Sialyltransferase of the 13762 Rat Mammary Ascites Tumor Cells

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ABSTRACT

The MAT-B1 and MAT-C1 ascites sublines of the 13762 rat mammary adenocarcinoma differ in morphology, agglutinability with concanavalin A, and xenotransplantability. Both cell lines contain a major mucin-type glycoprotein, but the MAT-C1 (xenotransplantable) subline contains a 3-fold-greater content of sialic acid on the glycoprotein than does the MAT-B1 (nonxenotransplantable) subline.

The present work indicates that whole cells of both lines incorporate radioactivity from labeled CMP-sialic acid into a component which comigrates with the major glycoprotein by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and that label incorporated by MAT-B1 cells is released by alkaline-borohydride treatment. Sialyltransferase can be purified from 250- to 400-fold by chromatography of a Triton X-100 extract of microsomes on CDP-agarose. The purified fraction of both cell lines has a $K_m$ for CMP-sialic acid of 0.40 ± 0.10 mw with asialofetuin as the acceptor, and gives 35 to 40% of the activity assayed with lactose yields only 3'-sialyllactose for both cell lines. The MAT-C1 extract showed higher sialyltransferase activity at a pH below 6.5 than did the MAT-B1 extract. Analysis of the products following incubation with lactose yields only 3'-sialyllactose for both cell lines. The results indicate that the differences in MAT-B1 and MAT-C1 sialyltransferase when assayed with glycoprotein acceptors are not large enough to account for the differences in sialic acid content of the two cell lines.

INTRODUCTION

Sialic acid, as a component of cell surface glycoproteins and glycolipids, is thought to play a role in cell-cell communication. For example, the presence of sialic acid increases the lifetime of circulating glycoproteins and cells by preventing uptake by the blood clearance system (6). The role of sialic acid in other biological processes, including cellular adhesiveness, binding of viruses to cells, activation of lymphocytes, and synaptic transmission, has been reviewed (6, 13). Studies have indicated that serum sialic acid levels are increased in cancer patients relative to healthy individuals (21), and the sialic acid content of tumor cells appears to correlate with metastatic ability (26). In mouse mammary tumor cells (TA3-Ha), a sialic acid-rich mucin, epigallocatechin, masks cell surface antigens, allowing the cells to grow in allogeneic hosts (5, 23). An understanding of the factors which regulate the content of cell surface sialic acid seems critical to understanding viral infection, the immune response, tumorigenesis, or metastasis at the molecular level.

The MAT-B1 and MAT-C1 sublines of the 13762 rat mammary adenocarcinoma are a suitable system for studying sialic acid metabolism. The 2 cell lines, originally derived from the same solid tumor, show marked differences in ability to be transplanted into mice, agglutinability with concanavalin A, and total sialic acid content (19). Greater than 70% of the protein-bound sialic acid in both cell lines is due to a high-molecular-weight mucin-type glycoprotein, ASGP-1 (16). The O-linked chains have a core structure Gal$(\beta 1\rightarrow 4)$GlcNAc$(\beta 1\rightarrow 6)[Gal(\beta 1\rightarrow 3)]$GlcNAc$3^\alpha$ where both galactose residues may be substituted with sialic acids linked $(\alpha 2\rightarrow 3)$. The MAT-C1 subline contains much more of disialylated hexasaccharide than does the MAT-B1 subline, whereas the MAT-B1 oligosaccharides are predominantly neutral but may contain sulfate as well as sialic acid (17). Thus, the sublines do not appear to differ in the amount of ASGP-1 but, rather, in the relative proportion of sialylated O-linked oligosaccharides in ASGP-1.

This report describes studies of sialyltransferase in MAT-B1 and MAT-C1 cell lines. Preliminary reports of this work have appeared (18, 20).

MATERIALS AND METHODS

All chemicals were reagent grade unless specified otherwise. [4-C$^{14}$]-CMP-N-acetylaneyracaminic acid, with specific activity of 1.6 mCi/mmol, was obtained from New England Nuclear. Unlabeled CMP-NeuAc was prepared by the method of Kean and Roseman (7) and was additionally purified by chromatography on Sephadex G-25 in ethanol:water (1:1). The glycoproteins fetuin and human transferrin were obtained from Sigma Chemical Co. and were desialylated by treatment with 0.05% H$_2$SO$_4$ for 1 hr at 80°, neutralized, dialyzed against distilled water, and lyophilized. OSM was prepared by the method of Carlson et al. (4) and was desialylated as described above. Galactosylated OSM was prepared by incubating asialo OSM with porcine submaxillary gland microsomes and UDP-galactose as described by Van den Eijnden et al. (22). Carbohydrate analysis of the various acceptors (12) indicated that the standard sialyltransferase assay (0.1 mg acceptor) contained asialofetuin (15.5 nmol galactose), asialotransferrin (0.67 nmol galactose), asialo-OSM (83.5 nmol GaINAc), or galactosylated OSM (17.2 nmol galactose and 77.6 nmol GaINAc).

Cells and Cell Fractions. MAT-B1 and MAT-C1 refer to ascites sublines of 13762 rat mammary adenocarcinoma which arose by passage of the MAT-B and MAT-C lines (Mason Research Laboratories, Worcester, MA) in the laboratory of K. L. Carraway. Tumor-bearing rats were kindly provided by K. L. Carraway, University of Miami School of Medicine. Cells were maintained by weekly transfer and through frozen stocks. The properties of these cell lines have been described previously (19). To ensure that no significant changes in the major glycoprotein had

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The abbreviations used are: GaINAc, N-acetylgalactosamine; OSM, ovine submaxillary mucin; Gal, galactose; GlcNAc, N-acetylglucosamine; SDS, sodium dodecyl sulfate; PBS, Dulbecco's phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 8.0 mM Na$_2$HPO$_4$, 1.44 mM KH$_2$PO$_4$, pH 7.4; NeuAc, N-acetylneuraminic acid.

occurred, ASGP-1 from each cell line was isolated, and the carbohydrate composition was determined (16). No significant differences in the current analysis and the findings reported previously were detected. In particular, the MAT-C1 glycoprotein still contained significantly more sialic acid (1.49 mol per mol of GalNAc) than did the MAT-B1 line (0.69 mol per mol of GalNAc).

Cells were washed and homogenized as described previously (16). Immediately after homogenization, the suspension was brought to a 3 mM concentration in MgSO4 by addition of 30 mM MgCl2 and 100 mM NaCl. The homogenate was centrifuged at 1,000 x g for 1 min, and the supernatant was centrifuged at 10,000 x g for 10 min. The supernatant following the second centrifugation is referred to as "crude extract," and the pellet following 100,000 x g x 60 min centrifugation of crude extract is referred to as "microsomes." Triton X-100 extracts of both fractions were prepared by incubation with 1% Triton X-100 and 10 mM Tris, pH 8.0, on ice for 30 min, and centrifugation at 100,000 x g x 60 min. Sialyltransferase Assay. The assay mixture contained the following components in a total volume of 70 μl: (a) [14C]CMP-A/-acetylneuraminic acid (63 nmol; 220,000 dpm); (b) sodium cacodylate buffer (10 nmol); (c) bovine serum albumin (20 μg); and (d) either a glycoprotein acceptor (0.1 mg; see above) or distilled water. The pH of the sodium cacodylate buffer was adjusted with acetic acid at room temperature prior to the assay, and the standard assay was performed in the presence of asialofetuin at a pH of 6.75.

The assay mixture was incubated at 37°C for 10 or 20 min, and the reaction was quenched with 1% phosphotungstic acid and 0.5 M HCl. Samples were filtered and counted as described by Paulson et al. (9) using a Beckman LS7500 scintillation counter. Under these conditions, at least 40% of the major glycoprotein ASGP-1 in both cell lines was precipitable. Duplicate values typically did not differ by more than 20%. Protein was estimated by the method of Lowry et al. (8).

Affinity Chromatography. CDP-agarose (6-carbon spacer; Sigma), which had been equilibrated with 10 mM sodium cacodylate, pH 6.5:50 mM NaCl:1% Triton X-100 (Buffer A), was used as the affinity absorbent. Crude extract or microsomal extract from approximately 1 to 2 x 109 ascites cells (25 to 40 ml; protein, 5 to 10 mg/ml) was mixed with 5 to 10 ml of CDP-agarose, allowed to stand at 4°C for 3 hr, and packed in a 0.7-cm column. Following elution of the sample, the column was washed with 10 bed-volumes of 10 mM sodium cacodylate, pH 6.5:50 mM NaCl:1% Triton X-100 (Buffer B) and 3 to 5 bed-volumes of 10 mM sodium cacodylate, pH 6.5:100 mM NaCl:0.3 mM CTP:1% Triton X-100 (Buffer C). Fractions of 1.5 ml were collected and assayed for sialyltransferase activity and protein.

Characterization of Endogenous Acceptor of Sialyltransferase. Ascites cells were washed 3 times with PBS and 1 x 109 cells in a total volume of 0.25 ml were incubated in PBS with 0.4 μmol each of MnCl2 and MgCl2 with [3H]CMP-sialic acid (2.3 μCi; 1 nmol) at 37°C for 10 or 20 min. Cold PBS (0.2 ml) was added, and the sample was immediately solubilized by addition of 0.4 ml of sample buffer (0.25 M Tris, pH 6.8:3% SDS:5% β-mercaptoethanol:10% glycerol) and immersion in a boiling water bath. Labeling of cells by periodate:[3H]borohydride, SDS:polyacrylamide gel electrophoresis, and fluorography were performed as described previously (19).

In a separate experiment, MAT-B1 ascites cells were incubated with [3H]CMP-sialic acid (0.3 μCi; 0.19 μmol) for 20 min under the conditions described above, and were solubilized with sample buffer. The sample was chromatographed on a Sephadex G-50-40 column (1.0 x 40 cm) in 0.5% deoxycholate:10 mM Tris, pH 8.0, to separate the product from unreacted [3H]CMP-sialic acid. Void volume fractions were pooled, diazoylated/concentrated against distilled water with a colloidion bag apparatus, treated for 16 hr at 45°C with 0.05 M NaOH and 0.01 M NaBH4, and chromatographed on a Bio-Gel P-4 column (1 x 100 cm) in 0.1 M pyridine acetate, pH 6.0. For comparison, MAT-B1 ASGP-1 was purified from cells which had been labeled with [3H]glucosamine (16) and chromatographed on the same Bio-Gel P-4 column following alkaline-borohydride treatment.

Analysis of Sialyllactose Product. Assay mixtures containing the acceptor lactose (58 mg/ml) were prepared as described above, except that a 3-fold-greater volume of all reagents was used. Following a 20-min incubation of 37°C and heat inactivation, the samples were treated as described by Paulson et al. (10); i.e., the reaction was quenched by addition of 1 ml of cold 5 mM sodium phosphate, pH 6.8. The mixture was applied to a small column of Dowex 1-X8 (phosphate) to remove unreacted CMP-sialic acid. The eluate was applied to a Dowex 1-X8 (acetate; 1 ml) column; washed with 2 ml of water; eluted with 2 ml 0.5 M pyridine acetate, pH 5.0; and lyophilized. The sample was taken up in 25 μl of distilled water containing 100 nmol sialylactose (mixed isomers) and 25 nmol N-acetyllactosaminic acid, and the entire sample was injected.

Separation of the oligosaccharides was performed on a Waters Model 272 liquid chromatograph with Model 480 variable-wave-length detector. A column of Bondapak NH (Waters radial cartridge) was used for all separations with conditions as described by Bergh et al. (2). Separation of sialylactose isomers was performed isocratically with acetonitrile:15 mM potassium phosphate, pH 5.2 (72:28) with a flow rate of 2 ml/min. The absorbance of the eluent was monitored at 195 nm. Beginning at 10 to 15 min postinjection, fractions were collected at 0.5-min intervals. The absorbance trace was aligned with the radioactivity profile by injection of the standard [14C]NeuAc.

RESULTS

Endogenous Acceptor of Cellular Sialyltransferase. Incubation of MAT-B1 and MAT-C1 cells with [3H]CMP-sialic acid resulted in incorporation of H into material precipitable with 1% phosphotungstic acid:0.5 M HCl. Preliminary experiments demonstrated that the incorporation is linear for up to 20-min incubation, and that the endogenous activity of MAT-B1 cells is greater than that of MAT-C1 cells (3.2 versus 1.0 pmol/min/mg cell protein). The endogenous activity decreased 3- to 10-fold by the addition of 1% Triton X-100.

Analysis of the endogenous acceptor by SDS:polyacrylamide gel electrophoresis (Fig. 1) indicated that the major acceptor in both MAT-B1 and MAT-C1 cells was a high-molecular-weight component with the same mobility as the major glycoprotein, ASGP-1. Further analysis of the MAT-B1 acceptor by chromatography of the alkaline-borohydride-treated product (Chart 1) showed that radioactivity from [14C]CMP-sialic acid had been incorporated into alkali-labile chains.

Activity of Various Fractions Following Cell Homogenization. Homogenates and fractions from MAT-B1 and MAT-C1 cells were assayed for sialyltransferase activity at pH 6.75 in the presence of Triton X-100 with asialofetuin as the acceptor. Typically, the incorporation with homogenates of both cell lines was not stimulated by the addition of asialofetuin, whereas microsomes gave a specific activity of 12.5 (MAT-B1) or 11.9 (MAT-C1) pmol/min/mg with asialofetuin when corrected for endogenous activity. At least 50% of the activity remained in the supernatant during the centrifugation used to pellet the microsomes. Thus, further studies were performed on microsomal and cytosolic material.

Affinity Chromatography on CDP-Agarose. The pattern shown in Chart 2 was obtained when crude extract from MAT-B1 cells was chromatographed on CDP-agarose. Essentially 3 peaks of activity were observed: (a) Peak I, eluting with 1 to 2 bed volumes of 50 mM NaCl; (b) Peak II, eluting with 8 to 10 bed-volumes of 50 mM NaCl; and (c) Peak III, eluting with 2 to 3 bed volumes of 0.1 M NaCl:0.3 mM CTP. These same 3 peaks of activity were observed for virtually all of the samples examined, and the relative recoveries of activity in the 3 peaks were 15 to
30% (Peak I), 5 to 15% (Peak II), and 3 to 10% (Peak III). No significant differences were detected between microsomes and crude extract or between fractions from MAT-B1 and MAT-C1 cells. Peak III had a specific activity of 3200 (MAT-B1) or 5400 (MAT-C1) pmol/min/mg protein, showing a 250- to 450-fold purification from microsomes. The $K_m$ for CMP-sialic acid with asialofetuin as the acceptor was in the range of 0.4 ± 0.1 mM for Peak III from both MAT-B1 and MAT-C1 cells. Peak III from both cell lines also showed activity toward the acceptor asialotransferrin, yielding 35 to 40% of the activity obtained with asialofetuin.

Activity with Various Acceptors as a Function of pH. Crude extracts were tested with various acceptors to determine whether the MAT-B1 and MAT-C1 sialyltransferases differed in specificity. With asialofetuin as the acceptor (Chart 3A), the extracts from both cell lines showed a broad plateau of activity from pH 6.0 to 7.5. Asialofetuin, which contains 3 O-linked oligosaccharides [Gal(β1→3)GalNAc] and 3 triantennary N-linked oligosaccharides [each with nonreducing terminus [Gal(β1→4)GlcNAc−)], can serve as an acceptor for a variety of sialyltrans-
ferases. The product formed by incubation (pH 6.75) of MAT-B1 crude extract with [14C]CMP-sialic acid and asialofetuin was alkaline-borohydride-treated and chromatographed on Bio-Gel P-4. Most of the radioactivity appeared in the void volume, indicating that incorporation was predominantly onto N-linked chains (data not shown). With the mucin acceptors, OSM (Chart 3C) and galactosylated OSM (Chart 3D), the MAT-B1 extract showed optimal activity around pH 7.5, whereas the MAT-C1 extract gave moderate activity from pH 6.0 to 7.5. OSM contains predominantly the side chain GalNAc, whereas galactosylated OSM contains GalNAc (78%) and Gal(β1→3)GalNAc (22%). Analysis of the products with OSM as the acceptor was performed by high-performance liquid chromatography following alkaline borohydride treatment (2, 15). None of the product NeuAc(α2→6)GalNAc was detected, although the formation of Gal(β1→3) [ NeuAc(α2→6)] GalNAc could not be ruled out by the analysis. Thus, it is likely that, with OSM, the Gal(β1→3)GalNAc chains preferentially serve as the acceptor, even though these chains comprise only about 5% of the total. With asialotransferrin, which contains N-linked chains terminating in Gal(β1→4)GlcNAc, both cell lines showed moderate activity, with the MAT-C1 extract more active at low pH than the MAT-B1 extract (Chart 3E).

Lactose as the Acceptor. Since the sialyltransferase from both cell lines showed activity with both N-linked and O-linked chains, it was possible that enzymes capable of forming 2 types of linkages to galactose ([α2→3] and [α2→6]) were present in the cell extracts. To test for these 2 classes of enzyme, microsomes were incubated with lactose and [14C]CMP-sialic acid, and the products were analyzed by high-performance liquid chromatography. The results (Chart 4) demonstrate that, for both cell lines at pH 6.55 and pH 7.40, 3'-sialyllactose is the only isomer formed. Furthermore, both cell lines incorporated more radioactivity (1.5 to 2 times as much) at pH 7.4 than at pH 6.55.

Incorporation of radioactivity from [14C]CMP-sialic acid into sialylactose was determined as a function of pH for MAT-B1 and MAT-C1 microsomes, using the method of Paulson et al. (10). The results confirmed the finding that both cell lines incorporated approximately twice as much radioactivity at pH 7.4 than at pH 6.55, although no differences in the specific activity of MAT-B1 and MAT-C1 microsomes toward lactose were detected (data not shown).

**DISCUSSION**

The results demonstrate that, with asialofetuin as the acceptor, no significant differences in MAT-B1 and MAT-C1 sialyltransferase could be detected by: (a) specific activity of microsomes; (b) CDP-agarose chromatography; or (c) activity and K_m for CMP-sialic acid of a partially purified sialyltransferase.

Although MAT-C1 sialyltransferase appeared more active than the MAT-B1 enzyme at a pH of 6.5 to 6.7, it is unclear whether this difference could result in increased sialylation under physiological conditions. With respect to specificity of the tumor cell sialyltransferases, both N-linked (asialotransferrin) and O-linked (OSM and galactosylated OSM) glycoproteins serve as acceptors. Neither cell line catalyzes the transfer of sialic acid to GalNAc side chains of OSM, and the sole product with lactose as the acceptor is 3'-sialyllactose.

Previous work has shown that rat mammary gland contains a sialyltransferase which forms 3'-sialyllactose (3). However, this enzyme was less active with orosomucoid than with lactose and was not active with sialidase-treated submaxillary mucins (3). It is unlikely that an enzyme with the same properties as normal rat mammary gland sialyltransferase participates in the synthesis of such large amounts of mucin as are found in the MAT-B1 and MAT-C1 cells.

It is also unclear as to whether one enzyme catalyzes the transfer of sialic acid to both the Gal(β1→3)GalNAc and Gal(β1→4)GlcNAc termini of ASGP-1. The Gal-R(α2→3) sialyltransferase active on the former acceptor was purified from porcine submaxillary glands (14) and was shown to be minimally active with the Gal(β1→4)GlcNAc termini of N-linked glycoproteins (11). Similarly, the Gal-R(α2→3) enzyme active on the latter acceptor as part of an N-linked oligosaccharide has been purified from rat liver (24) and shown to be inactive on antifreeze glycoprotein which contains Gal(β1→3)GalNAc (25). Furthermore, a sialyltransferase in embryonic chicken brain catalyzes the transfer of sialic acid to both glycolipid termini Gal(β1→4)GlcNAc and Gal(β1→3)GalNAc, but is not active on the O-linked Gal(β1→3)GalNAc found in mucin (1). Thus, it appears that either the MAT-B1 and MAT-C1 sialyltransferase is of unusually broad specificity, accepting lactose and O-linked termi Gal(β1→3)GalNAc and Gal(β1→4)GlcNAc, or that different enzymes are required in the synthesis of ASGP-1.

The present work suggests that, with the acceptors used, no dramatic differences in sialyltransferase activity were detected between the MAT-B1 and MAT-C1 cell lines. Other aspects of sialic acid metabolism, including CMP-sialic acid synthetase and neuraminidase, are currently under investigation.

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