Identification of Further Elongation and Branching of Dimeric Type 1 Chain on Lactosylceramides from Colonic Adenocarcinoma by Tandem Mass Spectrometry Sequencing Analyses

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Mammalian glycan chain elongation is mostly based on extending the type 2 chain, Galβ1–4GlcNAc, whereas the corresponding type 1 chain, Galβ1–3GlcNAc, is not normally extended. In a broader context of developing high sensitivity mass spectrometry methodologies for glycomic identification of Lea versus Leb and linear versus branched poly-N-acetyllactosamine (polyLacNAc), we have now shown that the dimeric type 1 glycan chain, as carried on the lactosylceramides of a human colonic adenocarcinoma cell line, Colo205, not only can be further extended linearly but can likewise be branched at C6 of 3-linked Gal in a manner similar to polyLacNAc. A combination of chemical and enzymatic derivatization coupled with advanced mass spectrometry analyses afforded unambiguous identification of a complex mixture of type 1 and 2 hybrids as well as those fucosylated variants found exclusively on linear and branched trimeric type 1 chain. We further showed by in vitro enzymatic synthesis that extended type 1 and the hybrid chains can be branched by all three forms of the human 1 branching enzymes (IGnT) currently identified but with lower efficiency and stringency with respect to branching site preference. Importantly, it was found that a better substrate is one that carries a Gal site for branching that is extended at the non-reducing end by a type 2 and not a type 1 unit, whereas the IGnTs are less discriminative with respect to whether the targeted Gal site is itself β3- or β4-linked to GlcNAc at the reducing end.

Type 1 (Galβ1–3GlcNAc) and type 2 (Galβ1–4GlcNAc) glycan chains represent the two most common peripheral back-bone sequences extending from the core structures of mammalian glycoconjugates to which linkage-specific additions of terminal fucose, sialic acid, and other glycosyl residues give rise to a variety of commonly found bioactive epitopes. Extension of type 2 chain with additional Galβ1–4GlcNAc (LacNAc) disaccharide units into polylactosaminoglycans (polyLacNAc) occurs readily with and without further branching (1, 2) and is often associated with onco-developmental activation (3, 4). In contrast, the type 1 disaccharide units are usually found attached directly to the glycan core or non-reducing termini of type 2 chains (5, 6) and not further elongated.

Among the first reported incidences of extended type 1 chains are those constituting the lacto series glycosphingolipids (GSL) of human meconium, which could be extended by one or two additional type 2 disaccharide units with varying degrees of fucosylation (7). A decade later, in the early 90s, fucosylated dimeric type 1 chains on lactosylceramides were identified as tumor-associated antigens in the form of Leα-Lea and Lea-Lea (8, 9). The colonic adenocarcinoma cell line, Colo205, was thought to exhibit such unusual glyco-phenotypes due to its abnormally high β3-galactosyltransferase (β3GalT) activities (10, 11) coupled with possibly a novel or up-regulated β3-N-acetylgalcosaminyltransferase (β3GnT) activity (12). More recently, another rare occurrence of extended type 1 chain was reported for the GSLs isolated from the small intestine of an individual who is of blood group O, Le(a-b-) nonsecretor status (13). Non-fucosylated dimeric type 1 chain on lactosylceramide was found to carry an additional fucosylated type 2 branch. This suggests that although the fucosylated dimeric type 1 chain, Leα/Lea, may be truly tumor-associated, type 1 chain exten-

2 The abbreviations used are: LacNAc, Galβ1–4GlcNAc; polyLacNAc, poly-N-acetyllactosamine; β3GnT, β3-N-acetylgalcosaminyltransferase; β3GalT, β3-galactosyltransferase; β4GnT, β4-galactosyltransferase; Fus, fucose; GSL, glycosphingolipid; Hex, hexose; HexNAc, N-acetylatedhexosamine; IgMt, β3-N-acetylgalcosaminyltransferases; LacCer, Galβ1–4–Glc (Lac) ceramide; NeuAc, N-acetylenuraminic acid; type 1, Galβ1–3GlcNAc; type 2, Galβ1–4GlcNAc; CID, collision induced dissociation; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; QIT, quadruple ion trap; Q/TOF, quadruple-time of flight; TOF/TOF, tandem time of flight; mAb, monoclonal antibody.
Extension and Branching of Dimeric Type 1 Chain

sion itself, either with another type 1 or type 2 unit, may have a wider occurrence on normal human tissues. It can be associated with a particular genetic background, developmental stage, or activation process whereby glycosyltransferases normally responsible for capping off type 1 chains are down-regulated coupled with elevated basal activities of β3GnT and β3GalT.

A true evaluation of the occurrence and functions of extended type 1 chain is nonetheless difficult, largely hindered by a lack of sensitive detection methods either by analytical chemistry approach or biological probes such as monoclonal antibodies with strict specificity. However, recent advances in mass spectrometry (MS) analysis have opened up new possibilities in revisiting these issues. In particular, efficient MALDI-MS/MS utilizing both low and high energy collision induced dissociation (CID) is now possible at sufficiently high sensitivity to allow meaningful analysis of real biological samples (14).

We now show that further extension or branching of dimeric type 1 chain carried on the lactosylceramides of Colo205 is possible. MS/MS fragmentation pattern was established from previous and this work, in which the signal assignment was validated by comparative analysis of authentic standards synthesized in vitro by enzymes. We further demonstrate that the human 1 branching β6-N-acetylgalactosaminyltransferase (IGnT) is capable of transferring a GlcNAc onto the branch point of an extended type 1 chain albeit with significantly lower efficiency. Interestingly, by testing against all four possible combination of dimeric type 1 and type 2 chains as acceptors, we show that branching is significantly more efficient if a terminal type 2 and not type 1 unit is at the non-reducing terminus of the Gal and less dependent on whether the Gal itself is β3- or β4-linked to GlcNAc at the reducing end.

EXPERIMENTAL PROCEDURES

Extraction and Fractionation of GSLs—The Colo205 cells (ATCC no. CCL-222) were cultured in RPMI 1640 medium containing 10% fetal calf serum in a 5-liter spinner. GSLs were extracted from harvested cells by 15 volumes of 55:25:20 (v:v:v) isopropanol:hexane:water and then subjected to a Folch partition in 4:2:1 (v:v:v) chloroform:methanol:water (8). The Folch upper layer containing the GSLs were dried and re-dissolved in 30:60:8 (v:v:v) chloroform:methanol:water for loading onto a DEAE column. Neutral GSLs were washed through and collected in 3 volumes of 30:60:8 (v:v:v) chloroform:methanol:water. Size fractionation of the neutral GSLs was performed on a silica gel column (1.6 x 5 cm) packed with latexbeads 6RS-8060 (Iatron Laboratories Inc.). The column was pre-equilibrated with 55:40:5 (v:v:v) isopropanol:hexane:water and stepwise eluted with decreasing ratios of 55:40:5 (v:v:v) to 55:20:25 (v:v:v) of isopropanol:hexane:water.

HPTLC of GSLs and Immunostaining—Neutral GSLs were spotted on HPTLC plate (silica gel 60 F254, Merck) with a mobile phase containing chloroform:methanol:water in a ratio of 50:40:10 (v:v:v). GSLs were stained by spraying 0.2% orcinol (Sigma) in 10% H2SO4 and incubated for 10 min at 110 °C in oven. For immunostaining, the HPTLC plate was first fixed with 0.5% polysobutylmethacrylate (GlycoTech, Gaithersburg, MD) in chloroform:hexane,1:9 (v:v), for 30 s followed by blocking for 10 min in 3% bovine serum albumin/phosphate-buffered saline. The plates were then washed by phosphate-buffered saline and incubated with primary antibodies CF4C4 (anti-Lea) and SH1 (anti-Leb) (both kindly provided by Glycogen Inc., Taiwan) at room temperature for 1 h followed by biotinylated secondary antibody at room temperature for 1 h. The plate was then further incubated with an avidin-biotin complex kit (Vector Laboratories, Burlingame, CA) at room temperature for 30 min to amplify signals from secondary antibody, followed by development with Immunostaining HRP-1000 kit (Konica Minolta).

Enzymatic Release, Digest, and Synthesis of Glycans—The glycan moiety of GSL was released from the ceramide core by digestion with 5 milliunits of ceramide glycanase (Calbiochem, catalog no. 219484) in 50 mM sodium acetate, pH 5.0, containing 0.1% sodium cholate at 37 °C overnight. The released glycans were separated from the ceramide, salt, and detergents by passing through a Sepaclean Envi-carb SPE tube (Supelco) pre-conditioned in the sequential order of 1 N NaOH, water, 30% acetic acid, water, 75% acetonitrile + 0.1% trifluoroacetic acid, and water. After extensive washing with water, the glycan sample was eluted with 3 ml of 25% acetonitrile, 0.1% trifluoroacetic acid.

The released glycans were first defucosylated by 48% aqueous hydrofluoric acid at 0 °C in the dark for 48 h before glycosidase digestions. β1–3-Galactosidase (10 milliunits, from recombinant Escherichia coli, Calbiochem, catalog no. 345795) digestion was performed at 37 °C for 16 h in 50 mM sodium acetate, pH 4.5, and endo-β-galactosidase (5 milliunits, from Escherichia freundii, Seikagaku catalog no. 100455) digestion was performed at 37 °C for 16 h in 50 mM sodium acetate, pH 5.8. Recombinant α1–3fucosyltransferase from Helicobacter pylori strain NCTC11639 was kindly provided by Dr. Chun-Hung Lin (Institute of Biological Chemistry, Academia Sinica, Taiwan) (15). The fucosylation reaction was carried out in 50 mM Tris-HCl, pH 7.5, containing 1 mM MnCl2, and 1 mM GDP-fucose (50 nmol).

For in vitro enzymatic synthesis of glycans with hybrid type 1 and type 2 chains, 20 μm lacto-N-tetraose and lacto-N-neotetraose (Calbiochem) were separately incubated with recombinant human β3-N-acetylgalactosaminyltransferase 2 (β3GnT2) in 25 mM HEPES buffer, pH 7.0, containing 10 mM MnCl2, and 2 mM UDP-GlcNAc at 37 °C for 24 h. The reactions were then terminated by boiling at 100 °C for 5 min. Subsequently, one half of each product was incubated with recombinant human β3-N-acetylgalactosaminyltransferase 5 (β3GalT5), and the other half was incubated with recombinant human β4-galactosyltransferase 1 (β4GalT1) along with 1 mM UDP-Gal for another 24 h at 37 °C. Glycans with linear extended type 1 or type 2 chains were synthesized by incubation with β3GalT5 and β3GnT2 or with β4GalT1 and β3GnT2, respectively, in 25 mM HEPES buffer, pH 7.0, containing MnCl2, UDP-GlcNAc, and UDP-Gal as described above. For the 1-branching β6-N-acetylgalactosaminyltransferase (IGnT) assay, the glycan chains were similarly incubated with recombinant human IGnT1–3 (16) in 25 mM HEPES containing 10 mM EDTA and 5 mM UDP-GlcNAc. All
recombinant human glycosyltransferases were produced in HEK293T cells and were used as described (17). On completion, the reaction mixtures were spun through an Ultrafree-MC filter unit (0.22 μm, polyvinylidene difluoride, Millipore), and the synthesized free glycans along with any unreacted acceptor were recovered from the filtrate by passing through the Envi-care SPE tube, as described above.

Fractionation and Purification of Trimeric Type 1 Chain—HPLC purification of the glycans released from GSLs was performed on a normal phase Type N PALPAK column (Takara, 250 × 4.6 mm). Buffer A was 75% acetonitrile in 200 mM triethylamine, and buffer B was 50% acetonitrile in 200 mM triethylamine. The sample was eluted at a flow rate of 0.8 ml/min with the program set at isocratic elution in 100% solvent B for 5 min, followed by a stepwise linear gradient to 50% solvent B in 5 min, to 85% solvent B in 40 min, and finally to 100% solvent B in 10 min. Aliquots from every five fractions were combined and screened by MS to locate eluted glycans. (Gal-GlcNAc)₃Lac was eluted at 22–24 min by this program.

Quantification of IgnT Transcripts—Total RNA of the Colo205 colon cancer cell line was prepared using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). The first-strand cDNAs were primed using oligo-dT primer and synthesized by SuperScript III reverse transcriptase (Invitrogen). The forward IAF16 (5'-GACACTCAACAGATTCCGGTGTTCC-3'), nucleotides +906 through +932 of exon 1A of the IgnT gene, translation initiation codon as nucleotides 1 +1 to 3), ICF18 (5'-CCAGTGTCCAAGGACACTTTCAGTCTGA-3', nucleotides +902 through +929 of exon 1B of the IgnT gene), and ICF20 (5'-CCTTAAATAGGTTTCAGGTTTCCCTGG-3', nucleotides +908 through +935 of exon 1C of the IgnT gene) primers were used to amplify IgnTA, IgnTB, and IgnTC cDNAs, respectively, by PCR. The reverse primer used, IRs (5'-CCAGCCTGGGTGTATCCGACAG-3', complementary to the sequence locating in exon 3 of the IgnT gene), was common for the 3 IgnT cDNAs. PCR for the β-actin cDNA was performed using the ACTF (5'-GCACACGGGGCTGATG-3') and ACTR (5'-GCTCCGGTCAGCAGCA-3') primers. The cDNA sample and 2 pmol of each forward and reverse primer were added to 20 ml of the SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR detection (GenAmp 7300 Sequence Detection System, Applied Biosystems) was used for quantification of the transcripts. Serial dilutions of the pGEM-T vector (Promega Co., Madison, WI) containing IgnTA, IgnTB, IgnTC, or β-actin cDNAs were used to generate standard curves for each transcript. The IgnTA, IgnTB, and IgnTC genes as described (18) encode for the IgnT1, IgnT1, and IgnT3 enzymes (19), respectively, used in this work.

MS and MS/MS Analysis—All glycan samples were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell et al. (20). Briefly, NaOH pellets were ground in DMSO until a slurry was formed. 0.5 – 1 ml of the DMSO/NaOH slurry was added to glycans dried down in screw-capped glass tubes followed by about 0.2 ml of methyl iodide (Merck). The reaction mixtures were vortexed and placed on the automatic shaker for 20 min at room temperature, after which the reaction was terminated by the addition of 1 ml of water. Permethylated sample was then extracted into 1 ml of chloroform and washed several times with water.

For MALDI-TOF MS profiling, permethylated samples were dissolved in acetonitrile and mixed 1:1 with 10 mg/ml 2,5-dihydroxybenzoic acid (Sigma) in acetonitrile for spotting onto the target plate. Data acquisition was performed on either a benchtop MALDI LR system (Waters Micromass) or a 4700 Proteomics Analyzer (Applied Biosystems), both operated in the reflectron mode.

MALDI-MS/MS analyses of permethylated glycan and GSL samples at low collision energy were performed on a Q/TOF Ultima™ MALDI (Waters Micromass) using α-cyano–4-hydroxycinnamic acid as matrix (5 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid mixed 1:1 with sample dissolved in acetonitrile). Argon was used as the collision gas with a collision energy manually adjusted between 100 and 200 V to achieve an optimum degree of fragmentation. Alternatively, multistage low energy CID-MSⁿ of the permethylated glycans were acquired on an AXIMA™-QIT (Kratos Analytical, Shimadzu) using 2,5-dihydroxybenzoic acid as a matrix as described (21, 22). High energy CID MS/MS analyses were performed on the 4700 Proteomics Analyzer TOF-TOF system using 2,5-dihydroxybenzoic acid as matrix. The potential difference between the source acceleration voltage and the collision cell was set at 3 kV. The indicated collision cell pressure was increased from 3 × 10⁻¹⁸ (no gas) to around 2 × 10⁻¹⁰ torr by letting in argon as the collision gas. All CID MS/MS data were acquired and processed manually.

RESULTS

The Overall Profile and Characteristic Patterns of GSLs from Colo205—Multifucosylated variants of extended type 1 chain were first identified in the form of Le⁵-Le⁷ and Le⁴-Le⁷ on GSLs isolated from tumors in nude mice implanted with Colo205 (8, 9). Although it was evident from HPTLC immunostaining that smaller and larger Le⁴-carrying GSLs were present (Fig. 1A, inset), a more definitive overall profile such as one that would be afforded by MS analysis was not presented. In this work, total and further fractionated GSLs similarly extracted from harvested Colo205 cell cultures were mapped by MALDI-MS before and after permethylation (Fig. 1A). Based on the mass shifts observed after derivatization and further MS/MS analyses, the GSLs detected can in general be defined as Fuc₃,(Hex-HexNAc)₇₋₉-LacCer, where (Hex-HexNAc)nₙ-LacCer refers to either type 1 or 2 units, and LacCer refers to Galβ1–4-Glc (Lac) linked to the ceramide (Cer) core (Table 1).

As expected, the Colo205 GSLs MS profile is clearly dominated by signal clusters corresponding to multiply fucosylated (Hex-HexNAc)n₋₉-LacCer. The larger and those sialylated ones were visibly present but would become more apparent only after charge and size fractionation (Fig. 1, A and B, upper spectra). Within the signal clusters, mass differences of 14 (CH2)n or 30 (OCH2) mass units indicated heterogeneity in the fatty acyl chain length and the additional presence of hydroxyl group, respectively, whereas 142 mass units is a common mass difference corresponding to a C₁₆:₀ to hydroxylated C₂₄:₀ substitution. Removal of the highly heterogeneous ceramide moity by
ceramide glycanase yielded a much simplified MS profile with distinctive \([M + Na]^+\) molecular ion signals for the released glycan structures that share the backbone composition, (Hex-HexNAc),-Lac (Fig. 1B, Table 1). Thus, the major signal at \(m/z\) 1100 corresponds to permethylated Fuc_c(Hex-HexNAc)_1-Lac, and those at \(m/z\) 1723 and 1897 correspond to permethylated Fuc_c,2,3(Hex-HexNAc)_2-Lac, which are consistent with previous identification of Leb_c-Lea-LacCer as the characteristic GSLs of Colo205. Importantly, the data also unambiguously revealed the presence of Fuc_c,2,4(Hex-HexNAc)_3-Lac, and those at \(m/z\) 2173 and 1897 correspond to permethylated Fuc_c,2,3(Hex-HexNAc)_3-Lac, which are consistent with previous identification of Leb_c,-Lea,-Leb_c, or Le_c.

**MALDI MS/MS Characteristics for Identification of Type 1 and 2 Chains**—We have previously demonstrated that the low and high energy CID MALDI MS/MS fragmentation pattern of complex glycans as acquired on the Q/TOF and TOF/TOF, respectively, are highly complementary. Although the overall sequence, branching pattern, core type, and terminal epitope substituents of an \(N\)- or \(O\)-glycan were found to be best determined via low energy CID MS/MS due to its simplicity, high energy CID MS/MS on a TOF/TOF can provide additional linkage information through a more complicated cleavage pattern (14). In general, a type 1 chain or Le_c epitope is distinguished from a type 2 chain or Lex via the C3-linkage specific cleavage. Low energy CID MS/MS of permethylated glycans on a Q/TOF would readily afford a Z ion via elimination of glycosyl substituent at C3 position (6, 23). Thus, a Z ion corresponding to AGlCNac-R would be afforded by -Gal-3GlcNAc-R and not -Gal-4GlcNAc-R. In the current context, the presence of an internal Le_c unit will, therefore, be easily identified by the corresponding Z ion, which would not be observed if it is a Lex unit. This is schematically illustrated with the cleavage pattern afforded by low energy CID MS/MS on Le_b,-Le_c,-Lac versus Le_b,-Le_c-Lac (Fig. 2(i), MS/MS data are not shown). The former is distinguished by the additional presence of the...
TABLE 1
Molecular composition of major GSLs from Colo205

| [M+Na]+ of released glycan[^a] | Glycan composition | [M+Na]+ of corresponding GSL[^a] | Probable ceramide composition[^b] |
|-------------------------------|-------------------|---------------------------------|---------------------------------|
| 838.2                         | NeuAc-Lac        | 1401.4                          | d18:1 h16:0                     |
| 1100.3                        | (Fuc)₃(LacNAc)₂-Lac | 1543.6                          | t18:0 h24:1                     |
| 1461.4                        | NeuAc-(Fuc)₃(LacNAc)₂-Lac | 1635.7                          | d18:1 h16:0                     |
| 1723.7                        | (Fuc)₃(LacNAc)₂-Lac | 1693.7                          | d18:1 h16:0                     |
| 1897.8                        | (Fuc)₃(LacNAc)₂-Lac | 1775.8                          | d18:1 h24:0                     |
| 2173.0                        | (Fuc)₃(LacNAc)₂-Lac | 1850.8                          | t18:0 h24:1                     |
| 2347.2                        | (Fuc)₃(LacNAc)₂-Lac | 1994.9                          | d18:1 h16:0                     |
| 2493.3                        | (Fuc)₃(LacNAc)₂-Lac | 2024.9                          | d18:1 h16:0                     |
| 2850.6                        | (Fuc)₃(LacNAc)₂-Lac | 2137.0                          | d18:1 h24:0                     |
| 2521.2                        | (Fuc)₃(LacNAc)₂-Lac | 2431.3                          | d18:1 h16:0                     |
| 2278.1                        | (Fuc)₃(LacNAc)₃-Lac | 2622.3                          | d18:1 h24:0                     |
| 2237.0                        | (Fuc)₃(LacNAc)₃-Lac | 2706.5                          | d18:1 h16:0                     |
| 2562.4                        | (Fuc)₃(LacNAc)₃-Lac | 2736.5                          | d18:1 h16:0                     |
| 2992.8                        | (Fuc)₃(LacNAc)₃-Lac | 2850.6                          | d18:0 h24:0                     |
| 3022.8                        | (Fuc)₃(LacNAc)₃-Lac | 3052.8                          | t18:0 h24:1                     |
| 3472.1                        | (Fuc)₃(LacNAc)₄-Lac | 3502.1                          | t18:0 h24:1                     |
| 2399.2                        | (Fuc)₃(LacNAc)₃-Lac | 2603.4                          | t18:0 h24:1                     |
| 2850.6                        | (Fuc)₃(LacNAc)₃-Lac | 2992.8                          | t18:0 h24:1                     |
| 3472.1                        | (Fuc)₃(LacNAc)₄-Lac | 2736.5                          | t18:0 h24:1                     |
| 3502.1                        | (Fuc)₃(LacNAc)₄-Lac | 2562.4                          | t18:0 h24:1                     |
| 2907.0                        | (Fuc)₃(LacNAc)₄-Lac | 2706.5                          | d18:1 h16:0                     |
| 2992.8                        | (Fuc)₃(LacNAc)₄-Lac | 2736.5                          | d18:1 h16:0                     |
| 3022.8                        | (Fuc)₃(LacNAc)₄-Lac | 3052.8                          | t18:0 h24:1                     |
| 2521.2                        | (Fuc)₃(LacNAc)₅-Lac | 3169.6                          | d18:1 h24:0                     |
| 2622.3                        | (Fuc)₃(LacNAc)₅-Lac | 3288.0                          | d18:1 h24:0                     |
| 2278.1                        | (Fuc)₃(LacNAc)₅-Lac | 2706.5                          | t18:0 h24:1                     |
| 2796.5                        | (Fuc)₃(LacNAc)₅-Lac | 2992.8                          | t18:0 h24:1                     |
| 2278.1                        | (Fuc)₃(LacNAc)₅-Lac | 2736.5                          | t18:0 h24:1                     |
| 2562.4                        | (Fuc)₃(LacNAc)₅-Lac | 2706.5                          | d18:1 h16:0                     |
| 2796.5                        | (Fuc)₃(LacNAc)₅-Lac | 2992.8                          | t18:0 h24:1                     |
| 2562.4                        | (Fuc)₃(LacNAc)₅-Lac | 2706.5                          | d18:1 h16:0                     |

[^a]: The m/z values tabulated refer to the monoisotopic masses detected for each of the sodiated molecular ions afforded by the permethyl derivatives, as labeled on Fig. 1. m/z 828 was also detected as a major signal but not included in the mass range shown in Fig. 1B.

[^b]: The ceramide moieties (sphingosine and fatty acid) for the major GSLs detected were mostly inferred from the molecular masses and Karlsson and Larson (7) and Stroud (8). The stereochemistry and the location of additional degrees of unsaturation bonds and hydroxylation were not defined in this work. For more abundant peaks, direct MS/MS on the intact GSLs were also performed to ascertain the major isomeric forms. For the larger GSLs, only those eluted into the fraction used for subsequent analysis are tabulated. d18:1, (4E)-sphingine; t18:0, (4D)-hydroxypropylsphingine or phytosphingosine; h, hydroxyl form of the fatty acids.

Previously established fragmentation pattern (14), including the linkage-specific satellite ions arising from concerted elimination of glycosyl substituents around the ring, coined as E and G ions (24), and the 1,3X cross-ring cleavage ions formed at every glycosyl residues, which facilitate a complete sequencing from the non-reducing terminus. Another diagnostic ion identified is m/z 243, which corresponds to a C‘/Y ion formed through a glycosidic cleavage at the non-reducing terminal Gal in concert with loss of the glycosyl substituent at C2 and, therefore, indicative of Fuc-2Gal epitope as found in Le^b or Le^y.

Based on these characteristic fragment ions, MALDI-MS/MS analysis of the larger Fuc_2-Gal(Hex-HexNAc)_3-Lac structures derived from Colo205 GSLs showed that in addition to a linear backbone chain, ions indicative of a branched Hex-HexNAc-(Hex-HexNAc)Hex-HexNAc-Lac backbone could be detected for the under-fucosylated structures, the inner Hex-HexNAc unit of which was mostly not fucosylated (data not shown). More significantly, apart from heterogeneity in the degree of fucosylation, the linkage-specific Z ions indicative of type 1 chain could be detected for each of the three Hex-HexNAc units (Fig. 3). However, D ions indicative of the type 2 chain could also be detected at least for the terminal and middle Hex-HexNAc units. Collectively, the MS/MS data were, therefore, suggestive of the occurrence of a trimeric type 1 chain but did not allow its unequivocal identification among the isomeric mixtures comprising different permutation of type 1 and 2 hybrids. We, thus, set out to determine specifically if a dimeric type 1 chain can be further extended linearly or branched with another type 1 or 2 unit.

**Identification of Linear and Branched Trimeric Type 1 Chain**—Focusing on the type 1 and 2 backbone chains, the fraction highly enriched with Fuc_2-Gal(Hex-HexNAc)_3-Lac was first treated with ceramide glycanase as before followed by defucosylation with hydrofluoric acid to yield a single major component corresponding to (Hex-HexNAc)_3-Lac, as defined by MALDI-MS of the permethylated product ([M+Na]+ molecular ion at m/z 1824). This was then isolated by normal-phase HPLC and subjected to endo-β-galactosidase digestion to degrade any chain with an internal type 2 unit. Under the same experimental conditions, the enzyme was independently shown not to act on an internal type 1 unit as tested against an enzymatically synthesized authentic standard of a trimeric type 1 linear chain (data not shown). A major degradation product was obtained that was identified by MS/MS analysis of the permethyl derivative ([M+Na]+ at m/z 1171) as comprising two structural isomers corresponding to endo-β-galactosidase cleavages at the respective two internal type 2 units. Thus, the first product, Hex-HexNAc-Hex-HexNAc-Hex, indicates that a type 2 chain can be extended by a type 1 unit (Fig. 4A (I)), which was resistant to digestion. More importantly, the second product, HexNAc-Hex-HexNAc-Lac, shows that a type 1 chain can be extended by a type 2 unit (Fig. 4A (II)). The internal type 1 unit is critically identified by the Z and C ion pair at m/z 690 and 504, which unlike other ions, cannot arise from other cleavages.

The resistant, non-digested product carrying internal type 1 unit was then isolated from the degraded products by nor-
mal phase HPLC and incubated with α3-fucosyltransferase from *H. pylori*. Isomers with a non-reducing terminal type 2 unit, which is not susceptible to endo-β-galactosidase, and/or internal type 2 unit that was incompletely digested would be α3-fucosylated and, thus, shifted in molecular weight after the reaction. Indeed, major [M + Na]+ molecular ion signals corresponding to mono- (m/z 1998) and difucosylated (m/z 2172) products were detected by MS analysis of the permethyl derivatives (data not shown) along with the remaining unreacted (Hex-HexNAc)α-Lac (m/z 1824), which is, thus, by definition, resistant to both endo-β-galactosidase and α3-fucosyltransferase and corresponds to a structure with trimeric type 1 chain (Fig. 4B and the supplemental figure for a more detailed schematic elaboration of the overall workflow and rationale). This was further verified by high energy CID MALDI-MS/MS analysis, in comparison
with the original defucosylated sample not subjected to sequential enzymatic treatments, and an authentic trimeric type 1 linear chain was synthesized enzymatically in vitro (Fig. 5).

As expected, the [M+Na]+ of the permethylated (Hex-Hex-NAc)3-Lac (m/z 1824) from all three samples afforded a full complement of the 1,5X and E ions at each residue together with the B and Y ions at HexNAc and the C and Z ions at Hex, which are fully supportive of a trimeric type 1 linear chain. More importantly, the D ions (m/z 921 and 1370) attributed to internal type 2 unit, the 3,5A ion at m/z 329, and the H ions (m/z 1544, 1095, and 646) indicative of C4-linkage, which were present in the original samples (Fig. 5A), could no longer be detected in the treated sample (Fig. 5B) and standard (Fig. 5C).

Furthermore, in comparison with the synthesized trimeric type 1 linear chain standard, the original and treated samples clearly gave two additional fragment ions at m/z 676 (D ion) and 574 (3,5A ion), which are indicative of a co-existing branched structure, as schematically illustrated (Fig. 5 (I)). Further digestion of the enzyme-treated product with β1–3-galactosidase under conditions that preserved the enzyme specificity succeeded in complete removal of one and two terminal β3-Gal residues, which gave more evidence for the linear and branched trimeric type 1 chain structures, respectively. MS/MS analysis of the permethylated glycan is schematically illustrated based on an extended linear type 1 chain structure to accommodate for the Z ion series. As noted under “Results”, the data cannot resolve the co-existence of type 2 based Le’2 units, which were in fact shown to be present by additional MALDI-TOF/TOF analysis (data not shown). Incomplete fucosylation introduced further heterogeneity, but a similar conclusion can be drawn with respect to the coexistence of both type 1 and 2 units.

Collectively, the data thus conclusively demonstrated that, in Colo205, the dimeric type 1 chain carried on its LacCer can be further extended linearly or branched in a manner similar to polyLacNAc of type 2 chain. Although the dimeric structures were shown to be predominantly of extended type 1 chain, a significant proportion of the trimeric structures are based on a hybrid of type 1 and type 2 chains. Our MS-based analytical strategy additionally enabled an unambiguous identification of both linear and branched structures that consist exclusively of trimeric type 1 units among the isomeric mixtures. An accurate quantification of the relative abundance of each isomer detected is not possible by this approach. However, based on the normalized relative MS signal intensities of the recovered products, a rough approximation indicates that at least about 20% of the isolated trimeric structures was degradable by endo-β-galactosidase digestion, and another 20% of the resistant product can be further acted on by the α3-fucosyltransferase (Fig. 4B).

In Vitro Synthesis of Branched Dimeric Type 1 and 2 Chains and Their Structure Characterization—The identification of branched structures on a dimeric type 1 chain implies that either the currently known I branching enzymes, β6-N-acetylglucosaminyltransferases (IGNT, β6GnT), which normally act on type 2 polyLacNAc chains, can act on one that is based on a hybrid or an extended type 1 chain or that a novel glycosyltransferase activity has yet to be identified. To this end, all combination of linear dimeric type 1 and 2 chains and their hybrids were enzymatically synthesized in vitro and used as acceptor substrates to assay for the branching activities of IGNTs (Fig. 6). Based on MS detection and MS/MS analyses of the permethyl derivatives of the reaction products, it was found that the human IGNT1, IGNT2, and IGNT3 could transfer one or more
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GlcNAc residues to all the synthetic substrates albeit with different efficiencies.

Strikingly, acceptors with a non-reducing terminal type 2 unit served as better acceptors than those with a non-reducing terminal type 1 unit. Under the same reaction conditions, the former were mostly converted to products with one to three GlcNAc residues added (Fig. 6, A and C, m/z 1620, 1865, and 2110), whereas a significant proportion of the latter remained unreacted (Fig. 6, B and D, m/z 1375). The relative susceptibility to a higher degree of GlcNAc branching as detected by MS monitoring provided a convenient indicator of reactivity from which it can be inferred that the type 2-type 2-Lac sequence served as the best acceptor substrate followed by type 2-type 1-Lac, type 1-type 2-Lac, and type 1-type 1-Lac in the order of decreasing reactivity (Fig. 6E). For a dimeric type 2 chain, the product with a single GlcNAc residue added on was shown by MS/MS analysis to be branched mostly at the Gal of the internal -3Gal-4GlcNAc unit as expected (data not shown). However, the Gal at the reducing end Lac unit and, to a lesser extent, the terminal Gal at the non-reducing end, could also be acted on, thus, giving rise to a maximum of three GlcNAc residues added.

In contrast, branching site preference was less apparent for the single GlcNAc residue added onto an extended dimeric type 1 chain, MS/MS analysis of which afforded critical ions that are indicative of GlcNAc addition at all three possible Gal sites (Fig. 7). In particular, GlcNAc addition to the Gal of internal type 1 unit (I) is established by the double cleavage ions at m/z 717, 944, and 490 in both the MS2 and MS3 spectra. Additional GlcNAc at the Gal of Lac (II) is defined by the presence of B and Y (or C and Z) ion pairs at m/z 935 and 708, respectively. GlcNAc addition at the non-reducing end Gal (III) is identified by the C2 and B3 ions at m/z 504 and 731, respectively. The relative low intensities of these ions in both MS2 and MS3 spectra and the lack of the corresponding Z ions (m/z 1139 and 699) indicate that this isomeric product is likely to be least abundant, consistent with it being the least preferred site of GlcNAc addition. Furthermore, none of these site-specific fragment ions indicative of GlcNAc addition at the non-reducing end Gal or Gal of Lac was observed in the corresponding products with single GlcNAc residues added.
FIGURE 5. Sequencing of permethylated (Gal-GlcNAc)_3Lac derived from Colo205 GSLs by MALDI-TOF/TOF MS/MS. A, original (Gal-GlcNAc)_3Lac derived from the total pool. B, remaining (Gal-GlcNAc)_3Lac after endo-β-galactosidase and α3-fucosyltransferase reactions. C, in vitro synthesized, linear (Galβ1-3GlcNAcβ)_3Lac structure serving as standard. D, branched (GlcNAc)_2Gal-GlcNAc-Lac structure from B after further β3-galactosidase digestion. Signals indicative of type 2 chain and branched isomer could be detected in the original (Gal-GlcNAc)_3Lac sample (A) in addition to those derived from linear trimeric type 1 chain, as schematically illustrated (I). Assignment for cleavage ions was additionally illustrated and annotated in D for the further-digested product (II). All symbols used are as defined in Fig. 2.
addition from acceptors where the non-reducing terminal unit is a type 2 chain. The results, therefore, support the notion that a type 1 chain at the non-reducing end of the Gal makes it a less reactive acceptor substrate than that extended by a type 2 chain and, consequently, reduces the overall branching site preference.

FIGURE 6. Branching activities of IGnT against (Gal-GlcNAc)_2Lac substrates in vitro. Equal amounts of the four different synthetic acceptors representing type 1-type 1 (A), type 1-type 2 (B), type 2-type 1 (C), and type 2-type 2 (D) chains were treated with the same amount of the branching enzymes (IGnT1–3) under the same experimental conditions. The reaction products were permethylated after sample clean-up and profiled by MALDI-MS. Only the dataset corresponding to the activities of IGnT3 is shown. Relative abundance of each of the products with one or more GlcNAc added (annotated as +mGn) based on peak areas are normalized to generate a representation of the respective activity profile of IGnT1–3 against the four substrates in terms of % conversion of the initial substrate to the branched products (E). The relative abundance of each of the IGnT mRNA transcript/β-actin transcript expressed in Colo205 cells, as detected by reverse transcription-PCR (IGnT1, not detected; IGnT2, 2.13 × 10^{-4} ± 3.04 × 10^{-5}; IGnT3, 3.16 × 10^{-5} ± 5.61 × 10^{-6}), is shown in F.
All three human IGnTs (IGnT1–3) demonstrated similar substrate and branching site preference, as can be concluded from the MS/MS analyses, with the IGnT2 enzyme preparation marginally more reactive against the type 1-type 1- and type 1-type 2-Lac substrates (Fig. 6E). True specific activities were, however, difficult to define given the different combinations of products which can only be distinguished by MS and MS/MS analyses. In vivo, only the transcripts of IGnT2 and IGnT3 can be detected by reverse transcription-PCR, with the former expressed at 6.7-fold higher (Fig. 6F) and, thus, most likely account for the observed branching activities. In a separate set of experiments, a mixture of the in vitro synthesized dimeric, trimeric, and tetrameric linear type 2 chains were found to mostly have 2, 3, and 4 GlcNAc residues added by IGnT2, respectively (Fig. 8A). In each case, MS/MS analysis of the major products localized the extra site of branching to Gal of the Lac unit (data not shown). Under the same reaction conditions, a majority of the corresponding dimeric and trimeric linear type 1 chains synthesized remained unreacted, with a small portion receiving only a single GlcNAc residue (Fig. 8B).

DISCUSSION

It has been observed repeatedly that malignant transformed cells and metastatic tumor cells often make and present more polyLacNAc chains than the parent cells or low metastatic counterparts (25). Because a type 1 chain is not normally extended, most descriptions pertaining to the functioning of polyLacNAc chain, either linear or branched, are referred to type 2 chains. It should, however, be pointed out that whereas detection of polyLacNAc chains may not be too daunting, detailed structural delineation to ascertain the absence of co-existing or co-polymerized type 1 units is not an easy task. In the absence of data suggesting otherwise, the expression of extended type 1 chain appears to be rather restricted, which to a certain extent may simply be due to its being under-investigated systematically and specifically. This is not helped by lack of specific mAbs for a rapid screening of tissue expression. All reported anti-fucosylated extended type 1 chain mAbs currently available (8, 26) demonstrate a certain degree of cross-reactivities against fucosylated type 2 units.

An important consideration for the polyLacNAc chain is that it presents various fucosylated and/or sialylated antigens, not only at the non-reducing termini but also internal ones at high valency. In this context, a monomeric versus dimeric or oligomeric and a homotypic versus a hybrid of ±sialyl ± sulfo Leα and Leα on a linear versus branched backbone chains are unlikely to be functionally equivalent. Although the subtle differences may

FIGURE 7. The branching sites of the extra GlcNAc added in vitro by IGnT onto the linear extended type 1 chain. The MALDI-QIT/TOF MS2 fragment ion at m/z 1180 produced by the sodiated parent ion m/z 1620 of the permethyl derivatives (A) was further selected for MS3 analysis (B). A fragmentation pattern afforded by Q/TOF was found to be similar to that of Q/TOF, namely dominated by B, Y, C, and Z ions, as illustrated schematically in (I), (II), and (III), corresponding, respectively, to GlcNAc addition on the three probable Gal sites. Signals at m/z 1361, 898, and 449 correspond to loss of terminal GlcNAc from the molecular and primary fragment ions at m/z 1620, 1157, and 708, respectively. Additional non-assigned ions correspond mostly to a further loss of 74 mass units from those assigned, the origin of which is not clear. Symbols used are as defined in Fig. 2.
not be apparent when assayed with simplified model systems, there are now indications that our natural immune system is capable of surveying this difference and translates it into proper immuno-reactive consequences. In one notable example, the mannan-binding protein, a serum lectin associated with innate immunity, was shown to recognize specifically tandem repeats of terminal Leb and Lea, and not Lex/y, carried on multiantennary complex type N-glycans of SW116 cells, another human colorectal carcinoma (6). This mannan-binding protein and Leb/a-dependent cell-mediated cytotoxicity has also been reported for other colonic carcinomas including DLD-1 and Colo205 (27), although the implicated ligand and its carriers were poorly characterized.

Technically, it is a challenging task for any glycomic analysis to rival the resolving power of biological systems in distinguishing the occurrence and positioning of type 1 and 2 chains, which underpins the need in developing the enabling MS methodologies required for definitive structural studies at high sensitivity. In this work we have capitalized on previously established fragmentation pattern afforded by both low and high energy CID MS/MS (14) to identify ions that are indicative of type 1 and 2 linkages. A plethora of fragment ions can normally be detected that can unambiguously establish the chain sequence and branching position. However, only a handful of these ions are truly linkage-specific. A fundamental problem relates to situations when the characteristic ion signals for both type 1 and 2 units can be detected for each of the Gal-GlcNAc unit along the chain. It is not possible to resolve if a strictly type 1 or type 2 chain exists among the many permutation of hybrids. Because the overall aim in the current work was to ascertain if an extended dimeric type 1 chain can be further extended or branched, we have relied on a strategy that removed the isomers with internal type 2 units. A strictly extended type 1 chain would then be positively identified by the respective type 1-specific ions (C and Z ions by Q/TOF and also TOF/TOF) in conjunction with not detecting the type 2-specific ions (D ions by TOF/TOF).

Using this analytical strategy, we provided convincing structural evidence for the occurrence of extended linear and branched trimeric type 1 chains as well as the hybrids. Although it is not unusual for a single type 1 unit to be found extending from type 2 LacNAc or polyLacNAc chains, giving rise to terminal Leb or sialyl Lea carried on a type 2 chain (5, 6, 28), the reverse is rather uncommon (7). Similar hybrid structures were also found among the LacCer with tetrameric GalGlcNAc units using similar analytical approach. In contrast to the presence of only a small quantity (5–10%) of type 2 chain in the more abundant dimeric species (8), our current data indicated that the proportion of the type 2 chain in those larger GSLs is significantly higher (up to 40% or more), most
of which constitutes hybrids of type 1 and 2 chains in either direction.

*Ad priori,* elevated expression of type 1 chain would necessitate enhanced or up-regulated activity of β3GalT because is the enzyme that is responsible for making a type 1 unit by adding a β-galactosidase to the 3-position of a β-GlcNAc, in competition against the more ubiquitous β4GalT. This has indeed been elegantly demonstrated by Holmes *et al.* for Colo205 (10). The β3GalT activity was detectable in only a few cell lines, such as Colo205 and SW403 (10, 11), whereas the β4GalT activity was found to be highly expressed in all tumor cell lines as well as normal colonic epithelial HCMC cells. More recently, Colo205 had further been shown to express high amount of β3GalT5 transcript relative to β4GalT1 (11). Thus, the synthesis of type 1 unit itself does not appear to be rate-limiting or responsible for the occurrence of extended type 1 chains in cell lines such as Colo205, which is endowed with abnormally high β3GalT activity. For a type 1 unit to be further extended, its terminal Gal needs to be equally susceptible to the addition of a β-GlcNAc by a β3GlcNAcT(β3GnT) activity that would normally extend a type 2 chain. To date, there are no data investigating and distinguishing this scenario from one in which there is an elevated expression of a particular β3GnT which would preferentially elongate a type 1 unit.

The specificity and regulated expression of β3GnTs is a complicated issue beyond the scope of current studies. Of those investigated, β3GnT2, -3, -4, and -5 were shown to be expressed in Colo205 (19), and β3GnT2, β3GnT3, and β3GnT5 all showed a higher activity against type 2 chain on lacto-N-neotetraose than type 1 chain on lacto-N-tetraose (29). In this work we have additionally demonstrated that β3GnT2 in the presence of β3GalT5 can act in concert to elongate the type 1 chain acceptor substrate, lacto-N-tetraose, to a series of extended type 1 chains of varying lengths. This *in vitro* synthesis using recombinant enzymes did not resolve the issue of which endogenous β3GnT(s) is responsible for *in vivo* synthesis using the natural Lc₃Cer (Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer) substrates. It simply demonstrated that if a type 1 unit could be readily extended to dimeric type 1 chain, it could be further oligomerized to give the trimeric extended type 1 chain identified in this study. Importantly, this provided a pure, authentic standard for us to validate our MS/MS sequencing data on the products revealed that a GlcNAc could also be added to both the Gal of the reducing end Lac unit and the non-reducing end terminal Gal. However, this apparent lack of preference is only evident against a dimeric type 1 chain. Against a dimeric type 2 chain, a single GlcNAc is clearly preferentially added to the expected middle Gal site, i.e., Galβ1→4GlcNAcβ1→3Galβ1→1R. Thus, it could be concluded that although the dimeric extended type 1 chain did not serve as an optimum substrate for the IGnTs, the occurrence of branched structures is supported by the expression of a significant level of IgnT2 mRNA transcript in Colo205. In other words, the expression of IgnT2 is capable of converting the natural type 2 polylactNAc substrate and possibly the aberrant glycosylation products, extended type 1 or the hybrid chains, into the branched structures identified in this work.

In summary, our data indicate that once a yet to be identified oncogenic event perturbs or subverts the normal constraints imposed on extending the type 1 chain, it can be further elongated and subsequently branched using the existing arrays of βGalTs and βGnTs despite presenting a somewhat less than ideal substrates. The high activity of β3GalT itself relative to β4GalT is a requisite for enhanced level of type 1 unit and with it Leα and sialyl Leα commonly associated with colon carcinoma cells (30, 31). However, it may or may not be the determining factor to tilt the balance in promoting the synthesis of extended type 1 chains, which is a relatively rare and unique feature of Colo205. The key to this aberrant glycosylation may additionally be dependent on the regulated activities of the β3GnTs expressed. The Colo205 and other colonic adenocarcinoma cell lines, therefore, provide a good model system to further investigate the relative specificities and activities of β3GalTs, β4GalTs, β3GnTs, and β6GnTs, in the biosynthesis of extended linear and branched glycan chains.

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