Purification and Characterization of a CMP–Sialic:LcOse$_4$Cer Sialyltransferase from Human Colorectal Carcinoma Cell Membranes†

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ABSTRACT: Purified lactotetraosylceramide (Galβ1→3GlcNAcβ1→3Galβ1→4Glc1-Cer) was tested for its ability to accept [^{14}C]sialic acid from CMP–[^{14}C]sialic into monosialoganglioside fractions in the presence of membrane fractions purified from human colorectal carcinoma cells (SW1116). Membrane fractions were isolated by three different methods: sucrose density centrifugation, CMP–agarose gel column chromatography, and LcOse$_4$ gel chromatography. We optimized the incubation conditions for detergent dependency (taurocholate), pH (6.3), and acceptor concentration. The sialyltransferase activity was dependent on membrane protein and linear for time up to at least 4 h. The LcOse$_4$ affinity chromatography of the crude microsomal membrane pellet from these cells yielded a membrane fraction that was 136-fold enriched in LcOse$_4$ acceptor specific activity compared to cell homogenates. The apparent K_m for the sialyltransferase activity with LcOse$_4$Cer acceptor in the presence of affinity-purified membranes was 20 μM and the V_{max} was 7 pmol h^{-1} (100 μg of protein)$^{-1}$. Acceptor capabilities of other core structures were 5–20-fold lower: LcOse$_4$Cer ≫ GgOse$_4$Cer > nLcOse$_4$Cer ≫ GbOse$_4$Cer. The enzymatic activity was purified further (900-fold) by a combination of LcOse$_4$ and CMP affinity gels. SDS–PAGE electrophoresis of this material showed a major set of closely migrating bands of M_r 58 000–54 000 compared to authentic proteins, as well as a minor band at 27 000. We analyzed picomole quantities of the radioactive product by convenient controlled short-term hydrolyses with an endoglycoceramidase and sialidases (from four different sources) in comparison to sialylated tetrasaccharides of known structure. We conclude that the major product of the sialyltransferase activity, present in these affinity column purified membrane fractions, is IV^{3}NeuNAcLcOse$_4$Cer and that the enzyme specifically recognizes lactotetraosylceramide. This enzymatic activity may control the level of, at least, two ganglioside antigens of the lacto series reported previously to be "tumor associated", Ca50 and 19-9. Our results are the first to present extensive purification of this enzymatic activity from human carcinoma cells.

The existence of *N*-acetylglucosamine-containing gangliosides was reported by Wiegandt and Schulze in 1969. Since then technical improvements in the isolation and characterization of these compounds from exiguous levels occurring in peripheral tissues have led to the notion of tumor-associated glycolipid antigens (Hakomori, 1984). Prominent among these has been the sialylated Lewis^a or fucosylated monosialoganglioside of the lacto series originally identified in the SW1116 colorectal carcinoma cell line (Magnani et al., 1982). This compound is associated with gastrointestinal carcinomas, but glycoproteins having identical sugar sequence and linkage may be present in mucins of the digestive system (Raux et al., 1983). On the basis of results with oligosaccharide substrates, biosynthesis of this glycolipid apparently occurs by α2→3 sialylation of the lactotetraosyl moiety and subsequent α1→4 fucosylation of the sialosyllactotetraosylceramide (IV^{3}NeuNAcLcOse$_4$Cer)[1] to sialylated Le^a (IV^{3}NeuNAcIII^{4}FucLcOse$_4$Cer) (Hanson & Zopf, 1985). Sialosyllactotetraosylceramide has recently been described as a novel tumor-associated glycolipid in colorectal carcinomas (Nilsson et al., 1985).

We here describe the characteristics of an enzymatic activity present in purified membranes of human colorectal carcinoma cells (SW1116) that specifically sialylates LcOse$_4$Cer. Our evidence indicates that the product is sialosyllactotetraosylceramide. This sialyltransferase may control the level of the sialylated Lewis^a because it synthesizes its direct precursor and appears to do so at a much lower rate of LcOse$_4$Cer utilization than the fucosyltransferase activities for Lewis^a and Lewis^b biosynthesis present in these cells (Liepkans & Larson, 1987). To our knowledge, this is the first characterization of this enzymatic activity in carcinoma cells from serum-free medium with a glycolipid acceptor of documented purity and structure (Karlsson & Larson, 1979). We report results on three methods of membrane-bound enzyme purification based on three different properties of the sialyltransferase activity: its localization in Golgi-enriched fractions, its affinity for CMP–agarose, and its affinity for a lactotetraosyl gel. We also present a convenient and rapid analysis of sialyltransferase

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[1] Abbreviations: LcOse$_4$Cer, lactotetraosylceramide, Galβ1→3GlcNAcβ1→3Galβ1→4Glc1-ceramide; nLcOse$_4$Cer, Galβ1→4GlcNAcβ1→3Galβ1→4Glc1-ceramide; GgOse$_4$Cer, Galβ1→3GalNAcβ1→4Galβ1→4Glc1-ceramide; GbOse$_4$Cer, GalNAcβ1→3Galα1→4Galβ1→4Glc1-ceramide; CST buffer, 0.025 M sodium cacodylate, 0.05 M NaCl, 1 mM taurocholate; IV^3 NeuNAcLcOse$_4$Cer, sialosyllactotetraosylceramide (NeuNAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc1-ceramide); IV^{3}NeuNAcIII^{4}FucLcOse$_4$Cer, 19-9 antigen, gastrointestinal carcinoma associated glycolipid antigen (NeuNAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glc1-ceramide); GM$_1$, II^3 NeuNAcGgOse$_4$Cer; HPTLC, high-performance thin-layer chromatography; CMP-sialic, cytidine monophosphate sialic acid. The glycolipid notation used here is according to the IUPAC–IUB nomenclature [(1977) *Eur. J. Biochem. 79*, 11–21].

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products using endoglycoceramidase in combination with bacterial and viral sialidases (Ito & Yamagata, 1985).

Materials and Methods

Cell Biology

SW1116 cells were cultured and harvested from serum-free media as described previously (Liepkans et al., 1985).

Materials

Lactotetraosylceramide ($LcOse_4Cer$) was purified from human meconium (donor blood type O) as described previously and was freed of $nLcOse_4Cer$ by chromatography (as the acetylated compound) on HPLC and Iatrobeads as previously described (Karlsson & Larson, 1979). H-1 fucolipid ($IV^2FucLcOse_4Cer$) and Le^a fucolipid ($III^4FucLcOse_4Cer$) were also obtained from human meconia (Karlsson & Larson, 1981). Cerebroside, gangliotetraosylceramide ($GgOse_4Cer$), globoside ($GbOse_4Cer$), GM_1 ganglioside, and ceramide trihexoside were purchased from Supelco (Bellefontaine, PA) and checked for purity prior to use. Detergent, sodium taurocholate, Triton X-100, Triton CF-54, Tween-80 and Nonidet-40 were from Sigma (St. Louis, MO). Galactose oxidase of *Polyporus circinatus* was purchased from P-L Biochemicals (Milwaukee, WI). CMP-[^{14}C]sialic acid (351 μCi/μmol) was purchased from Amersham and CPM-[^{3}H]sialic acid (18.9 mCi/μmol) and GDP-[^{14}C]fucose (254 Ci/mmol) was obtained from NEW (Boston, MA). CMP–sialic for the dilution of specific radioactivity was from NEN. C50 antibody and GM1F ($II^3NeuNAcIV^2Fuc\ GgOse_4Cer$) and authentic $IV^3NeuNAcLcOse_4Cer$ were obtained through the good offices of Dr. Lars Svennerholm of the University of Göteborg, Sweden. C50 Mab is specific for the lacto series of monosialogangliosides. Authentic $IV^6NeuNAcnLcOse_4Cer$ was obtained from Dr. Olle Nilsson of the University of Göteborg. Authentic $nLcOse_4Cer$ was obtained from Dr. Niklas Stromberg of the University of Göteborg and was the product of fucosidase digestion of type 2 H fucolipid from dog intestine.

Methods

Assay for Glycolipid-Dependent Sialyltransferase. Purified membrane fractions containing sialyltransferase activity were obtained from human colorectal carcinoma SW1116 cells by sucrose density centrifugation as previously described (Liepkans & Larson, 1987). Standard conditions for incubation were the following: 70 μg of taurocholate (sodium salt) dissolved in chloroform/methanol (2/1) was dried with 0.2–5 μg of $LcOse_4Cer$ or test glycolipid in a conical assay tube; 30–45 μL of (final concentrations) cacodylate (0.15 mM), sucrose (0.3 M), $MgCl_2$ (1 mM), and CDP–choline or ATP (0.5 mM) were then added. After the addition of membranes (2–10 μg of protein), 10 nmol of CMP-[^{14}C]sialic was added (sp. act. 32–351 μCi/μmol), and the mixtures were incubated for 2 h at 37 °C, final volume 50–80 μL, pH 6.3. CDP–choline or ATP was added as irrelevant nucleotide to retard CMP-[^{14}C]sialic donor hydrolysis. For some incubations we used CMP-[^{3}H]sialic donor in order to obtain radioactive products of higher specific activity for analysis.

Membrane Fractions Obtained by Affinity Chromatography. Two milliliters of affinity gel, CMP–agarose (Sigma) or $LcOse_4$-fractogel (Bio-Carb, Lund, Sweden) were washed in CST buffer (0.025 M cacodylate, 0.050 M NaCl, 0.05% taurocholate) by suspension and centrifugation at 1000*g*, 6 × 6 mL. Human colorectal carcinoma cells cultured in serum-free medium were obtained as described previously (Liepkans et al., 1985): the cells were homogenized and sonicated (15 s, 4 bursts at +4 °C) in CSM buffer (0.2 M cacodylate, 0.2 M sucrose, 1 mM $MgCl_2$) and then centrifuged at 1000*g* for 1 h. The pellet of nuclei unbroken cells and debris was discarded, and the supernatant was applied to a sucrose gradient (p = 1.10–1.18) in CSM buffer (Liepkans & Larson, 1987). After the sucrose gradient, the total membranes from this centrifugation (excluding the pellet) were recovered by dilution 2-fold with 0.15 M cacodylate, pH 6.2, and centrifugation at 150000*g* for 2 h. The resulting final microsomal pellet, which was devoid of mitochondria, was suspended in CST buffer by repeated gentle regurgitation with sterile Pasteur pipets. The opalescent suspension was applied to 1 × 3.5 (CMP–agarose) or 1 × 2.5 ($LcOse_4$ fractogel) cm column at a concentration of 1 mg/mL in a volume of 2 mL at 4 °C.

The material was collected from the column and reapplied six times. The column was then washed with 5 × 2 mL of CST buffer and 1 mL of CST buffer containing 1 M NaCl. Then 2 mL of CST buffer containing 1 M NaCl was applied, and the resulting fractions were collected and dialyzed in Spectrapor 6 sterile dialysis bags against CST buffer for 12 h at 4 °C. Affinity gels could be regenerated by the following series of washes: 10 mL of CST buffer, 20 mL of deionized H_2O; 5 mL of 6 M urea; 10 mL of SDS 0.5%; 20 mL of H_2O; 10 mL of 0.1 M NaCl/0.1 M borate/0.1 M NaOH, pH 8.9; 10 mL of H_2O; 10 mL of 0.1 NaOAc, pH 4.1; 20 mL of H_2O; 5 mL of 50 mM cacodylate, pH 6.2; and finally 5 × 2 mL washes of CST buffer containing 0.02% NaN_3. For recovery of enzymatic activity from CMP–agarose columns of fractions that had previously been obtained from $LcOse_4$ gel columns, the detergent concentration of CST was decreased in proportion to the decrease in protein concentration (about 8–10-fold).

Analysis of the Radioactive Products of the Incubations. The incubations were terminated by the addition of 0.5 mL of chloroform/methanol (H_2O) [1/2 (3%)] and allowed to sit overnight at 4 °C to permit precipitation of insoluble components. After the addition of 1 μg of GM_1 ganglioside carrier to each extract, the solutions were spotted on 3MM Whatman paper for ascending chromatography in 1% sodium tetraborate, pH 9.1, for 90 min (Basu et al., 1982).

The chromatogram was then dried thoroughly, and the origins (1.5 cm^2) were cut out and placed in conical tubes for extraction with chloroform/methanol/H_2O (30/60/8 v/v/v) 3 × 2 mL (Ledeen & Yu, 1982). These extracts were then applied and reapplied to a DEAE-Sepharose Cl-6B (acetate form) columns (1.5 × 0.5 cm) that were eluted with 3 × 2 mL 0.03 M NH_4OAc, and this fraction was dried in scintillation vials for radioactivity determinations. We added 0.25 mL of methanol to each vial to dissolve the small amounts of salt and counted each sample in 10 mL of Insta-gel (Packard, IL) against two averaged (minus glycolipid) controls for times up to 1 h depending on statistical validity and chemiluminescence interference, automatically determined by the LKB Rack β counter. Control experiments showed that essentially all of the GM_1 could be collected in the 0.03 M NH_4OAc fraction. Control experiments also showed that when the $LcOse_4Cer$ concentration was varied over a 20-fold range, the nonlipid extractable radioactivity remaining at the origins of paper chromatograms varied with a standard deviation of ±4%.

High-performance thin-layer chromatography for the lacto series of gangliosides was performed as described by Nilsson et al. (1981) and Hanada and Konno (1985) in the solvent systems chloroform/methanol/2.5 M NH_3 (60/40/9 v/v/v) (I), chloroform/methanol/0.25% KCl (60/35/8) (II), and propanol/H_2O/NH_3 (75/25/5) (III). In these systems

$IV^3NeuNAcnLcOse_4Cer$ runs ahead of ganglioside GM_1 ($II^3NeuNAcGgOse_4Cer$), and GM_1 runs ahead of $IV^6NeuNAcnLcOse_4Cer$. Galactose oxidase treatment of $LcOse_4Cer$ was performed as previously described (Liepkans & Larson, 1987).

Immunochemistry. Immunochemistry of meconium glycolipid antigens was studied by the method modified from Magnani et al. (1982) and Mori et al. (1982).

Neuraminidase Assays. Bacterial neuraminidases were purchased from Behringer Mannheim (*Arthrobacter ureafaciens*, *Clostridium perfringens*, and *Vibrio cholerae*). Influenza virus "HAM" mutants were kindly provided by Dr. J. J. Skehel of the National Medical Research Institute, London.

The following concentrations of enzymes were used in each incubation: 3 mg of sialidase from *A. ureafaciens* (+10 μg of taurocholate); 25 milliunits of sialidase from *C. perfringens* (minus detergent); 50 μg of *Influenza viral* protein; 10 microunits of *V. cholerae* sialidase, each in 22 μL total volume of 0.05 M NaOAc buffer, pH 5.5.

$IV^3NeuNAcLcOse_4$, $III^6NeuNAcLcOse_4$, $IV^6NeuNAcnLcOse_4$ substrates (20 μg/assay) were purchased from Bio-Carb. After incubations of 10 min–4 h, protein was precipitated by the addition of $CHCl_3/CH_3OH$ (1/2) (3% H_2O), 0.2 mL, and the extract was dried under N_2, spotted on HPTLC plates, and chromatographed in butanol/AcOH/H_2O (2/1/1). This system gives excellent separation of pentasaccharides, from tetraoses, from sialic acid (Ito & Yamagata, 1986; Corfield et al., 1987; Jolif & Liepkans, 1988).

SDS–PAGE Electrophoresis. SDS–PAGE electrophoresis was performed in a miniprotein II apparatus (Bio-Rad) with 4% gel stocked onto 12% polyacrylamide in Tris-based migration buffer under 150 V for 90 min as adapted from Laemmli (1970). We stained the gel with 0.1% Coomassie blue in 40% CH_2OH, 10% AcOH for 2 h.

Results

The incorporation of radioactivity into monosialoganglioside fractions was linearly dependent on gradient purified SW1116 membrane protein concentration of the incubations up to about 10 μg/100 μL under the standard conditions of incubation containing 4 nmol of $LcOse_4Cer$ and 10 nmol of CMP–[^{14}C]sialic/80 μL. Generally, we incubated for 2 h, but incorporation into monosialoganglioside was linearly time dependent for up to at least 4 h.

The transferase activity appeared to have a specific detergent preference. Of the five detergents tested (taurocholate, Triton X-100, Triton CF-54, Tween 80, Nonidet 40) at the concentration tested (0–2 mM), taurocholate appeared to stimulate activity most effectively.

The pH optimum for purified $LcOse_4Cer$ acceptor capability was 6.3. These experiments (Figure 1) were reproducible at two specific activities of CMP–[^{14}C]sialic differing by 10-fold. The pH optimum for purified $nLcOse_4Cer$ acceptor activity was similar to that of $LcOse_4Cer$ in sucrose density purified membranes.

Migration of the radioactive product(s) of these incubations on high-performance thin-layer chromatograms (HPTLC) in relation to standards is shown in Figure 2. The ^{3}H-labeled product (lane B) of these incubations ($LcOse_4Cer$ acceptor), which was isolated by lipid extraction, paper chromatography, and DEAE-Sepharose column chromatography, migrated with authentic $IV^3NeuNAcLcOse_4Cer$ (lane C) just ahead of $II^3NeuNAcGgOse_4Cer$ (lane E) and not with $IV^6NeuNAcnLcOse_4Cer$ (lane D). Treatment with *C. perfringens* sialidase for 8 h changed the migration of the radioactivity to an R_f GM_1 = 0.29 (lane A).

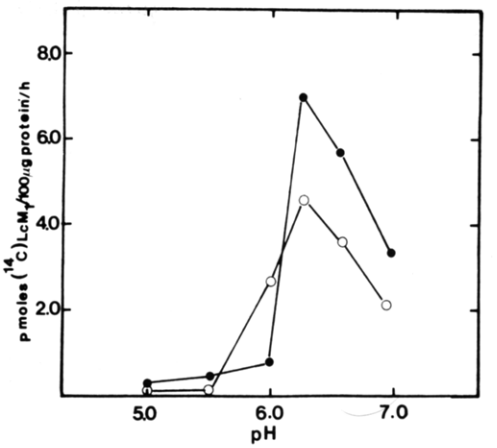

FIGURE 1: Incorporation of [^{14}C]sialic acid into monosialoganglioside fractions in the presence of $LcOse_4Cer$ or $nLcOse_4Cer$ as a function of the pH of the incubations. Each incubation contained 5.5 μg of protein, 100 μM CMP–[^{14}C]sialic, and 40 μM $LcOse_4Cer$ or $nLcOse_4Cer$ for 2 h at 37 °C. The pH at 5.0 had been adjusted with citric acid. (●) $LcOse_4Cer$ substrate; (○) $nLcOse_4$ substrate. LcM_1 is shorthand for monosialoganglioside.

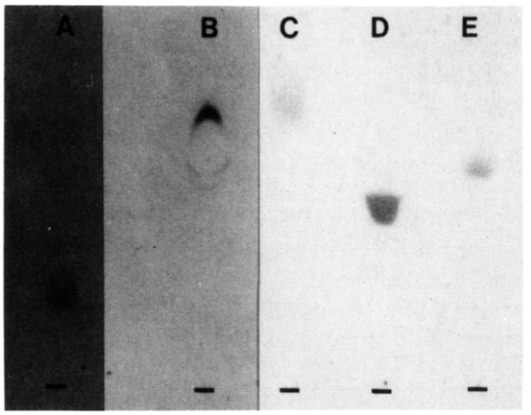

FIGURE 2: High-performance thin-layer chromatography of the ^{3}H-labeled monosialoganglioside (lane B) fraction (450 dpm) obtained by incubation of $LcOse_4Cer$ (3 μg/100 μL) and CMP–[^{3}H]sialic (18.9 mCi/mmol) in the presence of human colorectal carcinoma cell membranes under the optimum conditions outlined under Methods and Results. HPTLC was performed in chloroform/methanol/2.5 M NH_3 (60/40/9). The plate was exposed to Kodak X-Omat film for 5 days after treatment with enhancer (fluorography). Lanes C–E were developed with resorcinol/Hcl spray for lipid sugar. The ^{3}H-labeled monosialoganglioside fraction was freed of salt by treatment with Sep-Pack reversed-phase chromatography (Ledeen & Yu, 1982). Lane A was spotted with ^{3}H-labeled transferase product(s) treated with *C. perfringens* neuraminidase in the absence of detergent. (Lane C) Authentic $IV^3NeuNAcLcOse_4Cer$; (lane D) authentic $III^6NeuNAcLcOse_4Cer$; (lane E) authentic $II^3NeuNAc$ $GgOse_4Cer$ (GM_1).

A more complete resume of the migrations of authentic compounds in relation to GM_1 in this HPTLC system is shown in Table I. The radioactive product also migrated with authentic $IV^3NeuNAcLcOse_4Cer$ in a $CHCl_3/CH_3OH$/KCl HPTLC system (see Methods).

Table II presents results of experiments designed to establish the type of sialosyl linkage in the ^{14}C-labeled enzymatic product.

Under controlled conditions of limited hydrolysis, the action of sialidases from four different sources on authentic pentasaccharides with α2→3 and α2→6 linkages was investigated. For incubations of less than 1 h *Influenza* and *V. cholerae* sialidase action on $III^6NeuNAcLcOse_4$ was incomplete. For incubation times of less than an hour the action of *V. cholerae* sialidase on $IV^6NeuNACnLcOse_4$ was also incomplete.

Table I: Migration of Sialyltransferase Products in Comparison to Standards on HPTLC

	R_f[a] GM_1	detection
^{14}C-labeled enzymatic product	1.21 ± 0.5 (n = 5)	fluorography
C50 antigenicity (meconium)	1.20	immunobinding ^{125}I
IV^{3}NeuNAcLcOse$_4$Cer (pure)	1.17	resorcinol
GM3	1.70	resorcinol
meconium monosialogangliosides (major)	1.75, 1.10	resorcinol, orcinol
(GM_1)	(1.0)	resorcinol, orcinol
GM_{1F}	0.87	resorcinol, orcinol
sialic acid	0.24	resorcinol
CMP–sialic	0.12	resorcinol
IV^{3}NeuNAcnLcOse$_4$Cer	1.47	Nilsson et al. (1981, 1985)
IV^{6}NeuNAcnLcOse$_4$Cer	0.80	resorcinol

[a] HPTLC system: chloroform/methanol/2.5 M NH_3 (60/40/9); Aluminum backed (90 min) and glass (50 min) silica plates.

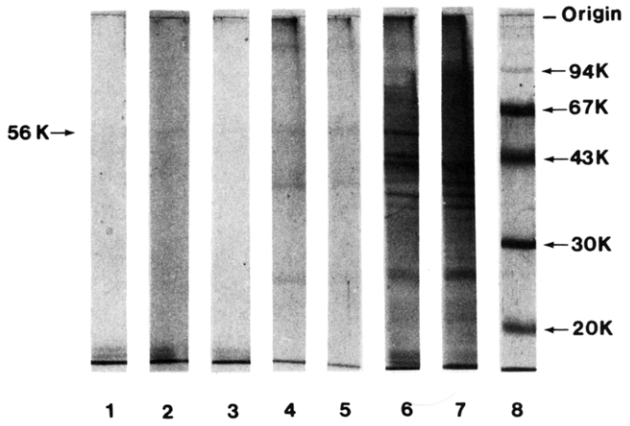

FIGURE 3: SDS–PAGE of membrane fractions and purified fractions of human colorectal carcinoma membranes (see Methods). (Lanes 1–3) Purified fractions obtained by affinity chromatography on LcOse$_4$ fractogel and CMP–agarose (6 μg per lane); (lanes 4 and 5) fractions obtained by LcOse$_4$ fractogel only (60 and 30 μg); (lane 6) sucrose density fraction 1.10–1.18 (100 μg); (lane 7) total microsomal protein from SW1116 cells (100 μg); (lane 8) molecular weight standards.

However, for incubations times within 30 min we obtained complete, or nearly complete, hydrolysis of the ^{14}C-labeled enzymatic product by *V. cholerae* and *Influenza* sialidases; the radioactive material was presented to the sialidases as the intact glycolipid or as the ^{14}C-labeled oligosaccharide from endoglycoceramidase pretreatment. This kind of lability was characteristic of the α2→3 linkage in that IV^{3}NeuNAcLcOse$_4$ was degraded 84–90% within 30 min by *Influenza* and *V. cholerae* sialidases. The concentrations of oligosaccharide presented to these sialidases were essentially identical in all incubations because the contribution of the mass of ^{14}C-labeled oligosaccharide compared to that of carrier was negligible (Table II).

We purified human colorectal carcinomas membranes, enriched in CMP–sialic:LcOse$_4$Cer sialyltransferase activity, by three different methods (Table III): sucrose density centrifugation, cytidine 5′-monophosphate–agarose (CMP–agarose) affinity column chromatography and LcOse$_4$ fractogel affinity chromatography. All three methods, each based on different characteristics of the enzymatic activity, yielded fractions that were useful and enriched more than 100-fold over activity in the homogenate and 5–8-fold over total microsomal pellets. We were able to obtain over 900-fold purification by combining LcOse$_4$ gel with CMP–agarose fractionation. Protein concentrations were rate limiting and detergent to protein ratios were kept as closely identical as possible in all comparative incubations (within 20%).

The specificity of the sialyltransferase enzymatic activity in the membrane fraction from LcOse$_4$ fractogel chromatography was then tested (Table IV). At glycolipid concentrations of 40 μM the enzyme(s) preferred the LcOse$_4$Cer acceptor over the GgOse$_4$Cer or the nLcOse$_4$Cer acceptor. GbOse$_4$Cer was a poor acceptor under these incubation conditions (Table IV, Methods).

When the LcOse$_4$Cer acceptor activity was tested as a function of acceptor concentration in the presence of LcOse$_4$ affinity column fractions, we obtained an apparent K_m of 20 μM for purified LcOse$_4$Cer and a V_{max} of 6.8 pmol h^{-1} (100 μg of protein)$^{-1}$; with gradient purified membrane we obtained a K_m of 9.6 μM for LcOse$_4$Cer. The K_m for the CMP–sialic donor, in the presence of gradient-purified membranes, was 10.3 μM. Despite the low acceptor activity with all concentrations of purified nLcOse$_4$Cer in the presence of LcOse$_4$ gel column purified membrane fractions, we were able to calculate an apparent K_m of 100 μM and a V_{max} of 1.2 pmol h^{-1} (100 μg of protein)$^{-1}$. However, we were unable to obtain significant linearity of $1/v$ vs $1/s$ with the GgOse$_4$Cer compound. Other purified glycolipids that proved to be poor or inactive acceptors at 40 μM were H-1 fucolipid, Lewis^a fucolipid, ceramide trihexoside, and galactose oxidase treated LcOse$_4$Cer (Liepkans & Larson, 1987).

The effects of divalent cations on the LcOse$_4$Cer activity were investigated. Concentrations of up to 1 mM Mg^{2+}, Mn^{2+}, and Zn^{2+} were not required for maximal activity under our assay conditions, with 40 μM LcOse$_4$Cer as acceptor, in the

Table II: Specificity of Neuraminidases from Bacterial and Viral Sources[a]

substrate	*A. ureafaciens*, 60 min	*C. perfringens*, 60 min	*Influenza* 10 min	*Influenza* 30 min	*Influenza* 60 min	*V. cholerae* 10 min	*V. cholerae* 30 min	*V. cholerae* 60 min
IV^{3}NeuNAcLcOse$_4$	66*	99	87	87	97	46	85	86
III^{6}NeuNAcLcOse$_4$	60	97	28	43	78	19	34	61
IV^{6}NeuNAcnLcOse$_4$	53	95	60	93	99	18	36	68
^{14}C-labeled enzymatic product	complete	complete	78	90			90	95
^{14}C-labeled enzymatic product as pentaose[b]			92				84	

[a] Percent of total sialic, as free sialic, after incubation for the times indicated and assayed on HPTLC silica gel plates [butanol/acetic acid/H_2O (2/1/1)], Followed by drying, resorcinol spray application, densitometry, and integration, or in the case of ^{14}C-labeled enzymatic product, autoradiography of the HPTLC plate, densitometry, and integration. After sialidase treatment, 100 pmol of IV^{3}NeuNAcLcOse$_4$Cer was added as carrier to each ^{14}C-labeled extract for HPTLC analysis. [b] About 500 cpm of ^{14}C-labeled enzymatic product and 100 pmol of IV^{3}NeuNAcLcOse$_4$Cer were dried with 7.5 μg of taurodeoxycholate, and 250 μunits of endoglycoceramidase (EGC) was added in a total volume of 20 μL of sodium acetate (pH 5.5), 0.05 M, and incubated at 37 °C for 90 min. Twenty micrograms of IV^{3}NeuNAcLcOse$_4$ was then added, and incubations were resumed as described under Methods. Control experiments showed about 50% of the gangliosidic pentaose had been released by EGC treatment (Ito & Yamagata, 1986).

Table III: Purification of CMP-Sialic:$LcOse_4Cer$ Sialyltransferase Activity from Microsomal Membranes by Three Methods

cell fraction	[protein]	sp. act.[a]	recovery of total act. (%)	purifn (x-fold)
homogenate	0.5 mg/mL	0.05		
150000g membranes (density 1.10–1.18)	1 mg/mL	1.1	158	22
sucrose density fraction 1.16–1.18 (4 °C)	0.85 mg/mL	5.4	92.1	108
CMP-agarose	40 μg/mL	7.8	35.5	156
$LcOse_4$ fractogel	80 μg/mL	6.8	62.8	136
$LcOse_4$ and CMP gels	10 μg/mL[b]	45.2	22	904

[a] Picomoles of [^{14}C]sialic from CMP[^{14}C]sialic incorporated into monosialoganglioside per 100 μg of protein per hour. Each assay tube contained 40 μM $LcOse_4Cer$, 60 μM CMP-[^{14}C]sialic, 1 mM taurocholate, and 2–4 μg of protein per 44-μL incubation in CST buffer, pH 6.2. Protein was determined by the method of Bradford (1976). [b] Protein was concentrated by lyophilization and/or Amicon dialysis under N_2 prior to assay.

Table IV: Sialyltransferase Activity in $LcOse_4$ Affinity Column Purified Membrane Fractions: Acceptor Capability[a] of Purified Glycolipids[b]

Galβ1→3GlcNAcβ1→3Galβ1→4Glc-Cer ($LcOse_4Cer$)	6.8 (5.3[c]) ± 0.6
Galβ1→3GalNAcβ1→4Galβ1→4Glc-Cer ($GgOse_4Cer$)	1.3 ± 0.2
Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer ($nLcOse_4Cer$)	0.8 ± 0.1
GalNAcβ1→3Galα1→4Galβ1→4Glc-Cer ($GbOse_4Cer$)	0.3 ± 0.04

[a] Measured as incorporation of [^{14}C]sialic from CMP-[^{14}C]sialic (pmol/100 μg of protein) into monosialoganglioside fractions (n = 3). Each incubation contained 40 μM exogenous purified glycolipid, 60 μM CMP-[^{14}C]sialic, 2 μg of affinity-purified membrane fraction in a total volume of 44 μL, CST buffer, pH 6.2. [b] Terminal disaccharide moieties have been underlined to emphasize structural differences between acceptors. [c] 30 μM CMP-[^{14}C]sialic.

presence of $LcOse_4$ fractogel purified membrane fractions.

SDS-PAGE electrophoresis of purified fractions containing sialyltransferase activity is shown in Figure 3. The gel that supported the migration of proteins from $LcOse_4$ gel affinity chromatography contained seven bands visible by Coomassie blue staining under M_r 100 000 and one major band at about M_r 120 000 (Figure 3, lanes 4 and 5). By a semilog plot these bands migrated characteristic of molecular weights 90 000, 78 000, 56 000, 39 000, 37 000, 35 000, and 27 000.

After a second affinity column of CMP-agarose the major molecular species remaining appeared at about M_r 56 000. Closer examination by densitometry revealed two or three bands between M_r 58 000 and 54 000 and a minor doublet that migrated at M_r 27 000; these bands were common to all of the membrane fractions we tested (Figure 3, lanes 1–7). The molecular weight authentic standards that we used were phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; and soybean trypsin inhibitor, 20 100.

Discussion

We conclude that a major product of the sialyltransferase activity in the presence of $LcOse_4Cer$ acceptor, CMP-[^{14}C]-sialic, and human colorectal carcinoma cell membranes is [^{14}C]$IV^3NeuNAcLcOse_4Cer$. This conclusion is based, first, on its chromatographic behavior (Figure 2, Table I), second, on its sensitivity to short-term sialidase action (Table II), and, third, on the specificity of the transferase activity after $LcOse_4$ affinity chromatography, (Table IV) vis-à-vis four major core structures. Some resistance of the ^{14}C-labeled products to total sialidase digestion (5–10%) may indicate the presence of a 2→6 transferase activity to $LcOse_4Cer$ or a minor glycolipid impurity with acceptor capacity. On autoradiograms of HPTLC plates we have observed a minor unidentified band trailing the major radioactive product (Figure 2).

The sialylation of $GgOse_4Cer$ to form $IV^3NeuNAcGgOse_4Cer$ in human brain extracts has also recently been reported by Ariga and Yu (1987) (Table IV).

The presence of a sialosyllactotetraosylceramide ($IV^3NeuNAcLcOse_4Cer$) (Ca5o antigen) in SW1116 cells has not previously been established. But the biosynthesis of the sialyl Le[a] monosialoganglioside (19-9 antigen) in these cells has been suggested to occur via the $LcOse_4$-→-$IV^3NeuNAcLcOse_4$-→$IV^3NeuNAcIII^4Fuc\ LcOse_4$- sequence (Hanson & Zopf, 1985). This is analogous to the biosynthetic pathway for sialylated Lewis[x] subsequently reported for lung carcinoma PC9 cells (Holmes et al., 1986). Nilsson et al. (1985) have reported that $IV^3NeuNAcLcOse_4Cer$ is a minor component of several different carcinomas including colorectal carcinoma cell line colo 205 and may itself be an "oncofetal associated" antigen. The presence of monosialosyl glycolipids with type 1 chains in human embryonal carcinoma cells has recently been reported by Fukuda et al. (1986).

Beyer et al. (1981) have reported the substrate specificity of an α2→3 sialyltransferase from porcine submaxillary gland. Using synthetic oligosaccharides, they showed that the purified enzyme had a preference for the Galβ1→3GlcNAc terminal sequence and that lacto-N-neotetraose was not an acceptor. Conversely, Holmes et al. have recently reported that the sialyltransferase from PC9 human lung carcinomas apparently is specific for the $nLcOse_4Cer$ and does not sialylate the $LcOse_4Cer$ glycolipid (Holmes et al., 1986). SW1116 membrane preparations also appear to have an enzymatic activity that sialylates $nLcOse_4Cer$ (Figure 1). This suggests the potential for sialosyl Lewis[x] (type 2 chain) biosynthesis in SW1116 cells.

Our study presents evidence that sialylation of $LcOse_4$ occurs in SW1116 cells via a specific enzyme and that the terminal galactose in the type 1 sequence is a preferred target for sialylation (Results, Tables III and IV). In addition, the 5-fold lower apparent K_m and higher V_{max} for $LcOse_4Cer$ acceptor vs $nLcOse_4Cer$ (after $LcOse_4$ gel chromatography) is evidence that there is an enzyme in this membrane fraction that specifically recognizes the nonreducing terminal Galβ1→3GlcNac moiety of lactotetraosylceramide (or the physical structure lent by that linkage). The purity of our $LcOse_4Cer$ sample from human meconium (97.6%) was determined on HPTLC by acetylation, chromatography, and densitometry in a system that specifically separates $LcOse_4Cer$ from $nLcOse_4Cer$ (see Methods). The nature of the core structure in our $nLcOse_4Cer$ sample (obtained by fucosidase digestion of type 2 H fucolipid from dog intestine) was determined by mass spectrometry, from which was observed a fragment, m/e 182, diagnostic of type II chains (Dr. N. Stromberg, personal communication). However, at concentrations approaching 100 μM minor impurities in the $nLcOse_4Cer$ sample on the order or 1 or 2% that had acceptor capacity could contribute. The fact that we obtained a lower K_m with gradient-purified membranes than with affinity-purified subfractions may reflect the presence of factors in intact membranes that affected the binding of $LcOse_4Cer$ acceptor

to the membrane-bound transferase.

Sialylation of $LcOse_4Cer$ acceptor appears to occur at a lower V_{max} and with a higher K_m than fucosylation of the $LcOse_4Cer$ previously reported in these cells (Liepkans & Larson; 1987, Liepkans et al., 1985a). In vitro incubation may not necessarily reflect conditions that obtain in the intact cell, but a low rate of sialylation of $LcOse_4Cer$ compared to fucosylation, under these experimental conditions, may explain why $IV^3NeuNAcLcOse_4Cer$ is only a minor component of carcinoma cells and suggest that the rate of biosynthesis of this recently reported ganglioside may be limiting to the synthesis of sialylated Lewis[a] (Ca 19-9 or gastrointestinal carcinoma associated antigen). Regulation of these pathways may also entail transport of $LcOse_4Cer$ and CMP–sialic into membrane subcompartments where these transferases are located, as well as the inherent kinetic properties of the transferase activities.

The sialyltransferase activity ($LcOse_4Cer$ acceptor) appears to be associated with a Golgi-enriched fraction that contains elements of the plasma membrane (density 1.16–1.18, Table III) (Liepkans & Larson, 1987). The bands visible after SDS–PAGE from the fractions obtained by $LcOse_4$ gel affinity chromatography (Figure 3, lanes 4 and 5) could include fucosyltransferases and lectins that recognize moieties of the lacto-*N*-tetraosyl sequence. If the bands at M_r 54 000–58 000 represent membrane proteins enriched by CMP affinity and $LcOse_4$ affinity (Figure 3), as well as enriched CMP–sialic:$LcOse_4Cer$ sialyltransferase activity (Table III), our estimate of the molecular weight of the sialyltransferase from SDS–PAGE is about 56 000. This is slightly higher than the molecular weight reported for the 2→3 sialyltransferase from porcine submaxillary glands (50 000) and significantly higher than that of the enzyme purified from rat liver (44 000) (Beyer et al., 1981; Weinstein et al., 1982). However, since high-sensitivity densitometry of the gel suggested more than one molecular species, the presence of other sialyltransferase activities, which we have been unable to detect in the double-affinity-purified fraction, cannot be excluded, for example, a 2→6 sialyltransferase (Weinstein et al., 1982). A minor band at M_r 27 000 may represent subunits of the major band at M_r 56 000.

Convenient and rapid isolation of purified carcinoma cell membrane transferases specific for core structures such as presented here may help resolve some of the inconsistencies arising from the notion of "tumor associated glycolipid antigens" in the future. The epitope specificities of monoclonal antibodies are not always immediately evident; nucleotide sugar transferases may, in many cases, be more specific than available monoclonal antibodies. Furthermore, since some glycolipid precursors may be "limiting substrates" for further glycosylation, they may not be accessible or in sufficient local concentration for antigen–Mab complex formation. Appropriate sugar nucleotide transferases are detectable in vitro by assay for their radiolabeled products even in picomole quantities. Further evidence for the structure of their products can be conveniently and rapidly obtained by analysis with an endoglycoceramidase and a series of sialidases, under controlled short-term incubations, with reference to authentic oligosaccharides, as presented in this paper.

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