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Butyrate induces sLe^x synthesis by stimulation of selective glycosyltransferase genes

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Abstract

Sialyl Lewis x (sLe^x) is an important tumor-associated carbohydrate antigen present on the cell surface glycoconjugates involved in leukocyte migration and cancer metastasis. We report the formation of sLe^x epitope in butyrate-treated human pancreatic adenocarcinoma cells expressing MUC1 and core 2 N-acetylglucosaminyltransferase (C2GnT). Butyrate treatment stimulates not only the transgene but also a group of endogenous glycosyltransferase genes involved in the synthesis of sLe^x. Current finding raises a concern about the proposed use of butyrate as a cancer therapeutic agent.

Keywords

sLe^x; glycosyltransferase; butyrate and pancreatic cancer

Introduction

Glycoconjugates located at the outer surface of mammalian cells are characteristics of cell-types and developmental stages [1]. Malignant transformation is often associated with abnormal glycosylation, resulting in the synthesis of altered carbohydrates. Some of these carbohydrate epitopes are involved in selectin-mediated adhesion of leukocytes [2] and cancer cells [3–5] to vascular endothelium. One such carbohydrate is sialyl Lewis^x (sLe^x), NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc→R, which is a selectin ligand involved in the binding of tumor cells to endothelial cells during metastasis [3–5]. However, regulation of the synthesis of this epitope in metastatic cancer is not completely understood.

sLe^x is found at the non-reducing terminus of glycoconjugates [6]. The synthesis and functions of mucin glycan-associated sLe^x are well characterized [6]. Synthesis of sLe^x on Oglycans begins with attachment of *N*-acetylgalactosamine (GalNAc) to serine or threonine as catalyzed by polypeptidyl N-acetylgalactosaminyltransferase (ppGalNAcT) [7]. These glycans are then extended by the formation of core 1 (Galβ1–3GalNAc), which is the obligatory precursor of core 2, Galβ1–3(GlcNAcβ1–6)GalNAc [8]. Formation of sLe^x on core 2 branch proceeds by

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addition of β1–4galactose to GlcNAc followed by attachment of α2–3 sialic acid or N-acetylneuraminic acid (NeuAc) to galactose and then α1–3fucose to GlcNAc [9]. Elaboration of sLe^x on core 1 branch occurs by same process after core 1 has been extended with β1–3GlcNAc [10]. Alteration of the activities of the enzymes involved in the synthesis of sLe^x and its precursors can affect the expression of sLe^x.

The expression of cell surface carbohydrate antigens are affected by various agents including sodium butyrate [11–13]. Butyrate can alter the activities of endogenous glycosyltransferases in several cell lines, such as CaCo-2 colon carcinoma cells [14] and Chinese Hamster Ovary cells [15]. Butyrate also can modulate the expression of intestinal mucin gene expression [16]. Because butyrate can induce apoptosis in various transformed and nontransformed cell types [17–19] and various cancerous cells [20], it has been considered as a therapeutic agent [21,22]. In this communication, we report that butyrate treatment of pancreatic adenocarcinoma cells expressing MUC1 and core 2 N-acetylglucosaminyltransferase results in the formation of sLe^x. This finding raises a concern about the use of butyrate as a cancer therapeutic agent.

Materials and methods

Materials

The MUC1-expressing Pancreatic adenocarcinoma (Panc1/MUC1f) cells stably transfected with pcDNA3.1/Zeo+ containing bovine (b) C2GnT-1 cDNA (Panc1/MUC1f-bC2GnT-1) were previously generated in our laboratory [8]. UDP-[^{3}H]GlcNAc (60 Ci/mmol), UDP-[^{3}H]Gal (60 Ci/mmol), and UDP-[^{3}H]GalNAc (15 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). Bond Elut C18 cartridges were from Varian (Sunny Vale, CA). Other chemicals were from Sigma (St. Louis, MO) unless indicated otherwise.

Cell culture conditions

Panc 1 / MUC1f-bC2GnT-1 cells were routinely grown in minimal essential medium supplemented with 10% fetal bovine serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). All cell culture reagents were from Hyclone (South Logan, UT).

Sodium butyrate treatment

Panc 1 / MUC1f-bC2GnT-1 cells grown to about 75% confluence in T-25 flasks were treated with varying concentrations of sodium butyrate (0–4 mM). At various time points (0–36 h) after treatment, cells were processed for analysis of glycosyltransferase activities and mRNA expression.

Assay of C2GnT, core 1 β3Galactosyltransferase (core 1β3Gal-T) and ppGalNAcT enzyme activities

C2GnT activity was assayed using total cell lysates as described previously [23]. Control and sodium butyrate treated Panc 1 / MUC1f-bC2GnT-1 cells from a T25 flask were scrapped into 250 μL of 0.25 M sucrose, and then disrupted by forcefully passing the cells successively through 20- and 26-gauge needles. Aliquots of the homogenates were measured for C2GnT activity using UDP-[^{3}H]GlcNAc (~3,500 dpm/nmol) as the sugar donor and 2 mM Galβ1,3GalNAcα-O-Benzyl (Toronto Research Chemicals Inc. Downs view, Ontario, Canada) as the acceptor. The products were isolated with C18 cartridges and quantitated by a Liquid Scintillation Counter (Packard, Meriden, CT). Protein concentration was measured with Coomassive Plus Protein Assay Reagent (Pierce, Rockford, IL) using BSA as the standard. Enzyme activity was calculated by subtracting the endogenous activity measured without exogenous acceptor from total activity and expressed as nmol of sugar donor transferred/h/mg protein. Core 1 β3Gal-T, which catalyzes attachment of galactose in β1–3 linkage to

GalNAcα-ser/thr, was assayed as described previously [24], except that 2 mM GalNAc-benzyl was used as the acceptor, and the product was isolated and quantified as described above for assay of C2GnT activity. ppGalNAcT activity was assayed as described [25], except that the reaction mixture contained 0.1 mM UDP-[^{3}H]GalNAc (10^5 dpm/nmol), 1 mg/ml BSA and 0.1 mg/ml acceptor peptide. The acceptor peptide is a synthetic 29 amino acid MUC2 peptide having the PTTTPITTTTTVTPTPTPTGTQTPTTTPI sequence. Although numerous isoforms of ppGalNAcT possess distinct acceptor substrate preferences [7], the MUC2 tandem repeat sequence can be utilized efficiently by several isoforms of ppGalNAcT [26] and should provide a reasonable measure of overall ppGalNAcT activity.

Western Blot Analysis

Lysates of Panc 1 / MUC1f-bC2GnT-1 cells treated with or without 2 mM sodium butyrate for 36 h were resolved on 6% SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto a PVDF membrane (Immobilon-P, 0.2 μm, Millipore, Bedford, MA) overnight at 4° C and 100 mA, then blocked with 5 % BSA in TBS (0.9 % NaCl, 10 mM Tris, pH 7.5) at room temperature (R.T.) for 1 h. The membrane was then incubated for 1 h at R.T. with mouse KM93 anti-sLe^x monoclonal antibody (Calbiochem, EMD biosciences, LA Jolla, CA) (1:100) in 5 % BSA. The membrane was washed with TBS-T for 5 min and two additional 5 min washes. The membrane was incubated with peroxidase-conjugated goat anti-mouse IgG antibodies (1:2000) in TBS containing 5 % BSA at R.T. for 1 h. The blot was then rinsed with TBS, treated with ECL reagents (Pierce, Rockford, IL) and exposed to ECL-sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden).

RT-PCR analysis of glycosyltransferase gene expression

Total RNA was extracted from control and sodium butyrate (2 mM) treated pancreatic cancer cells using Tri reagent (MRC, Inc, USA). For cDNA synthesis, 2μg of total RNA were used as the template in a 20μl RT reaction mixture by using Reverse-iT^{TM} 1st Strand synthesis kit (ABgene, UK). The PCR reaction was performed in a Eppendorf Mastercyclerpersonal using 100ng of cDNA template in a total volume of 25μl. PCR analysis of mRNAs of glycosyltransferases and β-actin was carried out by using forward and reverse primers listed below; for core 1 β3Gal-T gene, 5’-GATCCTCATGCAAGGCATTC-3’ and 5’-GCTCCTCCACTCATGTAGCC-3’; for β3GnT-3 (core 1 extending enzyme) gene, 5’-TTCTTCAACCTCACGCTCAAGCAG-3’ and 5’-AGCATCTCATAAGGTAGGAAG CGG-3’; for C2GnT-1 gene, 5’-ACTCGAAACACCTCTCTTTTCTGGC-3’ and 5’-GGT CAGTGTTTTAATGTCTCCAAAG-3’; for C2GnT-2 gene, 5’-TGCATTACTTGTGGG CTCTG-3’ and 5’-TCTGGGGACTTCTCATCCAC-3’; for bC2GnT-1 gene, 5’-CATC GCATCCTGCTTCAGTA-3’ and 5’-ATGTGTCTTTTGCCCACTCC-3’; for β4GalT-1 gene, 5’-AGTGACGTGGACCTCATTCC-3’ and 5’-CCGATGTCCACTGTGATTTG-3’; for β4GalT-4 gene, 5’-GCTGTTGACTTTGTGCCTGA-3’ and 5’-GCCTGGTGGAT GACGTAGAT-3’; for ST3Gal-III gene, 5’-ACTCCAGTGGGAGGAGGACT-3’ and 5’-CGTGACCCCAGAGACTTGTT-3’; for ST3Gal-IV gene, 5’-CTAGCCATCACCAG CTCCTC-3’ and 5’-CCATGAAGAAGGGGTTGAGA-3’; for ST3Gal-VI gene, 5’-TTGCCTCTCTGCTGAGGTTT-3’ and 5’-TCACCCATCAACAATTCCAA-3’; for FucT-IV gene, 5’-GAGAGGCTCAGGCCGTGCTTTT-3’ and 5’-GCAGGAGCCC AATTTCGGGCAC-3’; for FucT-VII gene, 5’-CACCTCCGAGGCATCTTCAACTG -3’ and 5’-CGTTGGTATCGGCTCTCATTCATG-3’; for β-actin gene, 5’-GTGGGGCG CCCCAGGCACCA-3’ and 5’-CTCCTTAATGTCCGGACGATTC-3’. The PCR reaction conditions for all the genes were as follows: 94 °C for 2 min (1 cycle); 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min (30 cycles); and 72 °C for 5 min. The PCR products were subjected to electrophoresis (130V, constant-voltage field) on a 1% agarose gel equilibrated in Tris-borate electrophoresis buffer containing ethidium bromide (1mg/ml). Gels were photographed under UV light.

Results

Effect of sodium butyrate on enzymes involved in the synthesis of mucin glycan core structures

Previously, we showed that sodium butyrate stimulated stably transfected transgenes [27]. In this communication, we confirmed and extended this observation in Panc 1 / MUC1f cells stably transfected with pcDNA3.1 containing bC2GnT-1 cDNA controlled by CMV promoter. As shown in Fig. 1a and 1b, butyrate treatment enhanced C2GnT activity in Panc 1/MUC1f-bC2GnT-1 cells in a time and butyrate concentration dependent fashion. Although 4 mM butyrate treatment yielded highest specific activity, excessive cytotoxicity observed at this concentration led to the decision of using 2 mM butyrate for subsequent study. The time course study showed that 24-h treatment with 2 mM butyrate produced maximal C2GnT activity. In addition, butyrate treatment stimulated not only bC2GnT-1 transgene (Fig. 1c) but also endogenous core 1 β3Gal-T gene (Fig. 1d). This enzyme generates core 1 structure, which serves as the obligatory substrate for C2GnT enzyme. However, butyrate did not have a significant effect on the endogenous ppGalNAcT activity (Fig. 1e), which initiates the synthesis of mucin glycans.

Sodium butyrate treatment produced sLe^x epitope

Since expression of sLe^x glycotope usually accompanies the expression of C2GnT-1 gene [28]. We decided to determine if elevation of C2GnT activity in butyrate-treated Panc 1 / MUC1f-bC2GnT-1 cells would lead to the production of this glycotope. As shown in Fig. 2, treatment of Panc 1 / MUC1f-bC2GnT-1 cells with 2 mM butyrate for 36 h generated sLe^x carbohydrate epitope.

Sodium butyrate treatment enhanced the expression of endogenous glycosyltransferase genes involved in the synthesis of sLe^x

To seek the explanation for the butyrate-induced production of sLe^x, we employed RT-PCR to measure the expression of bC2GnT-1 and endogenous glycosyltransferase genes involved in the synthesis of sLe^x with and without butyrate treatment. As shown in Fig. 3, butyrate treatment of Panc 1 / MUC1f-bC2GnT-1 cells stimulated not only bC2GnT-1 transgene (Fig. 3b) but also the following endogenous human glycosyltransferase genes: core 1 β3Gal-T, β3GnT-3 (or core 1 extension enzyme) (Fig. 3a), C2GnT-2, C2GnT-3 (Fig. 3b), α2–3 sialyltransferase (ST3GalT)-IV and -VI (Fig. 3d) and Fucosyltransferase (FucT)-VII (Fig. 3e) as compared to those of untreated Panc 1 / MUC1f-bC2GnT-1 cells. Butyrate treatment did not affect the expression of the following glycosyltransferase genes: β4galactosyl transferases (β4GalT)-1 and -4 (Fig. 3c), ST3Gal-III (Fig. 3d), and FucT-IV (Fig. 3e). Expression of FucT-III gene was not detected in either control or butyrate-treated cells.

Discussion

Carbohydrate ligands containing sLe^x are involved in leukocyte migration and homing [29] and cancer metastasis [30]. sLe^x has been found to be elevated in many advanced cancers [30]. In this communication, we report that sLe^x can be generated by butyrate treatment. Butyrate has been considered as a cancer therapeutic agent because of its ability to induce apoptosis of cancer cells [20]. Given the role of this glycotope plays in cancer metastasis, current observation raise a concern about this therapeutic approach.

Generation of sLe^x requires a coordination of several enzymatic steps. As depicted in Figure 4, mucin glycan-associated sLe^x epitope can be synthesized on core 1 and/or core 2 branch elaborated by a group of glycosyltransferases. sLe^x on core 1 branch is built on the extension of core 1 by β3GlcNAc as catalyzed by β3GnT-3 [14] while sLe^x on core 2 branch is extended

from core 2 β6GlcNAc structure formed by C2GnT [15]. Subsequent steps involved in the synthesis of sLe^x include sequential actions of GlcNAc: β4galactosyltransferase (β4Gal-T), Galβ1–4GlcNAc: (NeuAc-Gal) α2–3 sialyltransferase (ST3Gal), and NeuAcα2–3Galβ1–4GlcNAc: (Fuc-GlcNAc) α1–3/4 fucosyltransferase (FucT). Core 1β3Gal-T is the only enzyme which can synthesize core 1 structure [31] and β3GnT-3 is the only core 1 extension enzyme [32]. Core 2 can be synthesized by three different isozymes, C2GnT-1, 2, and 3 [33]. Galβ1–4GlcNAc on core 1 and core 2 branchs can be synthesized by β4GalT-1 & 4, respectively. Three different isozymes, ST3Gal-III, IV & VI, can synthesize NeuAcα2–3Galβ1–4GlcNAc from Galβ1–4GlcNAc. sLe^x is synthesized from NeuAcα2–3Galβ1–4GlcNAc as catalyzed by FucT-III, IV & VII. FucT-VII only uses NeuAcα2–3Galβ1–4GlcNAc as the substrate and is the most efficient enzyme for synthesis of sLe^x [34]. On the other hand, the primary function of FucT-III is to synthesize sLe^a although it also can synthesize sLe^x with low efficiency [35]. The primary products synthesized by FucT-IV are Le^x and poly Le^x although it is capable of producing sLe^x with low efficiency [36]. Butyrate treatment enhances not only the enzyme activities for synthesis of core 1 and core 2 structures but also the expression of the genes for extending core 1 structure and for synthesizing sLe^x. It is noted that expression of several genes, including β4GalT-1 & 4, ST3Gal-III & IV, and FucT-IV, which are also involved in the synthesis of sLe^x is not stimulated by butyrate. FucT-III gene is not expressed in this cell line. Since sLe^x is not detected before butyrate treatment, the genes that are activated by butyrate are likely responsible for the production of sLe^x. Although β4GalT-1 & 4 genes are not affected by butyrate, high basal expression of these two genes would ensure production of sufficient amount of N-acetyllactosamine for generation of subsequent intermediate and final product. With the exception of bC2GnT-1, all other enzymes are generated by endogenous genes. This point is of particular significance when we try to evaluate the potential side effects of using butyrate as a cancer therapeutic agent.

Butyrate is known to modulate mucin gene expression in the intestinal epithelial goblet cells [16]. It also can enhance sLe^x production in HT29 cells through stimulation of FucT-III but not FucT-IV gene [37]. FucT-VII gene is not expressed in HT29 cells. Expression of other glycosyltransferases was not examined in this study. It is not clear whether they are affected by butyrate treatment. Our study provides a comprehensive analysis of the expression pattern of all glycosyltransferase genes involved in the synthesis of sLe^x in Panc 1 / MUC1f-bC2GnT-1 cells. As compared with HT29 cells, Panc 1 / MUC1f-bC2GnT-1 cells express FucT-IV and VII genes but not FucT-III gene. Butyrate treatment stimulates FucT-VII but not FucT-III and FucT-IV genes. Therefore, FucT-VII is likely the FucT gene responsible for the conversion of NeuAcα2–3Galβ1–4GlcNAc to sLe^x in these cells. Stimulation of FucT-VII and ST3Gal-VI along with bC2GnT-1 and core 1 β3Gal-T genes in response to butyrate treatment could explain the production of sLe^x in Panc 1 / MUC1f-bC2GnT-1 cells treated with butyrate.

Our results show that it takes up to 12 h for the butyrate effect to reach the peak. The result suggests that butyrate treatment affects gene expression rather than posttranslational modification, such as phosphorylation. Inhibition of histone deacetylation results in altered chromatin structure and gene expression [38], is one mechanism proposed for butyrate effect. However, it is not clear if same mechanism is responsible for the stimulation of endogenous glycosyltransferase genes involved in the synthesis of sLe^x. Since butyrate effect appears to be selective because not all glycosyltransferase genes are activated, it is predicted that the glycosyltransferase genes that are stimulated by butyrate may share common signaling pathway and/or transcription factors. Further study is needed in order to prove this hypothesis.

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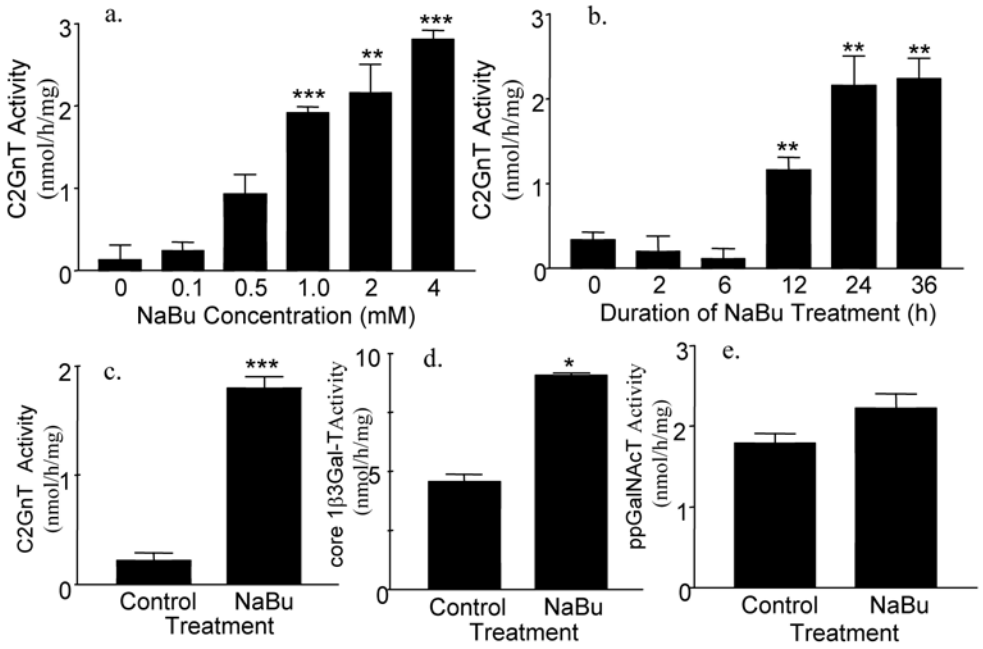

Fig. 1.

Glycosyltransferase activities in Panc 1 / MUC1f-bC2GnT-1 cells treated with sodium butyrate (NaBu). (a) C2GnT activity in Panc 1 / MUC1f-bC2GnT-1 cells treated with 0-4 mM mM NaBu for 24 h, b) C2GnT activity in Panc 1 / MUC1f-bC2GnT-1 cells treated with 2 mM NaBu for 0-36 h, c) C2GnT activity in Panc 1 / MUC1f-bC2GnT-1 cells treated with and without 2 mM NaBu for 24 h, (d) core 1 β3Gal-T activity in Panc 1 / MUC1f-bC2GnT-1 cells treated with and without 2 mM NaBu for 24 h, and (e) ppGalNAcT activity in Panc 1 / MUC1f-bC2GnT-1 cells treated with and without 2 mM NaBu for 24 h. Results of c-e were obtained from same cell homogenates. *, $p<0.05$; **, $p < 0.01$; ***, $p<0.001$. n = 3 independent T25 flasks.

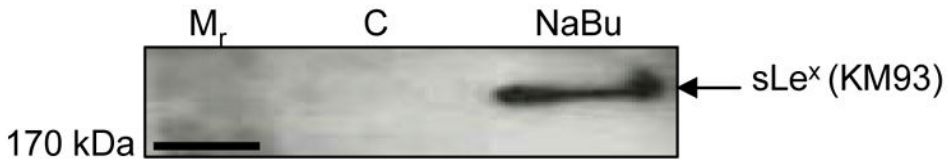

Fig. 2.

Production of sLe^x in Panc 1 / MUC1f-bC2GnT-1 cells treated with sodium butyrate. Panc 1 / MUC1f-bC2GnT-1 cells were treated with 2 mM NaBu for 36 h. Proteins were resolved in 6% SDS-PAGE, transferred to PVDF membrane, and probed with sLe^x-specific monoclonal antibody KM93. The ~250 kDa band, which corresponded to MUC 1 band [7], was detected only in butyrate-treated cells.

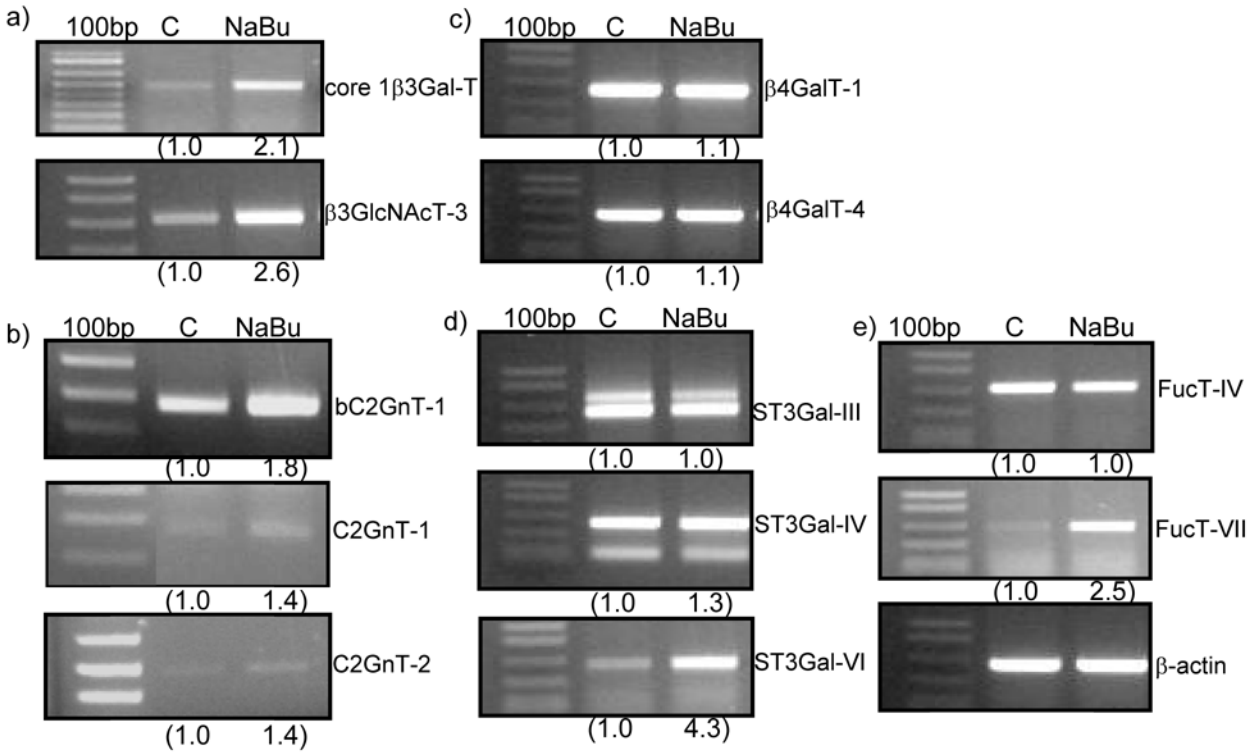

Fig. 3.

RT-PCR analysis of the expression pattern of glycosyltransferase genes involved in the synthesis of core 1, core 2, and sLe^x. Panc 1 / MUC1f-bC2GnT-1 cells were treated with and without 2 mM NaBu for 24 h and then analyzed by RT-PCR for the expression of the bovine (b) and human genes involved in the synthesis of core 1, core 2, and sLe^x. The expression of the genes upregulated by butyrate includes: core 1 β3Gal-T, β3GnT-3, bC2GnT-1, C2GnT-1, C2GnT-3, ST3Gal-VI, and FucT-VII. The expression of the genes not affected by butyrate includes: β4GalT-1 & 4, ST3Gal-III and IV, and FucT-IV and expression of FucT-III was not detected.

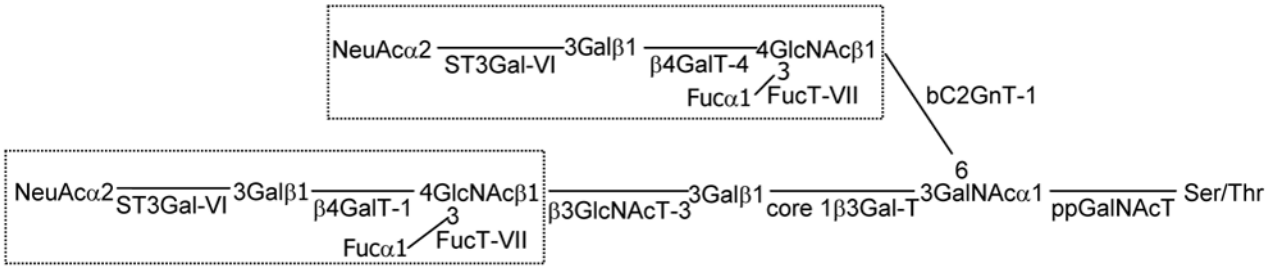

Fig. 4.

Schematic diagram of the biosynthetic pathway of biantennary sLe^x located on core 1 and core 2 mucin glycans. Only key glycosyltransferases involved in the synthesis of sLe^x on MUC1 of Panc 1 / MUC1f-bC2GnT-1 cells are shown.