Carbohydrate Research, 209 (1991) 261–277
Elsevier Science Publishers B.V., Amsterdam

Biosynthesis *in vitro* of core *lacto*-series glycosphingolipids by *N*-acetyl-D-glucosaminyltransferases from human colon carcinoma cells, Colo 205*

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(Received September 22nd 1989; accepted for publication, in revised form, April 17th, 1990)

ABSTRACT

Two *N*-acetyl-D-glucosaminyltransferases have been detected in human colon carcinoma Colo 205 cells. These enzymes catalyze the biosynthesis *in vitro* of the core-glycolipid of Type 1 and Type 2 *lacto*-series antigens and of the polylactosamine-containing longer chain antigenic structures, respectively. The first enzyme, GlcNAcT-1, which catalyzes the formation of lactotriosylceramide [$LcOse_3Cer$, β-D-Glc*p*NAc-(1→3)-$LcOse_2Cer$, the core for all *lacto*-series Type 1 and Type 2 chains] from lactosylceramide [β-D-Gal*p*-(1→4)-D-Glc*p*-Cer, $LcOse_2Cer$] and UDP-GlcNAc shows optimum activity in the presence of nonionic detergent Triton CF-54. The other enzyme, GlcNAcT-2, which catalyzes the biosynthesis *in vitro* of $iLcOse_5Cer$ [β-D-Glc*p*NAc-(1→3)-$nLcOse_4Cer$, the core for polylactosamine-containing antigens] from $nLcOse_4Cer$ [β-D-Gal*p*-(1→4)-$LcOse_3Cer$] and UDP-GlcNAc, is optimally active with the zwitterionic detergent, Zwittergent 3-14, when membrane-bound. Both of these activities, however, can be extracted from the membrane by use of a nonionic detergent, Triton X-114, with nearly the same efficiency. These two transferases showed different pH optima, different cation and anion effects, and differential heat-inactivation patterns at 55°. Permethylation studies of the radioactive products isolated from both of the enzyme-catalyzed reactions using respective ^3H-substrates and nonradioactive UDP-GlcNAc showed the presence of 2,4,6-tri-*O*-methylgalactose in the hydrolyzed products. This indicated the presence of a (1→3)-linked β-D-Glc*p*NAc group at the nonreducing end in both cases. The linkage of the β-D-Glc*p*NAc group to the subterminal D-Gal residue in the two products was confirmed by an almost 90% cleavage of the terminal [^3H]GlcNAc group by purified clam and papaya β-D-hexosaminidases.

INTRODUCTION

Lacto-series glycosphingolipids (unlike *globo*- and *ganglio*-series GSLs[2]) are characterized by having a 2-acetamido-2-deoxy-D-glucosyl residue as the third sugar linked to the terminal galactosyl group of lactosylceramide [$LcOse_2Cer$; β-D-Gal*p*-(1→4)-D-Glc→Cer] in their core structures. The presence of a D-Glc*p*NAc-(1→3)-D-Gal-R group is not restricted only to glycolipids; it is also present in glycoproteins and mucins and is the main precursor structure for both Type 1 and Type 2 chain-associated antigenic glycoconjugates of ABH, Lewis, and P-blood group families[3]. The presence of

*This investigation was supported by Grants CA-33 751 and CA-14 764. KKD was supported by the National Cancer Institute, National Institutes of Health. A preliminary report has been published[1].
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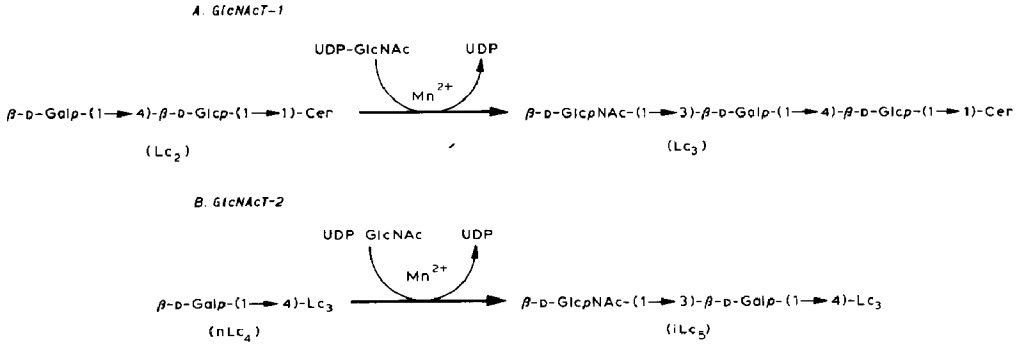
0008-6215/91/$ 03.50

poly(lactosamino)glycans, which are characterized by the presence of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3) repeating units, has been shown to occur in several animal cell surfaces[4,5], and has been associated with the development of erythroleukemic cells[6]. Glycoconjugates containing a polylactosamine repeating-unit have also been characterized in Ii-antigenic structures, which are implicated in the oncofetal antigens[7].

The branched structure, β-D-Glc*p*NAc-(1→3)-[β-D-Glc*p*NAc-(1→6)]-D-Gal*p*→R, present in the Ii-antigen, has been of particular interest in recent years with the emphasis on the synthetic route of this glycolipid. We previously reported the presence of both β-D-(1→3)- and β-D-(1→6)-*N*-acetylglucosaminyltransferases[8] in a detergent-soluble fraction obtained from mouse lymphoma P-1798, which catalyzes the biosynthesis *in vitro* of iLcOse$_5$Cer [β-D-Glc*p*NAc-(1→3)-nLcOse$_4$Cer] and β-D-Glc*p*NAc-(1→6)-nLcOse$_4$Cer, respectively. The same reactions were also reported for Novikoff ascites tumor cells and hog gastric mucosa [9–11]. The GlcNAcT-1 reported herein was tentatively characterized in rabbit bone marrow[12]. However, this enzyme could not be detected in mouse lymphoma P-1798 under our assay conditions. We report herein the characterization of two *N*-actylglucosaminyltransferases present in human colon carcinoma cells, Colo 205, which catalyze two reactions illustrated in Scheme 1.



Scheme 1. *N*-Acetylglucosaminyltransferases in human colon carcinoma cells (Colo-205). Lc$_3$: Core-GSL (for all Lc-family, blood-group antigens). iLc$_5$: Core-GSL for poly(*lacto*)-series glycolipids.

EXPERIMENTAL

Materials. — Labeled and unlabeled UDP-GlcNAc were purchased from the American Radiolabeled Company (St. Louis, MO) and Sigma Chemical Co. (St. Louis, MO), respectively. The following materials were obtained from other commercial sources: Triton CF-54, Triton X-114 (Sigma Chemical Co.); Zwittergent 3–14 (Calbiochem.); SG-81 papers (Scientific Products); Silica Gel G and t.l.c. plates (Brinkman Instruments); tissue culture flasks (Corning Glass Works); and RPMI-1640 medium, fetal calf serum, penicillin, and streptomycin (Gibco). The glycosphingolipids used in this study were prepared in our laboratory by published methods. Lactosylceramide was prepared from bovine erythrocytes[13] and lacto-*neo*-pentaglycosylceramide [α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc→Cer,

nLcOse$_5$Cer] was prepared either from bovine or rabbit erythrocytes[14,15]. The substrate nLcOse$_4$Cer for the GlcNAcT-2 enzyme was prepared by hydrolyzing the terminal D-galactopyranosyl group from nLcOse$_5$Cer with fig α-D-galactosidase[16]. Pure β-D-hexosaminidases used for product characterization were prepared from clam homogenate[17] and from papaya.

Cell culture. — Human colon carcinoma cells, Colo 205 (ref. 18), were obtained from ATCC and are routinely maintained in our laboratory. The cultures were grown in 250-mL plastic T-flasks in an RPMI-1640 medium supplemented with 10% fetal calf serum, 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°. The medium was changed once or twice before harvesting. The cells were subcultured with 0.25% trypsin when the monolayer reached confluence.

Preparation of enzymes. — All steps of the enzyme preparations were carried out at 4°. The packed cells were homogenized in 0.32M sucrose in 10mM HEPES buffer, pH 7.0, containing mM EDTA, and 0.1% mercaptoethanol (4 vols.) with a glass–Teflon homogenizer. Both of the *N*-acetyl-D-glucosaminyltransferases (GlcNAcT-1 and GlcNAcT-2) present in the homogenate preparation were solubilized from the membrane by extracting with a nonionic detergent, Triton X-114, at a protein-to-detergent ratio of 5:1. The detergent-soluble supernatant (DSS), obtained after centrifugation of the detergent-treated homogenate at 100 000*g* for 2 h, contained more than 80% of both GlcNAcT activities. The DSS fraction had a protein concentration of 3–4 mg/mL (measured by the method of Lowry *et al.*[19]) and was stored in 30% 1,2-ethanediol at −18°. The enzyme seemed to be stable up to 4–6 months under this condition.

Enzyme assay. — The incubation mixture for *N*-acetyl-D-glucosaminyltransferase-1 (GlcNAcT-1) assay contained the following components in a final volume of 0.04 mL: Acceptor glycolipid, LcOse$_2$Cer, 0.01 μmol; Triton CF-54 detergent, 30–40 μg for membrane-bound enzyme and 20 μg for DSS fraction; HEPES buffer, pH 7.2, 10 μmol; $MnCl_2$, 0.25; UDP-[^{3}H]GlcNAc, 0.025 μmol (3–4 10^6 c.p.m./μmol); and enzyme protein, 120–150 μg for membrane enzyme and 50–60 μg for DSS. After 2 h at 37°, the reaction was stopped with EDTA, after which the whole mixture was spotted onto SG-81 paper and chromatographed in descending fashion[20,21] in 1% $Na_2B_4O_7$. The incorporation of [^{3}H]GlcNAc in the product was determined by counting the radioactivity of the appropriate areas (origin plus 2.5 cm each behind and ahead of origin) of the chromatogram by a liquid-scintillation technique using a Beckman Scintillation Counter, Model LS 1801.

In the incubation mixture for *N*-acetyl-D-glucosaminyltransferase-2 (GlcNAcT-2) assay, the acceptor glycolipid nLcOse$_4$Cer was added at a concentration of 15 nmol/incubation volume and Zwittergent 3–14 detergent was used (20 μg) for the membrane-bound enzyme. However, no detergent was required for the DSS fraction. The remainder of the components for the incubation mixture and the assay methods were identical to those described above for GlcNAcT-1 enzyme.

Isolation of the radiolabeled products. — Two incubations with the proportions of components increased 20-fold were performed for the isolation of both GlcNAcT-1-

and GlcNAcT-2-catalyzed reactions using Lc_2 and nLc_4 as acceptor glycolipids, respectively. After 4–6 h of incubation at 37°, EDTA was added to stop the reaction and the mixture was then deposited on SG-81 paper and chromatographed as described above. The radioactive products, which remained at the origin, were eluted from the paper with 60:35:8 chloroform–methanol–water. Each of the products, [^{3}H]GlcNAc-$LcOse_2$Cer (GlcNAcT-1-catalyzed product) and [^{3}H]GlcNAc-$nLcOse_4$Cer (GlcNAcT-2-catalyzed product), was further purified on a Biosil column (200–400 mesh). The purity of each product was monitored by t.l.c. in different solvent systems. The appropriate areas of each chromatogram were scraped and the radioactivities were determined quantitatively by use of a liquid-scintillation technique.

Hexosaminidase treatment of the radioactive products. — [^{3}H]GlcNAc-$LcOse_2$Cer and [^{3}H]GlcNAc-$nLcOse_4$Cer ($\sim$ 2000 c.p.m., 1.0–3.0 nmol each) products were treated with purified hexosaminidases from clam or papaya[17,21] in the presence of sodium taurodeoxycholate[22] at pH 4.5–5.0. After incubation for 8–10 h at 37°, the reaction mixture was deposited on SG-81 paper and chromatographed in 1% $Na_2B_4O_7$. The free [^{3}H]GlcNAc moved with the solvent front and the unreacted radioactive products remained at the origin. The radioactivity in both areas was quantitatively determined by a liquid-scintillation technique[8].

Determination of the positional linkage of the terminal GlcNAc group in the radioactive product by permethylation technique. — The GlcNAcT-1- and GlcNAcT-2-catalyzed reaction products were isolated from 20-fold incubations using nonradioactive UDP-GlcNAc with [6-^{3}H]Gal→GlcCer ($LcOse_2$Cer) and [6-^{3}H]Gal→GlcNAc →Gal→Glc→Cer ($nLcOse_4$Cer) as substrates, respectively, which were prepared[23,24] by oxidation with D-galactose oxidase, followed by reduction with $NaBT_4$. After incubation at 37° and chromatography on SG-81 papers, the respective products were eluted as previously described and purified on a Biosil column. The purity of the radioactive products was monitored by t.l.c. which showed a single radioactive band cochromatographying with authentic, corresponding standards. Each product ($\sim$ 50 000–60 000 c.p.m.) was subjected to Hakomori permethylation, followed by acetolysis and hydrolysis[25,26]. The permethylated, hydrolyzed products were chromatographed with standard *O*-methyl-D-galactose samples in 50:1 acetone–5M NH_4OH as described previously[8]. Radioactivity was determined by scraping the appropriate areas, followed by counting in a liquid-scintillation system.

RESULTS

Effect of detergents on the solubilization of glucosaminyltransferases. — Various detergents were tested to extract both the membrane-bound GlcNAcT activities present in Colo-205 homogenate. The homogenate was treated with various detergents as indicated in Fig. 1 (a and b) for 2 h at 4°, and a protein-to-detergent ratio of 5:1. After the indicated time, the resulting mixture was spun at 100 000*g* for 2 h and the supernatant solution (DSS) removed. The enzyme activity was measured in the DSS fractions and also in the pellet suspended in HEPES buffer, pH 7.0. As seen in Fig. 1, activation of

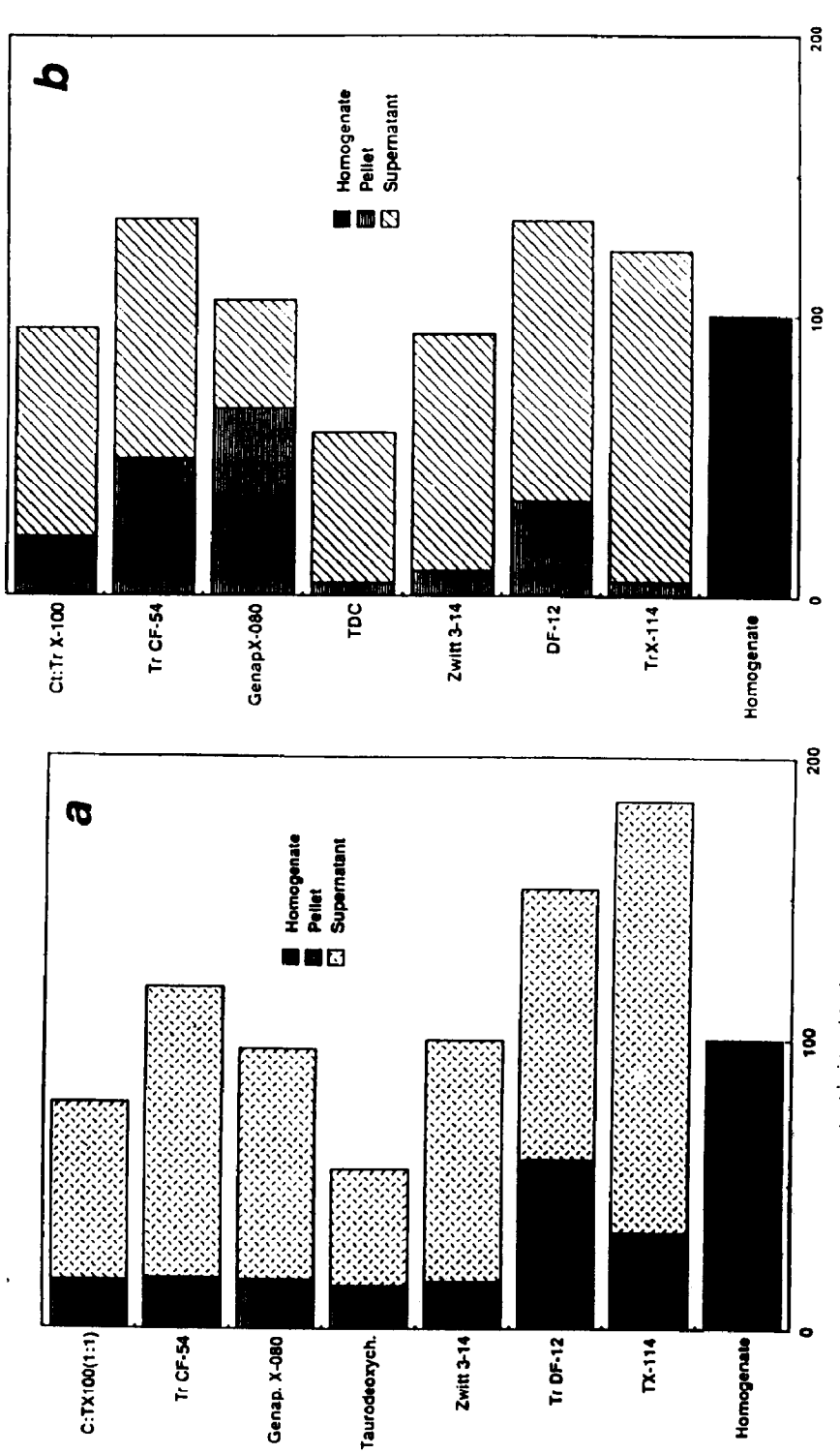


Fig. 1. Differential extraction of GlcNAcT-1 (a) and GlcNAcT-2 (b) by various detergents. The Colo-205 cell homogenate was treated with the indicated detergents for 2 h at 4°, after which time the mixtures were spun at 100 000*g* for 1 h. The homogenate, together with both detergent supernatants and pellets, were assayed for both GlcNAcT-1 and GlcNAcT-2 activity according to the conditions described for Table I.

both GlcNacT-1 and GlcNAcT-2 was observed when the activities from both DSS (obtained from different detergent extracts) and pellets were combined and compared to the homogenate. Triton X-114 was selected as the detergent for extraction of both GlcNAcT-1 and GlcNAcT-2 activity from the membrane because of the maximum activation of GlcNAcT-1 in DSS, and also because of its moderate extraction of GlcNAcT-2 in the supernatant solution. However, Triton CF-54 and Zwittergent 3–14 were used in the incubation for the determination of GlcNAcT-1 and GlcNAcT-2, respectively.

Requirements for GlcNAcTs. — Exogenous substrates $LcOse_2Cer$ and $nLcOse_4Cer$ were essential for GlcNAcT-1 and GlcNAcT-2 activity, respectively, with a Colo-205 DSS fraction as enzyme source. As seen in Table I, the GlcNAcT-1 activity required the presence of a detergent even after being extracted from the membrane. On the other hand, the incorporation of GlcNAc into the acceptor nLc_4 was stimulated by almost 50% when the detergent was removed from the incubation mixture. Both enzymes, however, showed optimal activity in the presence of Mn^{2+}, whereas EDTA completely inhibited both reactions. Product formation was proportional to the protein concentration up to 1.6 mg/mL for GlcNAcT-1 and GlcNAcT-2 enzymes, and both activities remained constant with time of incubation up to 4 h.

Effect of pH and divalent cations on GlcNAcTs. — The two *N*-actylglucosaminyltransferases showed two different pH optima under respective assay conditions. A broader pH range (between 6.5–8.0) in HEPES buffer was observed for GlcNAcT-1 activity, and that for the GlcNAcT-2 enzyme in HEPES buffer was found to be between 7.3–8.0.

TABLE I

Requirements for solubilized GlcNAcT-1 and GlcNAct-2 (Colo 205)[a]

Conditions	*[^{3}H]GlcNAc incorporated (nmol/mg/2h)*	
	GlcNAcT-1	*GlcNAcT-2*
Complete	1.5[b]	3.2[c]
Minus acceptor	0.6	0.9
Minus added detergent[d]	0.2	4.8
Minus Mn^{2+}	0.1	0.7
Plus EDTA	0.1	0.2
Heat-killed enzyme	0.1	0.3

[a] The complete incubation mixtures contained the following components (μmol) in a final volume of 0.04 mL: Substrate ($LcOse_2Cer$ for GlcNAcT-1 and $nLcOse_4Cer$ for GlcNAcT-2), 0.015; Triton CF-54, 20 μg (for GlcNAcT-1); HEPES buffer, pH 7.0, 10; $MnCl_2$, 0.25; UDP-[^{3}H]GlcNAc, 0.025, (3×10^6c.p.m./μmol); and enzyme protein, 50 μg. After 2 h of incubation, the mixtures were assayed by the chromatographic method described in the Experimental section. Under these conditions, rate of both reactions remained constant for 2 h and were proportional to protein concentrations up to 3.0 mg/mL. Values given were substracted from the background value (60 c.p.m.). [b] Contains Triton CF-54 (20μg). [c] Contains Zwittergent (20μg). [d] Contains Triton X-114 (20–30μg; 25μL of dit. sol.sup.).

A differential effect was observed for the two enzymes when the activities were measured in the presence of various divalent metal ions. As mentioned above (Table I), Mn^{2+} was essential for optimal activity of both enzymes. However, GlcNAcT-2 was found to be 33 and 15% as active with Cd^{2+} and Co^{2+}, respectively, as compared to Mn^{2+}, whereas no other metal ion was effective in activating GlcNAcT-1, except for Mn^{2+} (data not shown). It has been previously observed[8] that P-1798 GlcNAcT-2 was 29% as active in the presence of Zn^{2+}, in addition to Mn^{2+}(100%), Co^{2+}(57%), and Ca^{2+}(46%).

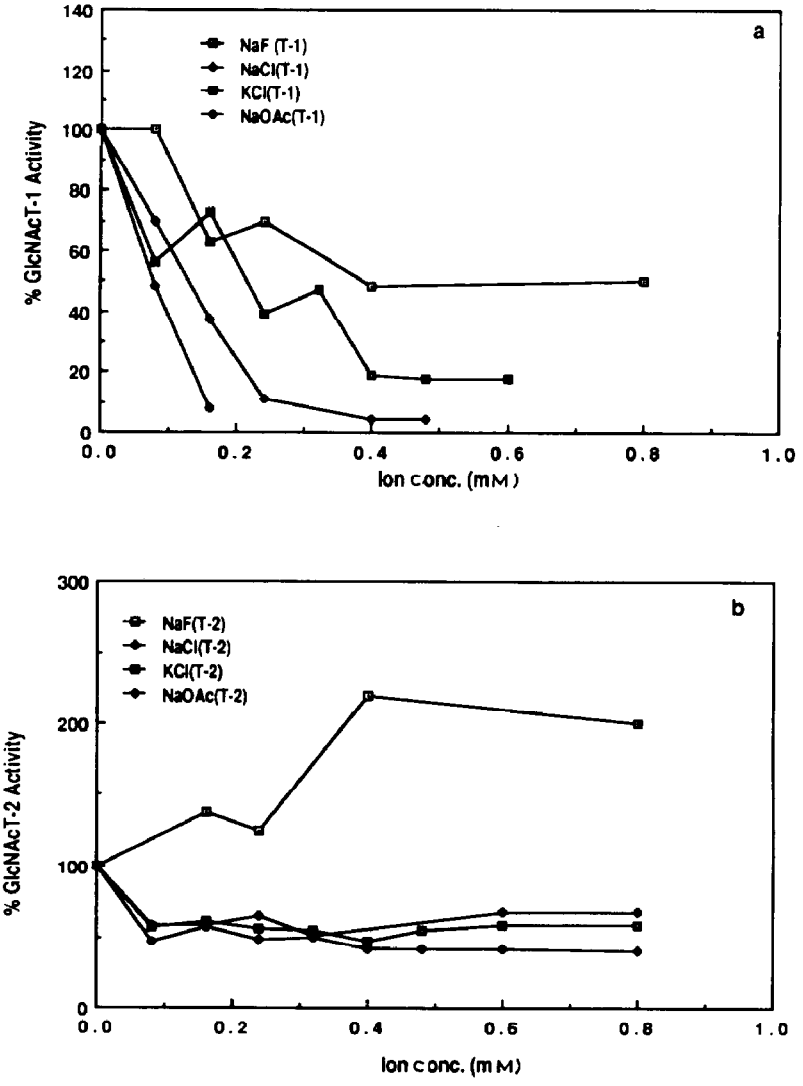


Fig. 2. Anion effect on GlcNAcT-catalyzed reactions. The enzyme activities were assayed according to the conditions described in Table I for each enzyme, except that various salts at the indicated concentrations were added in the incubation mixtures; (a) GlcNAcT-1 activity; (b) GlcNAcT-2 activity.

Effect of anions on GlcNAcTs. — The activity of both GlcNAcT-1 and GlcNAcT-2 enzymes was tested in a Colo-205 DSS fraction, in the presence of various anions at various concentrations, as shown in Fig. 2 (a and b). As seen in Fig. 2a, sodium acetate completely inhibited the GlcNAcT-1 activity, even at very low concentration. Among other salts tested, sodium fluoride was almost 50% inhibitory for GlcNAcT-1, up to a concentration of 0.8M, whereas other salts were more potent inhibitors at lower concentration. On the other hand, sodium fluoride stimulated the GlcNAcT-2 activity to almost 220% at a concentration of 0.4M, and the activity remained almost the same even at a concentration of 0.8M (Fig. 2b). All the other salts were more than 50% inhibitory under the same conditions.

Substrate concentration and acceptor specificity of GlcNAcTs. — The effects of various concentrations of respective substrates on corresponding GlcNAcT activity are shown in Fig. 3 (a and b). It is apparent from these data that the V_{max} value for the GlcNAcT-2-catalyzed reaction is 2.5 times higher than that catalyzed by GlcNAcT-1. The K_m values of GlcNAcT-1 (30μM; Fig. 3a) and GlcNAcT-2 (60μM; Fig. 3b) activity were determined with the Colo-205 DSS fraction under optimum conditions for each enzyme. Various glycolipids were tested as potential acceptors for the GlcNAcT-catalyzed reaction with the detergent-soluble supernatant solution as the enzyme source, in the presence of externally added detergent. As seen in Table II, in addition to $LcOse_2Cer$ and $nLcOse_4Cer$, $GgOse_3Cer$ was found to be almost equally active as Lc_2Cer. $GbOse_3Cer$ was also partially active as acceptor, whereas the other seemed to have either very little or negligible activity. The K_m value for UDP-[^{3}H]GlcNAc was found to be 330μM when tested under GlcNAcT-1 conditions. This value was similar to that observed[8] for GlcNAcT-2 activity in mouse lymphoma P-1798.

Competition studies were performed (Table III) with the detergent-soluble supernatant solution in order to specify the coexistence of both GlcNAcT-1 and GlcNAcT-2 in the enzyme preparation. The incorporation of [^{3}H]GlcNAc into $LcOse_2Cer$ and $nLcOse_4Cer$ was determined separately, and with mixed substrates at various concentrations as shown in Table III. The experimental values obtained at various mixed-substrate concentrations were closer to the calculated values expected for two specific enzymes than that for one nonspecific enzyme[27].

Differential heat inactivation of GlcNAcTs. — Additional experimental evidence that the GlcNAcT-1 and GlcNAcT-2 reactions might be catalyzed by two different proteins came from differential heat-inactivation profiles of these two activities shown in Fig. 4. The incubations were conducted with a Colo-205 DSS fraction under both conditions after aliquots of the enzyme were heated to 55° for the indicated times. As seen in Fig. 4, ~50% of the GlcNAcT-1 activity was eliminated after only 9 s of heat treatment, whereas GlcNAcT-2-remained 85% as active for that time. However, 50% of the GlcNAcT-2 activity was lost after 15 s of heat treatment. These data suggested that the protein which catalyzes the GlcNAcT-2 reaction might be more stable than that catalyzing the GlcNAcT-1 reaction.

Effect of amino acid modifiers on GlcNAcTs. — Several amino acid modifiers were tested for inhibition of both GlcNAcTs in an attempt to understand the involvement of

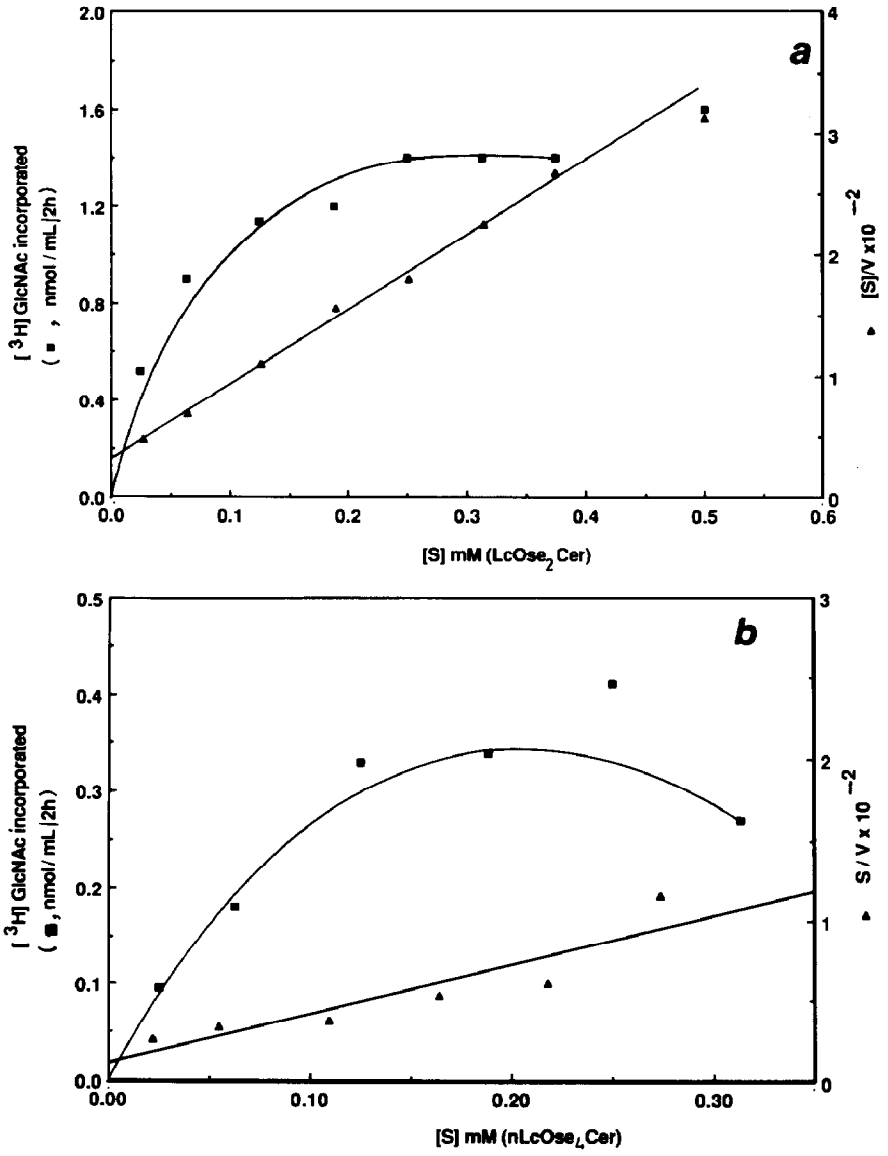


Fig. 3. Effect of substrate concentrations on rate of reactions. Experimental conditions for both GlcNAcT-1 (a) and GlcNAcT-2 (b) remained the same as described in Table I, except that various concentrations of respective substrates were used. The mixtures were assayed by chromatography as described in the Experimental section.

amino acids in the active sites of the enzymes. Preliminary studies indicated that GlcNAcT-1 activity was inhibited by 54% when assayed in the presence of *N*-ethylmaleimide, an inhibitor of the sulfhydryl group in protein, whereas inhibition of GlcNAcT-2 activity was only 13% (Table IV). Interestingly, 5,5′-dithiobis(2-nitrobenzoic acid), another cysteine inhibitor, totally abolished both of the activities. The same

TABLE II

Acceptor specificity of GlcNAcT from Colo 205 DSS[a]

Acceptor (0.4 mM)		*[^{3}H]GlcNAc incorporated (nmol/mg/2 h)*
Structure	*Abbrev.*	
β-D-Gal*p*-(1→4)-D-Glc*p*-Cer	$LcOse_2Cer$	1.6
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-Lc_2Cer	$nLcOse_4Cer$	3.4
α-D-Gal*p*-(1→4)-Lc_2Cer	$GbOse_3Cer$	0.9
β-D-Gal*p*NAc-(1→4)-Lc_2Cer	$GgOse_3Cer$	1.7
β-D-Glc*p*NAc-(1→3)-nLc_4Cer	$iLcOse_5Cer$	0.0
α-NeuAc-(2→3)-nLc_4Cer	LM_1	0.0

[a] The complete incubation mixture contained the same components as described in Table I for GlcNAcT-1 condition, except that various glycolipid acceptors were used as indicated in the Table at a 0.6mM concentration. The mixtures were assayed after incubation for 2 h by the chromatographic methods described in the Experimental section. The endogenous value (0.5 nmol/mg/2 h) was substracted from each experimental value.

TABLE III

Substrate competition of GlcNAcT-1 and GlcNAcT-2[a]

Substrate	*Conc. (mM)*	*[^{3}H]-Product formed*		
		Found	*Theoretical[b] for*	
			One enz.	*Two enz.*
			(nmol/mg/2 h)	
β-D-Gal*p*-(1→4)-D-Glc*p*-(1→1)-Cer (Lc_2)	0.125	0.8		
	0.25	1.2		
β-D-Gal*p*-(1→4)-D-Glc*p*NAc-Lc_2 (nLc_4)	0.125	1.6		
	0.25	2.8		
Lc_2 + nLc_4	0.125 + 0.125	2.5	0.9	2.4
	0.125 + 0.25	3.1	1.6	3.6
	0.25 + 0.25	3.3	1.6	4.0

[a] Conditions were the same as described in Table I, except that the indicated substrates or substrate mixtures were used. The mixtures were assayed by the chromatographic method as indicated in the Experimental section. [b] Theoretical values were calculated by use of the following equations[45]. For one enzyme: $v_t = [v_a\,(a/Ka) + v_b\,(b/Kb)]/\,(1 + a/Ka + b/Kb)$; for two enzymes, $v_t = v_a + v_b$.

result was observed in the presence of diethyl pyrocarbonate, a histidine modifier. As shown in Table IV, differential inhibition was observed with different arginine modifiers for each of the transferases. Further experiments are underway to establish a better understanding of this aspect of enzyme kinetics.

Characterization of the radioactive products. — The radioactive products cata-

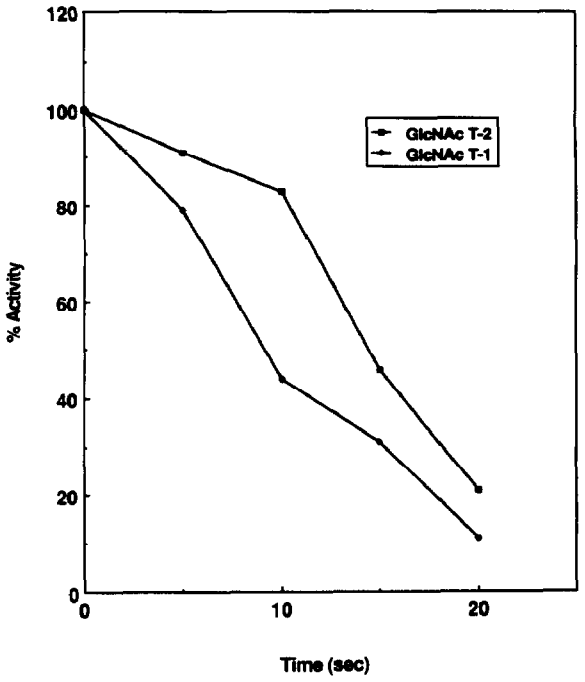


Fig. 4. Heat inactivation of GlcNAcT activities. Aliquots of a DSS fraction of Colo-205 cells were heated at 55° for indicated times and then used as enzyme source. The incubation conditions remained the same as those described in Table I for each enzyme.

TABLE IV

Effect of amino acid inhibitors on GlcNAcT-1 and GlcNAcT-2[a]

Inhibitor (*mM*)	*Inhibition* (%)[b]	
	GlcNAcT-1	*GlcNAcT-2*
None	0	0
Phenylglyoxal	100	100
1,2-Cyclohexadiene	66	32
1,3-Butadiene	84	68
Diethyl pyrocarbonate	100	100
5,5'-Dithiobis(2-nitro-benzoic acid)	100	100
N-Ethylmaleimide	46	13

[a] Complete incubations were done under conditions identical to those described for Table I, except that different active-site modifying agents were added at the concentrations indicated. The mixture were assayed by standard chromatographic method as described in the Experimental section. [b] 100% Activity of GlcNAcT-1, 1.2 nmol/mg/2 h. 100% Activity of GlcNAcT-2, 3.6 nmol/mg/2 h.

lyzed by these two enzyme proteins were isolated from two specific, 20-fold incubations and purified as described in the Experimental section. The purified radioactive product, [^{3}H]GlcNAc→LcOse$_2$Cer, catalyzed by the GlcNAcT-1 enzyme, comigrated (>78%) with authentic LcOse$_3$Cer on t.l.c. as a single band (Lane 2, Fig. 5), as evidenced by counting the radioactivity of the appropriate areas in a liquid-scintillation system after scraping. On the other hand, the GlcNAcT-2 catalyzed product, [^{3}H]GlcNAc→nLcOse$_4$Cer, moved (>92%) as a single band on t.l.c. close to authentic iLcOse$_5$Cer

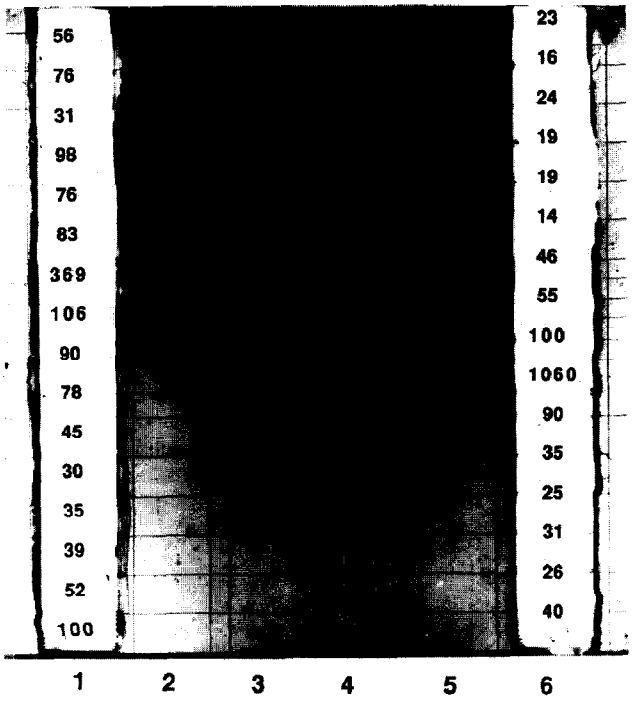


Fig. 5. Thin-layer chromatography of radioactive products. Purified radioactive products (2000 c.p.m.) from GlcNAcT-1- and GlcNAcT-2-catalyzed reactions were cochromatographed in Lanes 1 and 6, respectively, with authentic standards, in 11:9:2 (v/v) chloroform–methanol–0.2% $CaCl_2$. Authentic GSL standards are as follows: Lane 2, LcOse$_2$Cer; lane 3, LcOse$_3$Cer; lane 4, nLcOse$_4$Cer; and lane5, nLcOse$_5$Cer. The standards were detected by spraying with the diphenylamine reagent.

(β-D-Glc*p*NAc-(1→3)-nLcOse$_4$Cer), as evidenced by counting the radioactivity of the appropriate scraped areas in a liquid-scintillation system (Lane 6, Fig. 5).

Each radioactive product was incubated with purified β-D-hexosaminidases from clam, papaya, and jack bean, as described earlier; 90, 70, and 79%, respectively, of the terminal GlcNAc group was released from the [^{3}H]GlcNAc→LcOse$_2$Cer product; and 75, 42, and 66%, respectively, from the other product, [^{3}H]GlcNAc→nLcOse$_4$Cer. These cleavage patterns indicated the formation of a β-D-GlcNAc group linked to the terminal D-galactosyl group of the respective substrates in both products.

The products of both enzyme-catalyzed reactions were isolated and purified by use of unlabeled UDP-GlcNAc and respective terminal D-galactose-labeled substrates.

The purity of the radioactive products, GlcNAc→[^{3}H]Gal→Glc→Cer (GlcNAcT-1 product) and GlcNAc→[^{3}H]Gal→GlcNAc→Gal→Glc→Cer (GlcNAcT-2 product), was monitored by comigration of single bands with authentic standards on t.l.c. Each product was permethylated and hydrolyzed after acetolysis. T.l.c. of the permethylated, hydrolyzed products from each reaction showed the presence of 2,4,6-tri-*O*-methylga-

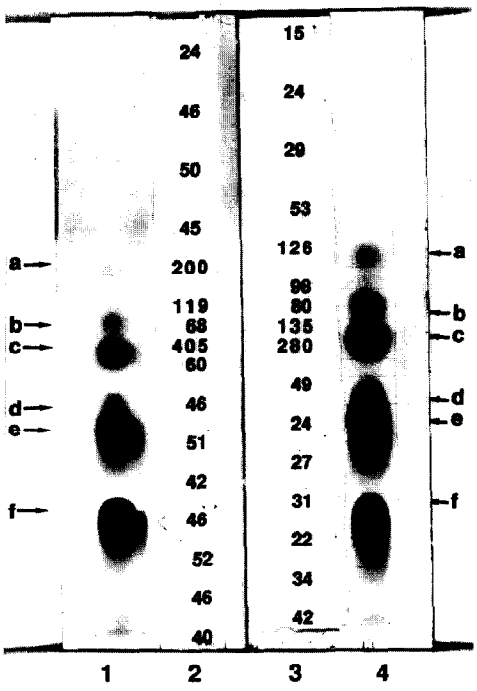


Fig. 6. Identification of ^{3}H-labeled methyl β-D-galactosides from radioactive products. Lanes 2 and 3, permethylated [^{3}H]galactose obtained from GlcNAcT-1- and GlcNAcT-2-catalyzed reaction products, respectively. Lanes 1 and 4, mixture of standard methylated galactose, (a) 2,3,4,6-tetra-*O*-methyl-, (b) 2,3,6-tri-*O*-methyl-, (c) 2,4,6-tri-*O*-methyl-, (d) 2,3,4-tri-*O*-methyl-, (e) 3,4,6-tri-*O*-methyl-, and (f) 2,3-di-*O*-methyl-galactose. After the plate had been developed with 50:1 acetone–5M NH_4OH, the spots were detected by spraying with the diphenylamine–aniline spray. Lanes 2 and 3 were scraped by comparing with the standards in lanes 1 and 4, followed by quantitative determination with a scintillation-counting system.

lactose when chromatographed in 50:1 acetone–5M NH_4OH in the presence of *O*-methylgalactose standards as indicated in Fig. 6 (Lanes 2 and 3). GlcNAcT-1- and GlcNAcT-2-catalyzed reaction products gave 50 and 68% of 2,4,6-tri-*O*-methylgalactose, respectively. The radioactivity in the 2,3,4,6-tetra-*O*-methylgalactose region of the chromatogram resulted from degradation products during methylation[28].

These data, in conjunction with the results of hexosaminidase degradation, suggested the formation of products containing a terminal β-D-Glc*p*NAc group (1→3)-linked in both cases to the terminal D-galactosyl group of the respective substrates.

DISCUSSION

The results presented herein have established the existence of two distinct *N*-acetylglucosaminyltransferases, GlcNAcT-1 and GlcNAcT-2, in human colon carcinoma Colo-205 cells. These enzymes catalyze two major steps in the biosynthesis of Type-1 and Type-2 *lacto*-series glycolipids and in polylactosamine-containing i/I antigenic glycolipids. *Lacto*-series glycolipids are the major structural glycolipids found in human erythrocyte membranes responsible for expressing ABH and Lewis blood-group determinants[3,5,7]. The accumulation of these glycolipids is also reported to be one of the major events during oncogenesis in different animal systems including humans[29–32].

The first enzyme reported herein, GlcNAcT-1, catalyzes the formation of $LcOse_3Cer$, the major core structure for both Type-1 and Type-2 *lacto*-series glycolipids, from lactosylceramide and UDP-GlcNAc, which was previously reported in a membrane-bound preparation from rabbit-bone marrow[8]. The studies of substrate specificity with human serum[11] and various carcinoma cells[33] showed some activity with lactosylceramide, but the radioactive products obtained in these reactions were not characterized by chemical methods (permethylation). Also, these activities were not characterized by further kinetic parameters. However, since $LcOse_3Cer$ is the major intermediate in the biosynthesis of many antigenic glycolipids, the enzyme that catalyzes its formation has to be present in almost all tissues, irrespective of tumor progression, as evidenced by its presence in other normal tissues[8,11]. The level of detergent-soluble GlcNAcT-1 activity from Colo-205 cells reported here is much higher than that reported from other normal tissues[8] or serum[11]. However, the level of the second enzyme, GlcNAcT-2, is considerably higher in the same detergent supernatant solution. GlcNAcT-2 catalyzes the biosynthesis *in vitro* of another major intermediate, $iLcOse_5Cer$, for polylactosamine-containing i/I antigenic glycolipids[8].

Kinetic studies with a partially purified enzyme from Colo-205 cells indicated the possible existence of two different proteins catalyzing these two reactions. Although Mn^{2+} was essential for optimum activities of both enzymes and could not be replaced with any other metals for GlcNAcT-1 activity, partial activation of GlcNAcT-2 by Cd^{2+} (33%) and Co^{2+} (15%) (data not shown) was observed, and GlcNAcT-2 was more resistant to different salts than GlcNAcT-1. Sodium fluoride, one of the salts tested in this study, was found to activate GlcNAcT-2 to almost 220% at a concentration of 0.8M, whereas GlcNAcT-1 was inhibited (50%) by this salt. Differential heat inactivation studies at 55° of the two enzymes also indicated that the protein which catalyzes the GlcNAcT-2 reaction is more stable than GlcNAcT-1 as the T-2 enzyme was still 50% as active even after being heated for 15 s, whereas 50% of GlcNAcT-1 activity was lost after only 8 s of heating. In a preliminary study with active-site modifiers, it was observed that SH-groups might be involved in the catalytic activity of GlcNAcT-2, as *N*-ethylmaleimide, an SH-modifying agent, inhibited this activity by almost 90%, whereas GlcNAcT-1 was still 50% as active under that concentration of *N*-ethylmaleimide. Further kinetic studies are underway to complete the characterization of these two enzymes.

N-Acetylglucosaminyltransferases play a major role in the biosynthesis of polylactosamine-containing, i-related glycolipids and poly(lactosamino)glycans as evidenced by several reports on the occurrence of these structures in a variety of animal cell surfaces of both normal and tumor origin[4,29–35]. The characterization, in Novikoff tumor cell ascites fluids, of a β-D-(1→3)-GlcNAcT that catalyzes the biosynthesis of i-active poly(lactosamino)glycans has very recently been reported[36]. The transition from a linear i-structure to the branched I-active glycolipids and to poly(lactosamino)glycans has been correlated to development and differentiation[7,37–39]. It has been speculated[8–10] that the branching to the I-structure is catalyzed by yet another transferase, β-D-(1→6)-GlcNAcT-3, after the linear chain with i-specificity has been formed. However, the presence of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) repeating units has been reported [40] in *O*-linked poly(lactosamino)glycans of human skim milk.

From the substrate-specificity studies presented herein, it is evident that, in addition to $LcOse_2Cer$ and $nLcOse_4Cer$, $GgOse_3Cer$ [β-D-Gal*p*NAc-(1→4)-$LcOse_2Cer$] exhibits a significant acceptor activity with the Colo-205 enzyme preparation (Table II). Both iLc_5Cer [β-D-Glc*p*NAc-(1→3)-$nLcOse_4Cer$] and LM1 [α-NeuAc-(2→3)-$nLcOse_4Cer$] were poor acceptors, and $GbOse_3Cer$ [α-D-Gal*p*-(1→4)-$LcOse_2Cer$] was only slightly active.

A novel structure containing both 2-acetamido-2-deoxy-D-glucose and -D-galactose in the same chain has been reported in murine leukemia cells and mullet roe[41,42]. *In vitro* biosynthesis of this hybrid structure, $LcGgOse_3Cer$ {β-D-Glc*p*NAc-(1→3)-[β-D-Gal*p*NAc-(1→4)]-β-D-Gal*p*-(1→4)-D-Glc*p*-(1→1)-Cer}, catalyzed by a *N*-acetylgalactosaminyltransferase from embryonic chicken brain with $LcOse_3Cer$ and UDP-GalNAc as acceptor and donor, respectively, has recently been reported[43,44]. An alternative biosynthetic route might be possible for this novel hybrid structure where a GlcNAcT might catalyze the transfer of GlcNAc from UDP-GlcNAc to the internal D-galactosyl residue of $GgOse_3Cer$, as evidenced from the substrate specificity studies of the Colo-205 enzyme, as the acceptor activity of $GgOse_3Cer$ is quite significant (Table II). Characterization of this reaction and of the enzymic product are in progress. However, the kinetic data presented herein, including the substrate-competition experiment (Table III), indicated the presence of two different proteins in the Colo-205 DSS enzyme preparation catalyzing GlcNAcT-1 and GlcNAcT-2 reactions. Experiments are underway for the separation of these two proteins by hydrophobic and substrate-bound affinity-column chromatography. Recently, a GalT-4 [UDP-Gal: $LcOse_3Cer$ or $iLcOse_5Cer$-β-D-(1→4)-GalT] activity has been observed in a soluble fraction from Colo 205 cells and the enzyme was purified 50 000-fold[45]. These results enable us to propose a stepwise biosynthesis of $iLcOse_6Cer$ starting from lactosylceramide in human colon carcinoma Colo 205 cells. Several *N*-acetylglucosaminyltransferases involved in glycoprotein biosynthesis act in a stepwise fashion according to the mode of synthesis of diverse structures of glycoproteins[46,47].

From recent advances in the study of glycosphingolipid structures and metabolism, it is anticipated that the important role of GlcNAcT enzymes in the biosynthesis of i/I-antigenic glycolipid as well as poly(lactosamine)-containing glycolipid will be estab-

lished. It is also important to note that GlcNAcT-1 is involved in the biosynthesis of the key intermediate, $LcOse_3Cer$, for all *lacto*-series glycolipids and, therefore, the level of this enzyme may determine the routing of the glycosphingolipid structures in normal, tumorigenic, and developmental stages.

ACKNOWLEDGMENT

The authors thank Prof. Subhash Basu for constant encouragement during the course of this work and invaluable help during the preparation of this manuscript.

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