer, was added, and incubated for 60 min at 37 °C. The wells were washed with washing buffer and then 200 μL of coloring solution (2 mg of *O*-phenylenediamine in 20 mL of water and 35 μL of 3% hydrogen peroxide) was added per well and incubated in the dark, at room temperature, until color developed (∼30 min). Absorbance at 405 nm was measured using a Titertek Multiscan Plus plate reader.

Cloning and Sequencing of Colo 205 SAT-3 cDNA Fragment (∼800 bp). The PCR primers (5′-end primer, 5′-CTTTGGCAACTACTCCCGGGATC; 3′-end primer, 5′-CCCATCTCCAGCATCCGCTTAAT) were selected from the sequence of STZ (SAT-3) cDNA from human placenta, using the PCR-Primer Selection Software (Epicenter Software, Pasedena, CA). The primers were used to PCR-amplify cDNA derived by reverse transcription from Colo 205 total RNA. Reverse transcription PCR (RT-PCR) was carried out utilizing a procedure described previously (Ghosh et al., 1992). Colo 205 total RNA template (10−20 μg) was first reverse transcribed to cDNA with recombinant MMLV-reverse transcriptase (United States Biochemicals, Cleveland, OH). The reverse transcription was primed with random primers in a final volume of 0.02 mL. After initial denaturation of RNA at 65 °C for 5 min and quick-chill, the reverse-transcription reaction was performed at 37 °C for 60 min, followed by incubation at 94 °C for 5 min to inactivate reverse transcriptase. The reaction mixture was then subjected to PCR in the same tube by adding the 5′- and 3′-end primers and other necessary reagents [reaction buffer, 0.05 M KCl, 0.01 M Tris-HCl, pH 8.4, 0.1 mg of nuclease-free BSA/mL; $MgCl_2$, 2.5 mM; 5′- and 3′-end primers 0.25 mM each; *Taq* polymerase (Promega, Madison, WI), 2−3 units] to a final volume of 0.1 mL. The thermocycling profile was as follows: 95 °C, 4 min, (95 °C, 1 min, 55 °C, 1 min, 72 °C, 2 min)$_{35cycles}$, 72 °C, 10 min. A negative control experiment was simultaneously carried out either without reverse transcriptase or without total RNA template. At the end of the reaction, PCR products were checked on 1%−1.5% agarose gels followed by staining with ethidium bromide. A ∼800 bp cDNA fragment was amplified by RT-PCR. The cDNA was cloned into pT7-Blue vector (Novagen, Madison, WI) according to the manufacturer's instructions. The double-stranded plasmids with the cloned DNA insert were alkaline-denatured and heated at 95 °C for 5 min before the DNA fragment was sequenced by the dideoxyribonucleotide chain termination method using Sequencase Version 2.0 kit supplied by United States Biochemicals (Cleveland, OH).

Production of Anti-GST-SAT-3 (ECB). A homolog of human STZ (SAT-3) cDNA was also cloned from embryonic chicken brain (ECB) using a similar method as described above. The cloned SAT-3 (STZ) partial cDNA (∼800 bp) was expressed in *Escherichia coli* as GST fusion protein (Ghosh et al., 1995a; Basu, S. S., et al., 1995). Polyclonal antibody was produced against affinity-purified GST-SAT-3 (62 kDa) fusion protein via inoculation of antigen into the popliteal lymph nodes of a New Zealand white rabbit (Siegel et al., 1983). A 25 μg amount of protein was injected in the first inoculation followed by three booster injections of 20 μg of protein each time. The final antiserum was collected 7 weeks after the first inoculation of the rabbit. ELISA and Western blot analysis showed that the anti-GST-SAT-3 antibody can bind to both SAT-3 (∼36 kDa) and GST (∼26 kDa) domains of the fusion protein (results to be published elsewhere; Basu, S. S., et al., 1996). Antisera were stored at −20 °C in 0.02% sodium azide.

RESULTS AND DISCUSSION

The two enzyme activities SAT-3 and SAT-4 can be detected in Colo 205 cells. In the homogenate of Colo 205 cell, the optimum pH values for SAT-3 and SAT-4 activities were found to be different, 6.4 for SAT-3 and 6.8 for SAT-4 activities. This was the preliminary indication of the possibility that the two activities are catalyzed by two different proteins. However, on solubilization with taurocholate detergent, the pH optimum of SAT-3 activity shifted to 6.8. This kind of shift of pH optimum was reported (Joziasse et al., 1985) with another sialyltransferase, ST3O (CMP-NeuAc:Galβ1-3GalNAc α2-3 sialyltransferase) from human placenta, as the enzyme was purified. The SAT-3 and SAT-4 activities in Colo 205 cells do not have requirement for divalent metal ions but are inhibited partially by Ca^{2+} and Co^{2+}, and almost completely by Ni^{2+}, Cu^{2+}, and Zn^{2+}, at 8 mM concentration.

Among the detergents tested, sodium taurocholate was found to be the most efficient in solubilizing SAT-3 and SAT-4 activities from Colo 205 cells (Figure 1) at a protein to detergent ratio of 2:1 (Figure 2). 100% or more of the SAT-3 activities can be recovered in the DSS prepared with taurocholate and Zwittergent; all other detergents tested failed to obtain complete solubilization of the SAT-3 activity. About 5-fold more activity was recovered in case of SAT-4 when taurocholate was used for solubilization. Triton X-100, Cutscum, and Zwittergent are also effective in solubilizing

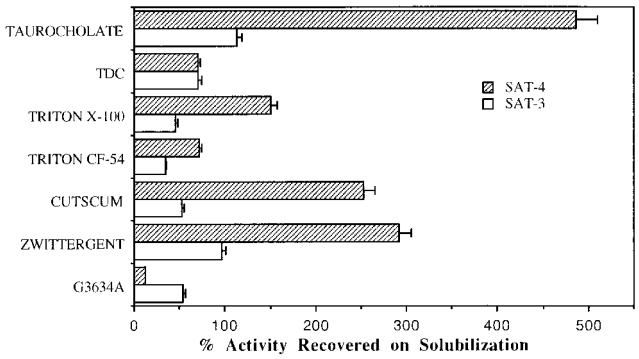

FIGURE 1: Solubilization of SAT-3 and SAT-4 activities with different detergents. Effect of different detergents on solubilization of the two sialyltransferase activities was tested as described in the materials and methods section. A protein to detergent ratio of 2:1 was maintained in all cases. The amount of the activities recovered in the DSS was expressed as the percentage of activities recovered with respect to activities found in the Colo 205 homogenate.

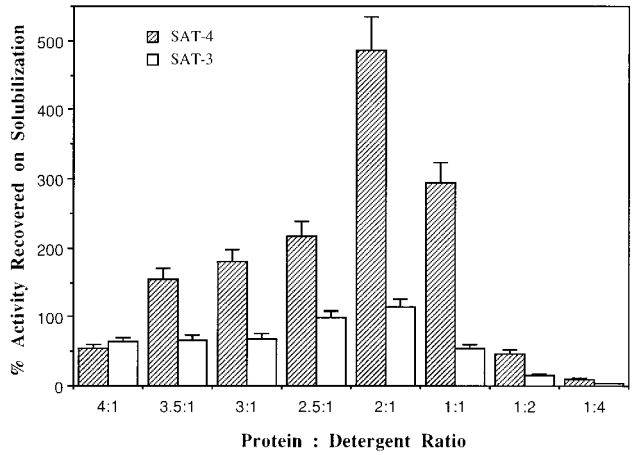

FIGURE 2: Determination of protein:detergent ratio for solubilization of SAT-3 and SAT-4 activities with taurocholate. To determine the optimum protein:detergent ratio for the solubilization of the two activities, Colo 205 homogenate was mixed with different amount of taurocholate and solubilization was carried out as described under materials and methods.

SAT-4 activity from Colo 205 homogenate. G3634A, a positively charged detergent, inhibited the activities of both enzymes but to different extents. During further study with the solubilized enzymes, no extra detergent was added to the incubation mixture. Most of the sialyltransferase activities so far reported were solubilized with neutral detergents, except CMP-NeuAc:Lc4Cer sialyltransferase from human colorectal carcinoma cell (Liepkans et al., 1988) and SAT-1 from rat liver (Melkerson-Watson & Sweeley, 1991) were solubilized with negatively charged detergents taurocholate and lauryldimethylamine oxide, respectively. Under SAT-3 and SAT-4 assay conditions the amount of detectable SAT-1 and SAT-2 activities was very little in the Colo 205 cell DSS.

The apparent K_m and V_{max} values for different glycolipid substrates were determined with detergent-solubilized supernatant. The kinetic constants for nLc4 are $K_m = 190\ \mu M$, $V_{max} = 1.3$ nmol/mg of protein/h; for Gg4, $K_m = 930\ \mu M$, $V_{max} = 13.0$ nmol/mg of protein/h; for GM1, $K_m = 450\ \mu M$, $V_{max} = 4.2$ nmol/mg of protein/h. These values were used for calculating reaction velocity (v_t) during competition kinetic studies to determine whether SAT-3 and SAT-4 activities were catalyzed by the same or different catalytic entities.

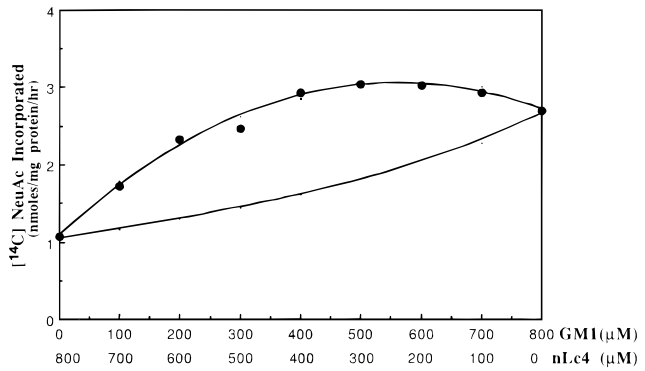

FIGURE 3: Competition between nLc4 and GM1 in sialyltransferase assay. As described, acceptor substrates nLc4 and GM1 were used in various partial concentrations, keeping the total concentration of the acceptor substrates at 800 μM. Total velocity experimentally determined (●) was compared with the total calculated velocities (−) for the different models elucidated in Results and Discussion are plotted versus the partial concentrations. The upper curve represents for the two-enzyme theory and the lower curve represents for the one-enzyme theory.

In competition kinetic studies, glycolipids nLc4 and GM1 or Gg4 and GM1 were used as acceptor substrates for sialyltransferase at various partial concentrations, keeping total acceptor substrate concentration at 800 μM. The assays were performed as described above. Total reaction velocities were determined experimentally or as calculated with the following equations (Dixon & Webb, 1979) using the corresponding K_m and V_{max} values (as mentioned above), or determined simultaneously with same solubilized enzyme fraction.

$$v_t = v_a + v_b = \frac{V_a}{1 + \frac{K_a}{[a]}} + \frac{V_b}{1 + \frac{K_b}{[b]}} + \ldots \quad (1)$$

$$v_t = v_a + v_b = \frac{V_a}{1 + \frac{K_a}{[a]}\left[1 + \frac{[b]}{K_b}\right]} + \frac{V_b}{1 + \frac{K_b}{[b]}\left[1 + \frac{[a]}{K_a}\right]} + \ldots \quad (2)$$

For two independent enzymes, each recognizing only one of the substrates (a and b), the total reaction velocity can be calculated as the sum of two partial velocities v_a and v_b given by their respective Michaelis−Menten equation (1). If both substrates are accepted by the same enzyme, *i.e.*, if the two substrates compete for the same active site, then each substrate behaves as the competitive inhibitor of the other, and the inhibitor constant (K_i) of either substrate would be equal to its K_m value. The total velocity is given by eq 2.

In competition between nLc4 and GM1 (Figure 3) the experimentally obtained v_t values clearly fit those calculated for two enzymes, while in the case of competition between GM1 and Gg4 (Figure 4) the v_t values correspond to those calculated for one enzyme. Thus, nLc4 and GM1 act as glycolipid acceptors in LM1 and GD1a synthesis catalyzed by two different sialyltransferase, SAT-3 and SAT-4, respectively. SAT-4 transfer sialic acid to both GM1 and Gg4 substrate to synthesize the GD1b and GM1b, respectively. However, when nLc4 and Gg4 were used as acceptor substrates in the competition kinetics experiment, the observed v_t values were more than that calculated for two enzymes.

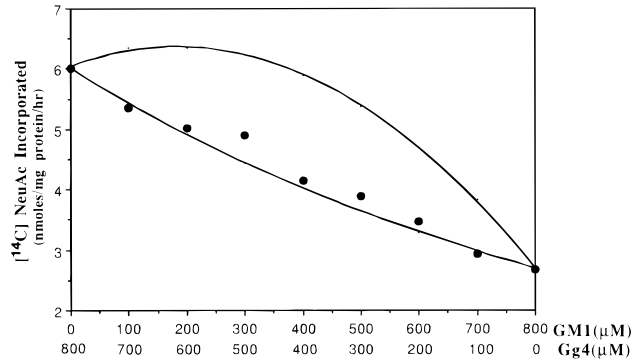

FIGURE 4: Competition between Gg4 and GM1 in sialyltransferase assay. In this experiment Gg4 and GM1 were used as acceptor substrates, and the experiment was performed in a way similar to that described in the legend of Figure 3. Total experimentally determined velocity (●) was compared with the total calculated velocity (−). The upper curve represents the two-enzymes theory, and the lower curve represents the one enzyme theory.

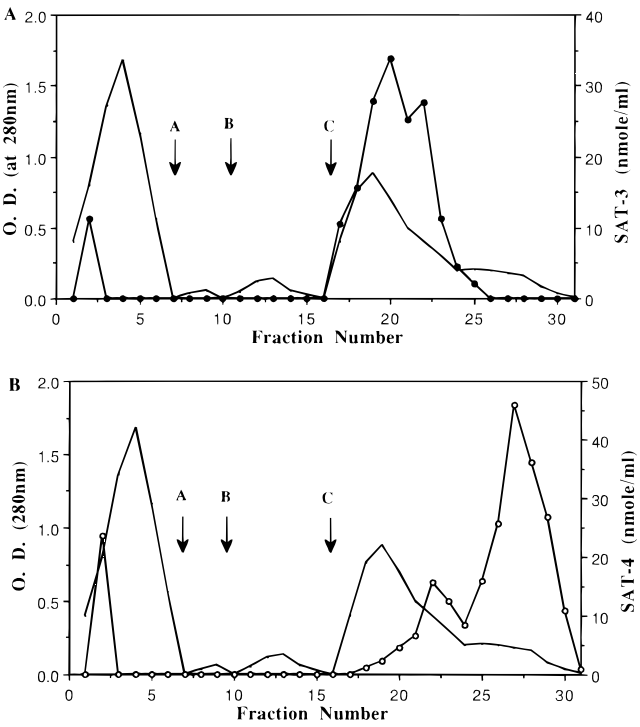

FIGURE 5: Resolution of detergent-solubilized SAT-3 and SAT-4 by chromatography on DEAE−Cibacron Blue column chromatography. The chromatography was run as described under materials and methods. The protein elution from the column was monitored continuously by measuring absorbance at 280 nm, depicted by a continuous line (−). 40 μL of each fraction was assayed for both SAT-3 (panel A, ●) and SAT-4 (panel B, ○), and the activity per mL of the fraction is plotted. Elution with buffer A was started at position marked A, elution with buffer B was started at B, and elution with a linear gradient of sodium acetate was started at C.

The SAT-3 and SAT-4 activities were inhibited to different extents with different amino acid modifiers (data not shown). Diisothiocyano-2,2′-disulfonic acid stilbene (DIDS, a specific, anion transport inhibitor) has been characterized as an irreversible inhibitor of number of glycosyltransferase (Ghosh, 1992). DIDS also inhibited both SAT-3 and SAT-4 activities, but the I_{50} values for the two activities were observed to be 85 and 165 mM, respectively. These findings further support that two different proteins are responsible for the two activities, SAT-3 and SAT-4.

Table 1: Comparison of Sialyltransferase Activities with nLc4 and Asialo α_1-Acid Glycoprotein in Colo 205 Enzyme Fractions[a]

	acceptors[b]		
enzyme fraction	nLcOse4Cer	α1-AGP	($S\bar{A}$) α1-AGP
Colo 205 homogenate	1.47	0	0.81
DEAE−Cibacron Blue column eluent	22.2	0	0

[a] The amount of radiolabeled NeuAc incorporated on the acceptor substrates was measured by performing sialyltransferase assay as described under Materials and Methods. Colo 205 cell homogenate and purified SAT-3 fraction from DEAE−Cibacron Blue column (contents of fractions 17−22; Figure 5) were used as enzyme sources. With both of the substrates high-voltage electrophoresis was done to separate the products from the radiolabeled CMP-NeuAc. [b] [3H]NeuAc incorporated (nmol/mg of protein/h).

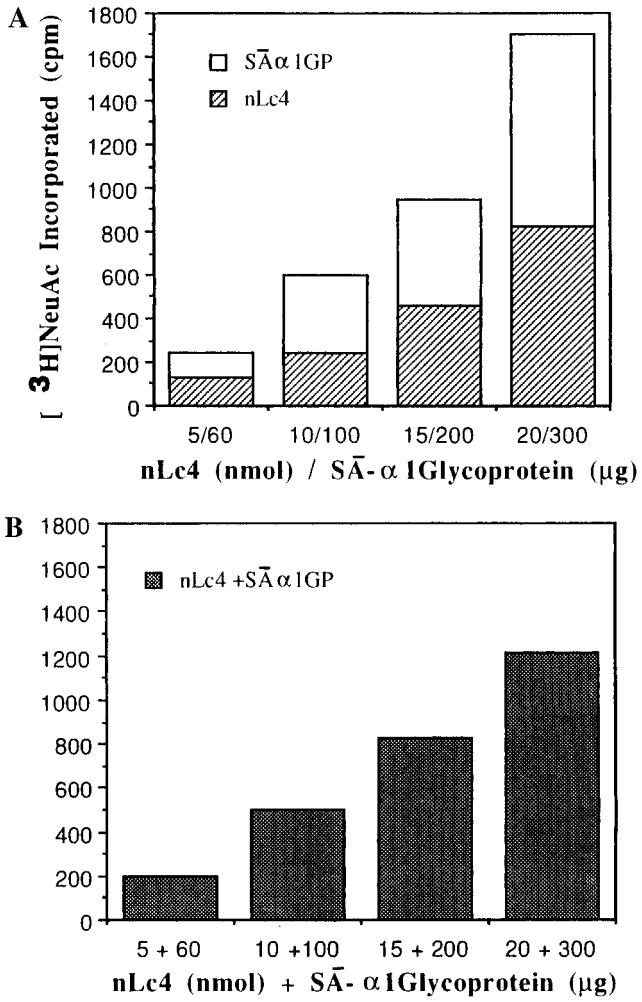

FIGURE 6: Competition between nLc4 and asialo α_1-acid glycoprotein in sialyltransferase assay. Panel A shows the summation of the amount of [3H]NeuAc incorporation in nLc4 and asialo α_1-acid glycoprotein when the two substrates at different concentrations (shown in the figure) were treated with a reaction mixture containing Colo 205 homogenate in separate incubation tubes. When both of the substrates at different concentrations were added to the same reaction mixture, the total amount of [3H]NeuAc incorporation is depicted in panel B.

SAT-3 and SAT-4 activities were resolved by DEAE-Cibacron Blue column chromatography (Figure 5). SAT-3 is eluted at a lower ionic concentration compared to SAT-4. SAT-4 shows two elution peaks, possibly due to the presence of different isoforms. This chromatography enriches the SAT-3 and SAT-4 activities by about 23- and 80-fold,

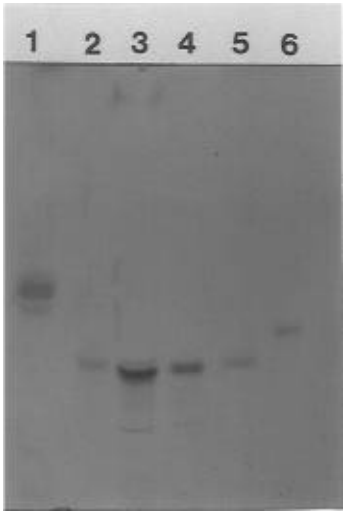

FIGURE 7: Thin-layer chromatography of the radiolabeled SAT-3 and SAT-4 enzymatic products ([^{14}C]NeuAc-nLc4 and [^{14}C]NeuAc-Gg4). The SAT-3- and SAT-4-catalyzed products were analyzed with TLC on a Whatman K6 silica gel plate developed in chloroform:methanol:water (60:35:8). 3000 cpm of the products ([^{14}C]NeuAc-Gg4 and [^{14}C]NeuAc-nLc4) was spotted in lanes 3 and 4 respectively, lane 1, Gg4; lane 2, GM1; lane 5, LM1; lane 6, nLc4.

Table 2: Release of Sialic Acid from the Enzymatic Products of SAT-3 and SAT-4 on Treatment with Neuraminidase[a]

neuraminidase (12 h incubation; Ca^{2+})	% of [^{14}C]NeuAc recovered	
	SAT-3 product	SAT-4 product
C. perfingenes	84	94
Newcastle disease virus	90	79

[a] The percentages of the total radioactivity in the products of SAT-3 and SAT-4 that were recovered after the treatment with the neuraminidase are shown in this table. The experiment was performed as described in the text.

respectively, over the Colo 205 homogenate. The yields for SAT-3 was 38%, and that for SAT-4 was 49%, with respect to that observed in the homogenate.

Comparison of the sialyltransferase activities with nLc4 and asialo α_1-acid glycoprotein as substrates (Table 1) shows the presence of both the activities in the cell-free extract of Colo 205 cell; but after solubilization of the SAT-3 activity with negatively charged taurocholate detergent and in the DEAE-Cibacron Blue column eluent we observed only the presence of enzymatic activity for efficient transfer of sialic acid onto nLc4. However, the competition kinetics study with cell-free extract, shown in Figure 6, does not exhibit an additive effect with regard to the total amount of radiolabeled NeuAc incorporated. These results indicate that the SAT-3 activity is different from the sialyltransferase, ST3N (Weinstein et al., 1982), that synthesizes NeuAcα2-3Gal linkage in asparagine-linked oligosaccharides of glycoprotein.

The enzymatic products after sialylation of nLc4 and Gg4, with sialyltransferases from the DSS, were then characterized to exclude any possibility of formation of α2-6 or α2-8 bond between sialic acid and the glycolipid substrates. The purified products were analyzed with thin-layer chromatography (Figure 7) on a Whatman K6 silica gel plate developed in chloroform:methanol:water (60:35:8). 3000 cpm of the products ([^{14}C]NeuAc-nLc4 and [^{14}C]NeuAc-Gg4) were spotted in lanes 3 and 4, respectively, along with standard

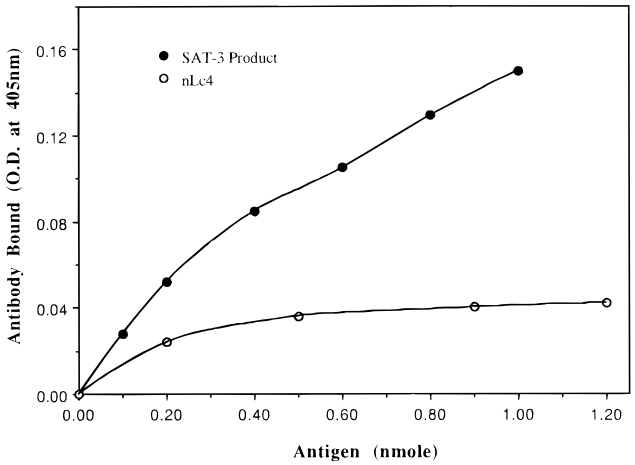

FIGURE 8: Binding of anti-LM1 to enzymatic product of SAT-3 ([^{14}C]NeuAc-nLc4). ELISA was done as described above with substrate, nLc4 (○), and the purified reaction product of SAT-3 (●), polyclonal antibody against LM1, was used in this case.

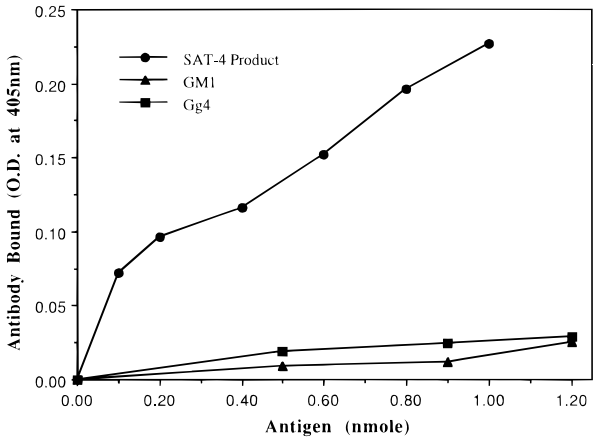

FIGURE 9: Binding of anti-SSEA-4 to enzymatic product of SAT-4 ([^{14}C]NeuAc-Gg4). ELISA was done with monoclonal antibody against NeuAcα2-3Galβ1-3GalNAc epitope to determine its binding to Gg4 (■), GM1 (▲), and the Colo 205 SAT-4-catalyzed reaction product (●).

glycolipids in other lanes (lane-1, nLc4; lane-2, LM1; lane-5, GM1; lane-6 Gg4). The radiolabeled product in lane 3 co-migrated with LM1, while in lane 4 the product moved little above standard GM1, as expected for GM1b.

Neuraminidase treatments of the purified products were carried out to determine the nature and position of the linkage formed between the radiolabeled NeuAc and the glycolipid substrates (nLc4 and Gg4). 80% or more of the [^{14}C]NeuAc was recovered (Table 2) from both SAT-3 and SAT-4 products after treatment with *C. perfingens* neuraminidase, which cleaves both terminal α2-3- and α2-6-linked sialic acid from a ganglioside but not internal sialic acid, or with Newcastle disease virus neuraminidase, which is specific for α2-3-linked sialic acid. Thus, the result indicates that these two enzymes, SAT-3 and SAT-4, catalyze formation of α2-3 linkage to the terminal galactose.

Further analysis of the products by ELISA using specific antibodies showed that the product of SAT-3 from nLc4 substrate is LM1 and that the product of SAT-4 from Gg4 is GM1a. Figure 8 shows the specific binding of anti-LM1 with SAT-3 purified enzymatic product; the antibody failed to bind to SAT-3 substrate, nLc4. Anti-SSEA-4, specific for NeuAcα2-3Galβ1-3GalNAc epitope (Kannagi et al.,

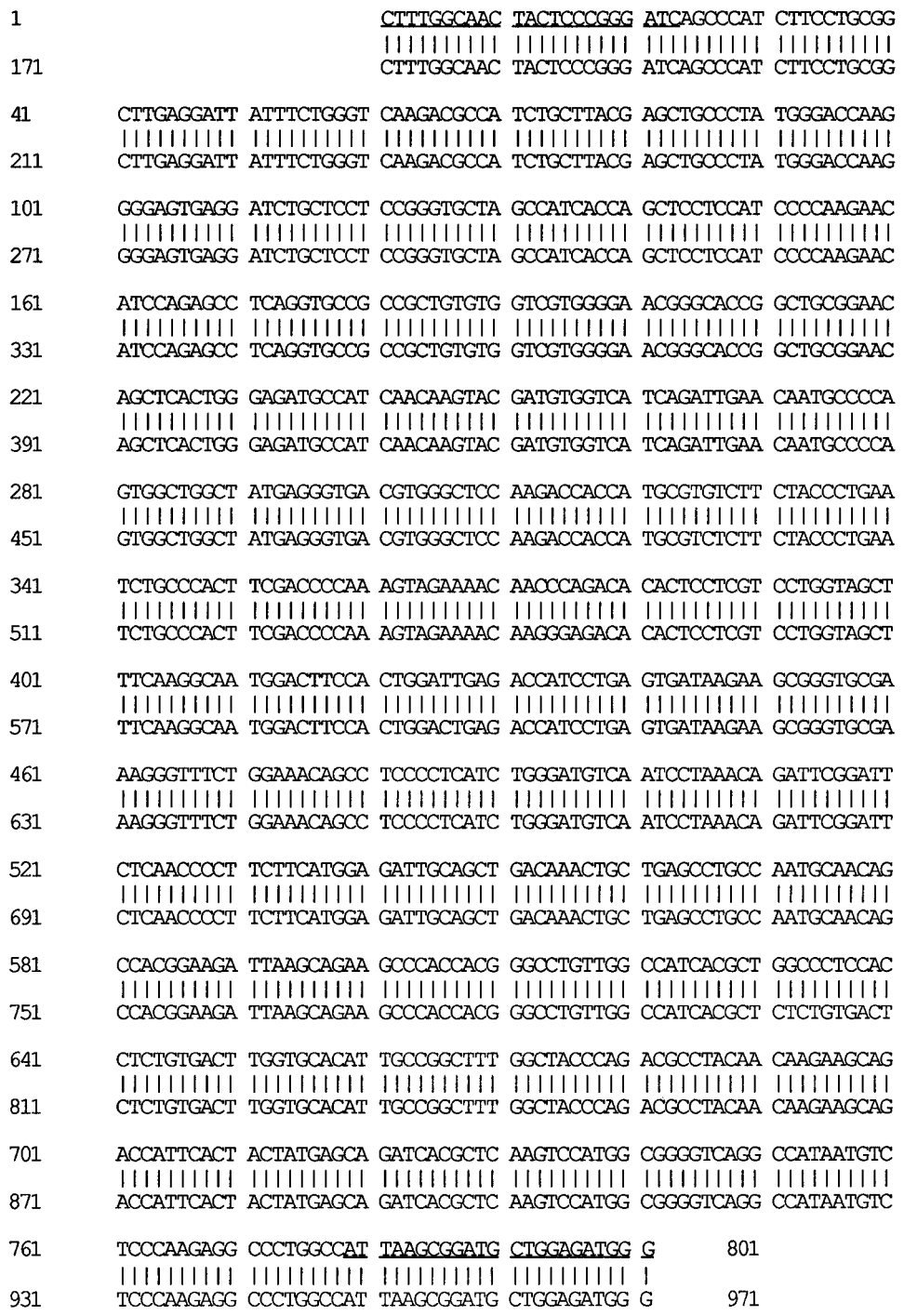

FIGURE 10: Sequence information of the RT-PCR-amplified cDNA fragment from Colo 205 cell. The nucleotide sequences of Colo 205 cell and human placenta SAT-3 are aligned within their regions of sequence homology. The upper sequence refers to the nucleotide sequence for the Colo 205 cell DNA, and the lower sequence corresponds to the sequence of the human placenta cDNA. The positions of the PCR primers are underlined.

1983), recognized SAT-4 product but not the substrates (Gg4 and GM1) of SAT-4 (Figure 9).

The results presented above clearly indicate that the two activities SAT-3 and SAT-4 are catalyzed by two different catalytic entities. These enzymes are specific for the terminal acceptor sugar galactose of the glycolipid substrates and α2-3 anomeric linkages formed during the reactions. The substrate specificity of SAT-3 and SAT-4 extends beyond the terminal galactose of the acceptor substrate to include the penultimate sugar. GlcNAc in nLc4 and GalNAc in GM1 and Gg4 are recognized by the active sites of SAT-3 and SAT-4, respectively. Whether the enzymes can recognize further beyond the penultimate sugar of the glycolipid substrate, as has been reported (Melkerson-Watson & Sweeley, 1991; Ghosh et al., 1995b) in the case of few other glycolipid-specific glycosyltransferases, CMP-NeuAc:Lac-Cer α2-3sialyltransferase (SAT-1) and UDP-Gal:GM2 β1-3galactosyltransferase (GalT-3), is yet to be answered in detail (Basu, S. C., 1991). Our result presented here raises the possibility that SAT-3 can distinguish between the terminal lactosamine epitope present on glycolipid and glycoprotein, and specifically transfers sialic acid to a glycolipid substrate.

SAT-4 has been purified from rat brain (Gu et al., 1990). On the basis of sequences of the highly conserved segment

Table 3: Inhibition of Colo 205 SAT-3 Activity by Anti-GST-SAT-3(ECB) Antibody[a]

		% activity	
serum	*n*-fold dilution	SAT-3	SAT-4
preimmune	0.167	97	101
anti-GST-SAT-3	−	100	100
	0.033	83	nd
	0.067	73	86
	0.100	53	nd
	0.133	35	nd
	0.167	19	65

[a] Polyclonal antibody was produced against affinity-purified GST-SAT-3 fusion protein. Isolation of the putative SAT-3 cDNA (partial) clone from embryonic chicken brain (ECB), prokaryotic expression of the cDNA as glutathione S-transferase (GST) fusion protein, and production of the rabbit polyclonal antibody will be reported elsewhere. Heat-treated (57 °C for 5 min) rabbit antiserum, in different dilutions, was added to the detergent-solubilized enzyme fractions from Colo 205 cells and Protein A-Sepharose and incubated for 1 h. After centrifugation of the immune complex, the supernatant was assayed for SAT-3 and SAT-4 activities by the standard methods. The residual activity was expressed as the percentage of the control enzyme activities, measured without addition of any serum. nd, not determined.

(sialyl motif) in all cloned sialyltransferases, a cDNA encoding an α2,3-sialyltransferase has been isolated from rat and mouse brain cDNA libraries (Lee et al., 1994). The cDNA when expressed in COS-7 cells exhibited SAT-4-like activity, *i.e.*, it transferred sialic acid more efficiently onto the terminal Galβ1-3GalNAc structure in Gg4 and GM1 than onto the same epitope in glycoprotein. Another α2-3 sialyltransferase (STZ or SAT-3), has been cloned from human melanoma cell WM266-4 using lectin resistance selection (Sasaki et al., 1993) and from human placenta using PCR homology cloning technique (Kitagawa & Paulson 1994). The cloned cDNA was shown to be involved in the *de novo* expression of sialyl-Le^x determinant on a human melanoma cell surface (Sasaki et al., 1993). When the putative catalytic domain coded by the human placenta cDNA was expressed as a Protein A fusion protein, the expressed protein transferred (*in vitro*) sialic acid onto the terminal galactose of nLc4 like the Colo 205 cell SAT-3 described here; but it also exhibits transferase activity toward Galβ1-4GlcNAc epitope in glycoprotein and toward Galβ1-3GalNAc in glycolipid and glycoprotein (Kitagawa & Paulson, 1994).

The expression of the transcript coding for this sialyltransferase in Colo 205 is shown by RT-PCR amplification of ~800 bp cDNA fragment using sequence specific primers. The nucleotide sequence identity was observed between this RT-PCR-amplified cDNA and the human placenta STZ, in the cloned 800 bp region (Figure 10). A 2.0 kb mRNA has been detected in Colo 205 cell by Northern analysis (Sasaki et al., 1993). A homolog of this cDNA was also isolated from ECB, expressed in *Escherichia coli* as a GST fusion protein, and rabbit polyclonal antibody was produced against the fusion protein (GST-SAT-3). An anti-GST-SAT-3 (rabbit polyclonal antibody) concentration-dependent decrease in residual SAT-3 activity in the Colo 205 supernatant (DSS) was observed after precipitation of the immune complex, while the preimmune serum did not decrease the residual SAT-3 activity compared to the control (Table 3). The binding of the antibody to the native Colo 205 SAT-3 protein was also demonstrated by ELISA (data not shown). About 35% of the SAT-4 activity was precipitated by this antiserum compared to 80% precipitation of the SAT-3 activity at an antibody dilution of 0.167. Partial inhibition of the SAT-4 activity may be due to the presence of antibodies against the conserved sialyl motifs common to all the members of sialyltransferase gene family, in the antiserum. From these results we can support the conclusion derived from the competition kinetic studies described above, *i.e.*, the SAT-3 and SAT-4 activities are the intrinsic properties of two different polypeptides.

Although differences have been observed between the acceptor substrate specificities of the Colo 205 SAT-3 described here and the proteins expressed from the cDNAs cloned from human melanoma cell WM266-4 (Sasaki et al., 1993) and from human placenta (Kitagawa & Paulson 1994), the preferential immunoprecipitation of the SAT-3 activity from Colo 205 by the antibody produced against the protein expressed from an homolog of the human placenta STZ (SAT-3) cDNA implicates close molecular similarity between them. This also raises the possibility of expression of other factor(s) involved in determining the substrate specificity of SAT-3 during carcinogenesis. Hence, further studies on the full-length SAT-3 native and expressed (heterologous expression) proteins from normal and cancerous cells are needed to understand the molecular basis of its substrate specificity.

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