neutral				
N1	Galβ1-4Glc-PA	lactose	2.9	4.6
N2	Galα1-4Galβ1-4Glc-PA	Gb$_3$	3.8	1.9
N3	GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	Gb$_4$	2.4	0.6
N4	Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	Lc$_4$	26.9	61.4
N5	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	nLc$_4$	2.5	3.7
N6-1	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	Type1H	33.5	5.2
N6-2	Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	Type2H	0.3	1.4
N7-1	$\mathrm{Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	Le^{x}	5.7	4.9
N7-2	$\mathrm{Gal\beta1\text{-}3}\underset{\substack{4\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	Le^{a}	0.2	0.2
N8	$\mathrm{Fuc\alpha1\text{-}2Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	Le^{y}	4.7	0.4
N9-1	$\mathrm{Fuc\alpha1\text{-}2Gal\beta1\text{-}3}\underset{\substack{4\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	Le^{b}	0.1	0.1
N9-2	Galβ1-3GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	Lc$_6$	1.0	2.8
N13	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	nLc$_6$	0	1.7
N10-1	$\mathrm{Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4GlcNAc\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	V^{3}Fucα-nLc$_6$	0.4	0.2
N10-2	$\mathrm{Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}3GlcNAc\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	V^{3}Fucα-$_{1,2}$Lc$_6$	0.8	1.8
N11	$\underset{\substack{2\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{Gal}}\mathrm{\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4GlcNAc\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	VI^{2}Fucα,V^{3}Fucα-nLc$_6$	0.1	0
N12	$\mathrm{Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4}\underset{\substack{3\\ \vert \\ \mathrm{Fuc\alpha1}}}{\mathrm{GlcNAc}}\mathrm{\beta1\text{-}3Gal\beta1\text{-}4Glc\text{-}PA}$	V^{3}Fucα,III^{3}Fucα-nLc$_6$	1.6	0.5

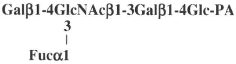
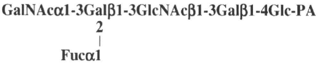
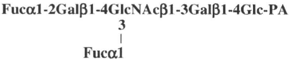
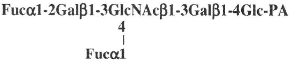
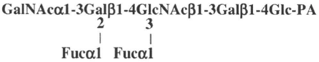
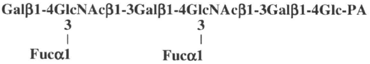
Lewis-negative individuals, very low levels of Le^{a} (N7-2) and Le^{b} (N9-1) were observed in normal epithelial cells of some Lewis-negative individuals (Figure 1A, Table II). This is probably because the highly sensitive methods employed in our study to analyze the oligosaccharide structures facilitated the detection of minute levels of Lewis antigens, which would otherwise be undetectable using conventional procedures. Secondly, a relatively high level of Le^{b} (N9-1) was found in cancer cells but not normal epithelial cells of some Lewis-negative individuals (Figure 1B, c, Table III). A molecular mechanism by which cancer cells of Lewis-negative individuals synthesize high levels of Le^{b} is proposed in the Discussion.

Structure of A8-2

Although A8-2 was expressed in three adenocarcinoma cells, it did not match any of the reference compounds on the 2D map accumulated prior to our studies. MS/MS (MS^2) analysis revealed that A8-2 is monosialylated monofucosylated hexasaccharide GSL having a tetrasaccharide backbone, Hex-HexNAc-Hex-Hex-PA. Hence, four GSLs, SLe^x, SLe^a, III^6NeuAcα,III^3Fucα-Lc_4, and ST2H were reasoned to be candidates of A8-2. However, the position of A8-2 on the 2D map did not match any of the four corresponding standards (Figure 2, closed and open circles). A8-2 was digested with neuraminidase from *Arthrobacter ureafaciens* and the products of the digestion subjected to 2D mapping. Analysis of the results showed that the products corresponded to reference compound Type1H but not the other monofucosylated pentasaccharides, Type2H, Le^a, and Le^x (Figure 2, closed and open triangles). From this result, the structure of A8-2 is tentatively predicted to be a sialylated

Table III. Estimated structures of acidic and neutral PA-oligosaccharides from pancreatic cancer and normal pancreatic epithelial cells of case 6 (C, cancer cells; N, normal epithelial cells)

Fraction	Structure	Abbreviation	Ratio (%)	
			C	N
acidic				
A1	Neu5Acα2-3Galβ1-4Glc-PA	GM3	29.0	63.7
A2	Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc-PA	GD3	0.6	0
A11	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA 3 | Neu5Acα2	GM1	0.5	0
A3	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	SLe^c	0.5	0.4
A4	Galβ1-3GalNAcβ1-4Galβ1-4Glc-PA 3 3 | | Neu5Acα2 Neu5Acα2	GD1a	1.2	0.4
A6	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	LST-c	1.5	0
A7	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA 6 | Neu5Acα2	IV^3NeuAcα,III^6NeuAcα-Lc_4	0.6	1.8
A8-1	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA 3 | Fucα1	SLe^x	0.4	0
A8-2	Neu5Acα2-6Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA 2 | Fucα1	ST1H	0.3	0
A13	Neu5Acα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	V^3NeuAcα-Gb_5	0	0.9
A14	Neu5Acα2-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glc-PA 6 | Neu5Acα2	V^3NeuAcα IV^6NeuAcα-Gb_5	0	0.9
neutral				
N1	Galβ1-4Glc-PA	lactose	1.6	0.6
N2	Galα1-4Galβ1-4Glc-PA	Gb_3	2.7	7.9
N3	GalNAcβ1-3Galα1-4Galβ1-4Glc-PA	Gb_4	1.5	1.5
N4	Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	Lc_4	0.4	0.5
N5	Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA	nLc_4	1.0	0.2
N6-1	Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc-PA	Type1H	43.3	1.6

N7-1	 $\text{Gal}\beta1\text{-}4\underset{\substack{3\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	Le^x	3.2	0.7
N14	 $\text{GalNAc}\alpha1\text{-}3\underset{\substack{2\\ \vert \\ \text{Fuc}\alpha1}}{\text{Gal}}\beta1\text{-}3\text{GlcNAc}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	Type1A	3.6	13.3
N8	 $\text{Fuc}\alpha1\text{-}2\text{Gal}\beta1\text{-}4\underset{\substack{3\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	Le^y	3.1	1.3
N9-1	 $\text{Fuc}\alpha1\text{-}2\text{Gal}\beta1\text{-}3\underset{\substack{4\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	Le^b	4.9	0
N15	 $\text{GalNAc}\alpha1\text{-}3\underset{\substack{2\\ \vert \\ \text{Fuc}\alpha1}}{\text{Gal}}\beta1\text{-}4\underset{\substack{3\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	ALe^y	0	4.4
N12	 $\text{Gal}\beta1\text{-}4\underset{\substack{3\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\underset{\substack{3\\ \vert \\ \text{Fuc}\alpha1}}{\text{GlcNAc}}\beta1\text{-}3\text{Gal}\beta1\text{-}4\text{Glc-PA}$	V^{3}Fucα,III^{3}Fucα-nLc$_6$	0.2	0

form of Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc (sialylated type1H). A8-2 was digested with α-fucosidase from bovine kidney, and the product of the digest did not coincide with the position of the any monosialylated pentasaccharide reference compound, SLe^c, LST-b, LST-c, SPG, or GM1 (Figure 2, closed and open diamonds). Linkage position of sialic acid could be determined by the specificity of α2,3-silalidase digestion as described in Materials and methods. Defucosylated product of A8-2 could not be digested with α2,3-sialidase under conditions where the enzyme specifically cleaves the α2-3 linkage (condition 1). However, digestion did occur with α2,3-sialidase under conditions where the enzyme cleaves the α2-3 and α2-6 linkages (condition 2). These results indicate that sialic acid is α2-6 linked to the terminal residue. In addition, the defucosylated desialylated product of A8-2 corresponded to Lc$_4$ but not other neolacto and ganglio series tetrasaccharides, nLc$_4$, asialo GM1 on the 2D map (Figure 2, closed and open squares). Hence, the structure of A8-2 is predicted to be NeuAcα2-6(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc (α2-6 sialylated type1H, abbreviated as ST1H), which has not been reported previously. This structure is consistent with the result from tandem mass analysis (Figure 3). The presence of a fragment ion at *m/z* 622 corresponding to [NeuAc+dHex+Hex+Na]$^+$ in the MS^2 spectra of A8-2 indicates that a fucose and sialic acid residues are linked to the outermost Hex residue. This structure has not been found in any normal colorectal and pancreatic epithelial cells, similar to ST2H.

Discussion

In our previous study, we found a novel GSL in colon cancer, NeuAcα2-6(Fucα1-2)Galβ1-4GlcNAcβ1-3Galβ1-4Glc (ST2H), which is expressed in colorectal and pancreatic cancer cells in about half of the patients, regardless of Lewis type (Korekane et al. 2007). In addition to ST2H, we also found a novel fucoganglioside, NeuAcα2-6(Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc (ST1H). Unlike ST2H, ST1H is believed to accumulate principally in cancer cells from Lewis-negative patients. Although there is a degree of similarity between ST1H and ST2H, the two molecules are fundamentally different. The terminal structure of both GSLs is the same, namely, sialic acid and fucose residue is linked to galactose via an α2-6 linkage and α1-2 linkage, respectively. However, the crucial difference is that the galactose of ST1H and ST2H is linked to GlcNAc via a β1-3 (type1) linkage and β1-4 (type2) linkage, respectively. This structural difference is similar to that between SLe^a and SLe^x, i.e., a sialic acid of both SLe^a and SLe^x is attached to the terminal galactose of Le^a (type 1) and Le^x (type 2) via an α2-3 linkage.

ST1H was found in cancer cells from two of 60 cases of colorectal cancer and one of five cases of pancreatic cancer. The incidence of this moiety in cancer cells appears to be very low. However, taking into consideration the highly likely possibility that this substance is preferentially synthesized in cancer cells of Lewis-negative patients, the incidence cannot be considered low. Specifically, this unique structure was found in two of four colorectal cancer patients and one of two pancreatic cancer patients that were Lewis-negative.

ST1H was not detected in normal colorectal and pancreatic epithelial cells. However, analyzed subjects were limited to cancer and normal epithelial cells of colorectal and pancreatic tissues. In future, the subject of analysis needs to be expanded to other tissues including fetal tissues, hyperplasia, adenoma, and inflammatory tissues to investigate whether ST1H is an oncofetal antigen.

Although the expression level of ST1H is quite low compared with major GSLs, it is nevertheless comparable with that of SLe^x (Tables II and III). Moreover, SLe^x is a well-known tumor-associated carbohydrate antigen, which has

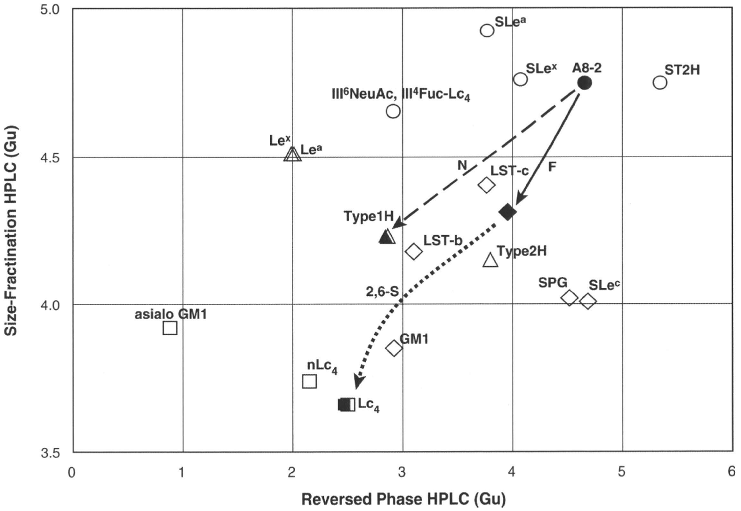


Fig. 2. Sequential digestion of A8-2. Circles, diamonds, triangles, and squares mark the positions of monosialylated monofucosylated hexasaccharides, monosialylated pentasaccharides, monofucosylated pentasaccharides, and tetrasaccharides, respectively. Closed marks indicate A8-2 (closed circle) and change after glycosidase digestion of A8-2 (closed triangle, diamond, and square). Open marks represent the positions of the standard compounds (shown as abbreviations). Lines indicate the direction of the change after glycosidase digestion of A8-2. Glycosidases are shown beside each line. Enzyme abbreviations are: N, α-sialidase (neuraminidase) from *Arthrobacter ureafaciens*; F, α-fucosidase from bovine kidney; 2,6-S, α2,3-sialidase from *Salmonella typhimurium* under conditions where the enzyme digests sialic acid at both α2-3 and α2-6 linkages.

been used as a serum tumor marker, and functions as a ligand for selectins. Therefore, the low level of ST1H expression per se does not necessarily imply an insignificant role in terms of cancer biology.

In addition to identifying novel structures in cancer cells of Lewis-negative individuals, we also found relatively high levels of expression of Le^b (N9-1) in some cancer cells (Figure 1B, c, Table III) even though they are judged to be Lewis-negative. Specifically, no or trace levels of Le^a expression were found in cancer cells and normal epithelial cells, with an undetectable level of CA19-9 in serum, null FUT3 (Lewis) gene and an undetectable level of α1-4 fucosyltransferase activity with Lc_4 (see Results section). We propose that FUT5, but not null FUT3, might be responsible for this reaction. FUT5 has predominantly α1-3 fucosyltransferase activity with type 2 chain acceptors, such as nLc_4 and type 2 disaccharide, and only a very low level of α1-4 fucosyltransferase activity with type 1 chain acceptors, such as Lc_4 and type 1 disaccharide (Weston et al. 1992; Nguyen et al. 1998). However, FUT5 showed comparable levels of α1-4 fucosyltransferase activity with Type 1H to convert to Le^b to FUT3 (Oulmouden et al. 1997). Furthermore, previous work suggests a requirement of Type 1H structures for FUT5 as precursors for type 1 Lewis antigen biosynthesis in cultured cells (Holgersson and Lofling 2006). Hence, even though FUT3 is inactivated, Le^b can be generated by FUT5 in the cells in which Type 1H is dominantly expressed, such as cancer cells of Lewis-negative individuals as described below (N6-1, Figure 1A and B). In agreement with this speculation, cancer cells of case 6 possess undetectable levels of α1-4 fucosyltransferase activity with Lc_4 to form Le^a. However, these cancer cells exhibited quite low, but nevertheless detectable, levels of α1-4 fucosyltransferase activity with Type 1H to form Le^b (data not shown).

Proposed synthetic pathways for the major groups of GSLs in cancer cells and normal epithelial cells are outlined in Figure 4. Based on the findings of our previous work (Misonou et al. 2009) and this study, we believe that the reason that ST1H is likely to be synthesized in cancer cells of Lewis-negative patients is as follows. Le^a and Le^b are the major products, and Type1H and Lc_4 are very minor in normal epithelial cells from Lewis-positive individuals (highlighted by square with thick line, Figure 4). Even though the levels of Le^a and Le^b decrease in carcinogenesis, the levels of Lc_4 and Type1H do not increase and remain quite low. ST1H is scarcely synthesized in cancer and normal epithelial cells from Lewis-positive individuals probably because active Lewis enzyme much prefers to act on Type1H (a precursor of ST1H) over α2-6 sialyltransferase; the pathway would flow to the synthesis of Le^b but not ST1H. α2-6 Sialylated Le^b and Le^a was not detected in our study. This is probably because α2-6 sialyltransferase cannot act on the terminal galactose of a type-1 lactosamine chain when the adjacent GlcNAc is fucosylated. While Lc_4, Type1H, and Type1H derivatives (Type 1A and/

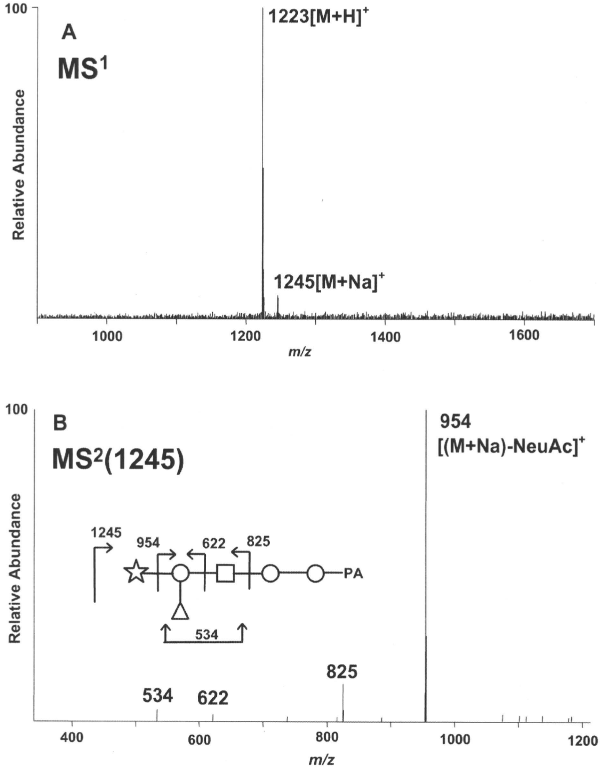


Fig. 3. MS^1 and MS^2 spectra of A8-2. (**A**) MS^1 spectra of A8-2. (**B**) MS^2 spectra of $[M+Na]^+$ precursor ion at *m/z* 1245 detected in MS^1 of panel **A**. Fragment ions with numbered mass values in panel **B** are sodium adduct ions. The MS/MS fragment ions were assigned as shown schematically. Symbols: Hex, open circle; HexNAc, open square; sialic acid, open star; dHex, open triangle.

or B) are the major products, Le^a and Le^b are absent or present at very low levels in normal epithelial cells from Lewis-negative individuals due to the lack of Lewis enzyme activity (highlighted by square with thin line, Figure 4). The common feature of the oligosaccharide structures of the three cancer cells (cases 1, 4, 6) expressing ST1H is that Type 1H is markedly increased to become the most abundant species (Figure 1A and B, N6-1). The marked increase in the level of Type1H from colorectal cancer cells is thought to be due to the elevation of activity of α1-2 fucosyltransferase during carcinogenesis (Misonou et al. 2009). However, the detailed mechanism of the upregulation of Type1H in pancreatic cancer cells of Lewis-negative patients is not clear. Furthermore, augmentation of the activity of α2-6 sialyltransferase during malignant transformation in colorectal cancer cells was demonstrated previously (Misonou et al. 2009). A similar elevation in the activity of α2-6 sialyltransferase in pancreatic cancer cells was also confirmed in our study (data not shown). Hence, the pathway would flow to the synthesis of ST1H in cancer cells from Lewis-negative individuals.

Because Lewis-negative individuals cannot produce the SLe^a epitope (CA19-9 epitope), serum levels of CA19-9 in these individuals is either undetectable or very low (i.e., under 1 U/mL). DU-PAN-2 (SLe^c epitope), which is a precursor structure of SLe^a, is another well-known tumor marker (Figure 4). Hence, measurement of CA19-9 and DU-PAN-2 is recommended to apply for Lewis-positive and Lewis-negative individuals, respectively (Narimatsu et al. 1998) (Figure 4). Both antigenic epitopes are known to be carried on mucins that are secreted into the plasma by cancer cells. Although the unique epitope of ST1H (NeuAcα2-6(Fucα1-2)Galβ1-3GlcNAcβ1-R) was found in GSLs in this study, it is highly possible that the ST1H epitope is also car-

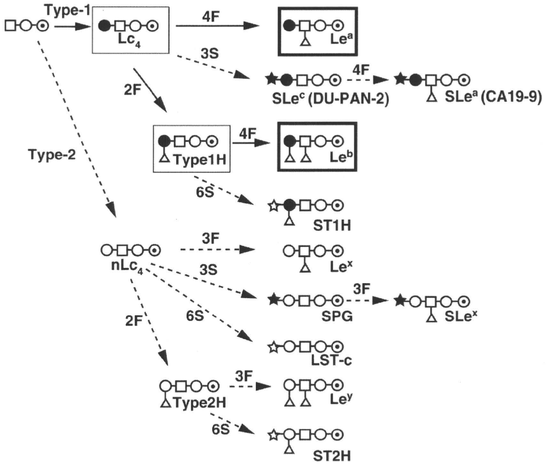


Fig. 4. Proposed synthetic pathways for major groups of GSLs in cancers and normal epithelial cells. Arrows indicate the pathways predominating in normal epithelial cells. Broken arrows indicate the pathways that are increased in carcinogenesis. Abbreviations: 4F, α1-4 fucosylation of GlcNAc (Lewis enzyme activity); 3F, α1-3 fucosylation of GlcNAc; 2F, α1-2 fucosylation of galactose; 3S, α2-3 sialylation of galactose; 6S, α2-6 sialylation of galactose. The structures of GSLs in normal epithelial cells from Lewis-positive individuals are composed of mainly Le^a and Le^b (highlighted by square with thick line). By contrast, the structures of GSLs in normal epithelial cells from Lewis-negative individuals are composed of mainly Lc$_4$ and Type1H (highlighted by square with thin line). In malignant transformation, the type-2 ratio, α2-3 and/or α2-6 sialylation, and α1-2 fucosylation are increased. These alterations result in increases in the amounts of, or the appearance of, a variety of oligosaccharides, such as Le^x, Le^y, LST-c, SLe^x, and ST2H as type 2 oligosaccharides, Type 1H, SLe^a, SLe^c, and ST1H as type 1 oligosaccharides. Note the difference in composition of type1 oligosaccharides between cancer cells from Lewis-positive and negative individuals, i.e., synthesis of SLe^a is increased in carcinogenesis and SLe^a becomes one of the major components of cancer cells from Lewis-positive individuals. However, SLe^a is not synthesized in cancer cells and normal epithelial cells from Lewis-negative individuals, but the levels of SLe^c and/or ST1H are increased in carcinogenesis. SLe^a epitope (NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-R) and SLe^c epitope (NeuAcα2-3Galβ1-3GlcNAcβ1-R) are recognized by CA19-9 and DU-PAN-2 antibodies, respectively. Schemes of representative oligosaccharides are shown. Symbols: open circle with a dot inside, glucose; open circle, galactose (β1-4 linkage, type 2); closed circle, galactose (β1-3 linkage, type 1); open square, GlcNAc; open star, sialic acid (α2-6 linkage); closed star, sialic acid (α2-3 linkage); open triangle, fucose.

ried on the mucins, similar to SLe^a and SLe^c epitopes. Furthermore, the synthetic flow of DU-PAN-2 and ST1H are different; i,e, DU-PAN-2 and ST1H are synthesized by α2-3 sialylation of Lc$_4$ and α2-6 sialylation of Type1H, respectively (Figure 4). The combination of ST1H and DU-PAN-2 determinants could serve as a highly sensitive tumor marker, especially for Lewis-negative individuals.

Materials and methods

All human specimens were obtained from Osaka Medical Center for Cancer and Cardiovascular Diseases. This study was approved by the Local Ethics Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Informed consent was obtained from the patients. The majority of experimental procedures including purification of cancer cells, isolation of GSLs, preparation and separation of PA-oligosaccharides, and mass spectrometry have been reported previously (Misonou et al. 2009). In brief, in order to improve the accuracy of analyses, cancer cells were highly purified from primary lesions of colorectal cancers and pancreatic cancers using the epithelial cell marker, CD326, and magnetic beads. The tissues were dissected into small blocks and incubated in DMEM/F12 medium containing 2 mg/mL collagenase (Sigma-Aldrich, St Louis, MO). The digested cells were resuspended in phosphate-buffered saline containing 2 mM EDTA and 0.5% bovine serum albumin, and CD326 positive cells were positively selected using magnetically labeled microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) according to the manufacturer's protocol.

The neutral and acidic GSLs were extracted from the cells and digested with recombinant endoglycoceramidase II from *Rhodococcus* sp. (Takara Bio Inc. Shiga, Japan) (Ito and Yamagata 1989). Released oligosaccharides were labeled with 2-aminopyridine (Natsuka and Hase 1998).

PA-oligosaccharides were separated on a Shimadzu LC-20A HPLC system equipped with a Waters 2475 fluorescence detector. Normal-phase HPLC was performed on a TSK gel Amide-80 column (0.2 × 25 cm, Tosoh, Tokyo, Japan). The molecular size of each PA-oligosaccharide is given in glucose units (Gu) based on the elution times of PA-isomaltooligosaccharides. Reversed-phase HPLC was performed on a TSK gel ODS-80Ts column (0.2 × 15 cm, Tosoh). The retention time of each PA-oligosaccharide is given in glucose units based on the elution times of PA-isomaltooligosaccharides. Thus, a given compound on these two columns provides a unique set of Gu (amide) and Gu (ODS) values, which correspond to coordinates of the 2D map. PA-oligosaccharides were analyzed by LC/ESI MS/MS. High-performance liquid chromatography was performed on a Paradigm MS4 equipped with a Magic C18 column (0.2 × 50 mm; Michrome BioResource, Auburn, CA). Mass spectrometry (MS) analyses were performed using a LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). In the LCQ method file, the LCQ was set to acquire a full MS scan between 400 and 2000 *m/z* followed by MS/MS scans in a data-dependent manner. Protonated ions were subjected to a further product ion scan for nonfucosylated PA-oligosaccharides. However, sodiated ions were subjected to a further product ion scan for fucose containing PA-oligosaccharides because of the following reason: intramolecular fucose rearrangements have been found in the CID spectra of protonated ions (but not in sodiated ions) produced from oligosaccharides derivatized at their reducing termini with aromatic amines, such as 2-aminobenzamide, which may lead to erroneous conclusions about oligosaccharide sequence (Harvey et al. 2002).

Standard PA-oligosaccharides

The structures, abbreviations, and glucose units of authentic PA-oligosaccharides used in this study are listed in Supplemental Table 1, which include PA-oligosaccharides purchased from a commercial source, kindly donated, or prepared during our previous study. Type 1-type 2 hybrid hexasaccharide, Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc are abbreviated as $_{1,2}Lc_6$, in order of linkage type of the fourth and sixth galactoses.

Glycosidase digestion

Sialyl PA-oligosaccharides were digested with 2 U/mL of α2,3-sialidase from *Salmonella typhimurium* (Takara Bio Inc.) or 2 U/mL of α-sialidase (neuraminidase) from *A. ureafaciens* (Nacalai, Kyoto, Japan) in 100 mM sodium acetate buffer, pH 5.5, for 2 h at 37°C (condition 1). Under these conditions, α2,3-sialidase specifically digests sialic acid α2-3 linked to the terminal residue but not sialic acid with an α2-6 linkage, while *Arthrobacter* α-sialidase digests both linkages independent of the linkage position. However, under conditions using 10 U/mL for 16 h (condition 2), even so-called α2,3-sialidase can hydrolyze sialic acid α2-6 linked to the terminal residue but not sialic acid linked to a non-terminal residue. Hence, we were able to conclude the linkage position of sialic acid using these two enzymes as follows: (1) When sialyl PA-oligosaccharide was cleaved by α2,3-sialidase in condition 1, sialic acid was concluded to be linked to the terminal residue through an α2-3 linkage. (2) When sialyl PA-oligosaccharide was cleaved by α2,3-sialidase in condition 2 but not in condition 1, sialic acid was concluded to be linked to the terminal residue through an α2-6 linkage. (3) When sialyl PA-oligosaccharide was cleaved by *Arthrobacter* α-sialidase in condition 1 but not by α2,3-sialidase in condition 2, sialic acid was concluded to be linked to a non-terminal residue.

PA-oligosaccharides were digested with 10 U/mL of α-fucosidase from bovine kidney (Sigma-Aldrich) in 100 mM sodium acetate buffer, pH 5.5, for 16 h at 37°C. All the reactions were terminated by boiling the solutions for 3 min.

Molecular cloning of Lewis gene (FUT3) alleles

PCR was used to amplify the coding region of the Lewis gene along with the flanking regions immediately 5′- and 3′- of the gene from six patients using the sense primer 5′-GAAACAGGAATAATAGCAGCTCC-3′ and antisense primer 5′-GTTGGCCACAAAGGACTCCAG-3′. Genomic DNA was extracted from normal rectal and pancreatic tissues with the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). Polymerase chain reactions consisted of 1× PCR buffer (TOYOBO, Osaka, Japan), 1 mM $MgSO_4$, 0.2 mM dNTP each, 0.02 U/μL KOD Plus DNA polymerase (TOYOBO), 0.5 μM each of sense and antisense primer, and Genomic DNA in a 20-μL reaction volume. The PCR program included hot start at 94°C for 2 min, followed by 35 cycles with 15 s at 94°C, 30 s at 56°C, and 1 min at 68°C. The PCR products were sequenced directly in their entirety using the BigDye Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems, Foster City, CA) with the 3100 Genetic Analyzer (Applied Biosystems). Furthermore, PCR products from a heterozygote were ligated into pTA2 TA-cloning vector (TOYOBO) according to the manufacturer's protocol. Positive clones were selected by blue-white screening, and both strands of their inserts were sequenced. We used oligonucleotides corresponding to flanking plasmid sequences and primers corresponding to internal sequences of the wild-type FUT3.

Lewis enzyme (α1-4 fucosyltransferase) activity

Highly purified cancer and normal epithelial cells were washed with phosphate-buffered saline, resuspended in 50 μL of 1% Triton X100, 20 mM HEPES (pH 7.4) and then subjected to sonication to obtain the cell homogenate. After centrifugation at 12,000 × *g* for 10 min, the supernatants were used as enzyme sources. The supernatants (1 μL) were assayed for fucosyltransefarse activities in 50 mM cacodylate buffer, pH 6.5, 5 mM ATP, 25 mM $MnCl_2$, 10 mM L-fucose, 75 μM GDP-fucose, and 1 mM PA-Lc_4 (a total volume of 5 μL). The reaction mixtures were incubated at 37°C for 2 h and terminated by boiling for 3 min. After centrifugation at 12,000 × *g* for 10 min, the supernatants were subjected to size fractionation HPLC.

Supplementary data

Supplementary data mentioned in the text is available to subscribers in *Glycobiology* online.

Acknowledgments

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Abbreviations

GSL, glycosphingolipids; Gu, glucose units; SLe^a, sialyl Le^a; SLe^x, sialyl Le^x; ST1H, α2-6 sialylated type1H; ST2H, α2-6 sialylated type 2H.

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Involvement of ST6Gal I in the biosynthesis of a unique human colon cancer biomarker candidate, α2,6-sialylated blood group type 2H (ST2H) antigen

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The α2,6-sialylated blood group type 2H (ST2H) antigen (Fucα1-2(NeuAcα2-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) is a fucoganglioside found in human colon cancer tissues. To elucidate an enzyme responsible for the ST2H antigen formation, we screened some partially purified candidate enzymes, α2,6-sialyltransferases, ST6Gal I and ST6Gal II, and α1,2-fucosyltransferases, FUT1 and FUT2 for their activities towards pyridylaminated type 2H (Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA) or LS-tetrasaccharide c (LST-c: NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA) as acceptor substrates. Here we show the ST6Gal I transfers NeuAc from the donor CMP-NeuAc to the terminal Gal of PA-type 2H, which formed the ST2H antigen, but the others could not synthesize it. Using a recombinant ST6Gal I, enzymatic reactions with two types of acceptors, PA-type 2H and PA-lacto-*N*-neotetraose (LNnT), were kinetically analysed. On the basis of catalytic efficiency (V_{max}/K_m), the specificity of ST6Gal I towards the PA-type 2H was estimated to be 42 times lower than that for PA-LNnT. The overexpression of ST6Gal I in human colon cancer DLD-1 cells effectively resulted in the ST2H antigen formation, as judged by LC-ESI-IT-MS. Many lines of evidence suggest the up-regulation of ST6Gal I in human colon cancer specimens. Collectively, these findings indicate that ST6Gal I is responsible for ST2H antigen biosynthesis in human colon cancer cells.

Keywords: α1,2-fucosyltransferase/α2,6-sialyltransferase/colon cancer/fucoganglioside/ST2H antigen.

Abbreviations: CID, collision-induced dissociation; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FUT, fucosyltransferase; GD3, NeuAcα2-8NeuAcα2-3Galβ1-4Glc; IT, ion trap; LC, liquid chromatography; Le^a, Galβ1-3(Fucα1-4)GlcNAc:Le^x, Galβ1-4(Fucα1-3)GlcNAc; Le^y, Fucα1-2Galβ1-4(Fucα1-3)GlcNAc; MS, mass spectrometry; nLc$_4$, lactoneotetraosyl-ceramide; NP, normal phase; PNGase F, peptide *N*-glycosidase F; RP, reversed phase; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SH, α2,6-sialylated blood group H; ST2H, α2,6-sialylated blood group type 2H (Fucα1-2(NeuAcα2-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc); ST6Gal, β-galactoside α2,6-sialyltransferase; Type 1, Galβ1-3GlcNAc; Type 2, Galβ1-4GlcNAc.

It is well known that glycans on a cell surface or in extracellular space play important roles in cellular differentiation, adhesion and proliferation (*1*, *2*). The biosynthesis of glycans is tissue-specific and is regulated under physiological and pathological conditions (*3*–*5*). Alterations in the oligosaccharide structures of glycosphingolipids (GSLs) in tumour cells occur in essentially all types of human cancer (*6*, *7*). A series of GSLs aberrantly accumulated in cancerous tissues have been isolated and characterized (*8*–*11*) and it has been revealed that each type of tumour is characterized by an accumulation of specific types of GSLs having types 1 or 2 chain derivatives such as Lewis^a (Le^a), Lewis^x (Le^x), Lewis^y (Le^y) or dimeric Le^x and their sialylated derivatives, observed in most human adenocarcinomas (*9*, *11*–*13*), while GD3 has been observed in melanomas (*14*). A subsequent series of studies have indicated the functional significance of aberrant gycosylation in cancer malignancy, such as metastasis and invasion (*6*, *7*, *15*). Furthermore, our previously designed studies showed that a unique human colon cancer biomarker candidate, α2,6-sialylated blood group type 2H, the ST2H antigen [Fucα1-2(NeuAcα2-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer], was first found in human colon cancer tissues (*16*) and

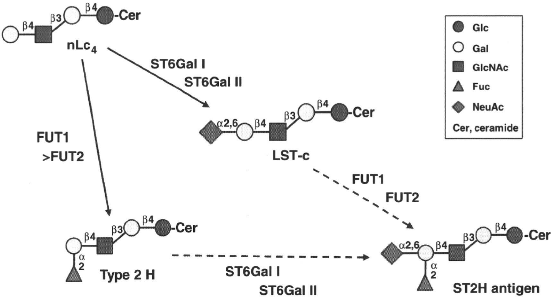

Fig. 1 Hypothetical pathway for ST2H antigen biosynthesis. Solid and dotted arrows indicate the pathways that are already known and unknown, respectively. Both FUT1 and FUT2 enzymes are active on the type 2 lactosamine chain structure and capable of making the type 2H structure, but FUT2 is known to show a little activity on the type 2 chain and a strong preference for the type 1 chain (*37*).

accumulated in colon cancer cells (*17*). The ST2H antigen was detected in the cancer cells of all 5 patients having liver metastasis and 4 other patients in whom liver metastasis had not been found, out of a total of 16 patients, though the biosynthetic process and the functional significance of this antigen has not yet been made clear. In view of its simple sugar chain structure, the biosynthetic pathway of the ST2H antigen is easily predicted, as shown in Fig. 1. Two β-galactoside α2,6-sialyltransferases, ST6Gal I (*18*) and ST6Gal II (*19*, *20*), and two β-galactoside α1,2-fucosyltransferases, FUT1 and FUT2 (*21*), are candidate enzymes for the ST2H antigen biosynthesis. There are two possible routes for the ST2H antigen formation. One is that FUT1 and/or FUT2 initially act on the nLc$_4$ structure, forming an α1,2-fucosylated nLc$_4$ (type 2H) structure. ST6Gal I and/or ST6Gal II subsequently act on the formed type 2H structure, resulting in the formation of the ST2H antigen. The other possibility is that ST6Gal I and ST6Gal II initially act on the nLc$_4$ as opposed to the first predicted route, forming an α2,6-sialylated nLc$_4$ (LST-c) structure. FUT1 and/or FUT2 subsequently act on the LST-c structure, resulting in the formation of the ST2H antigen. Though the first steps of these two possible pathways, namely the processes of type 2H and LST-c formations, are already well known (*22*, *23*), the final steps of the pathways, that is, ST2H antigen formation by α2,6-sialylation of type 2H or α1,2-fucosylation of LST-c by the candidate enzymes, are really hypothetical and have not yet been characterized. Thus, it is unclear which pathway actually works in an *in vivo* situation.

In this study, we screened the candidate enzymes, ST6Gal I, ST6Gal II, FUT1 and FUT2, for their activities in making the ST2H antigen as to whether or not the enzymes actually exhibit such an activity to form the ST2H antigen. Among the four glycosyltransferases screened, ST6Gal I was found to possess an activity that transfers a NeuAc from a donor CMP-NeuAc to the terminal Gal of a pyridylaminated (PA) type 2H structure in an α2,6-linkage, resulting in the formation of a PA-ST2H antigen. Using a purified recombinant ST6Gal I, kinetic parameters related to ST2H antigen formation were also compared with those associated with LST-c formation. The ST2H antigen expression was found to be induced by overexpressing the ST6Gal I in the human colon cancer cell line, DLD-1 cells. An activity forming the ST2H antigen, that is, α2,6-sialylation of the terminal position of Gal in PA-type 2H, was found to be certainly detectable in the homogenate of human colon cancer tissue. ST6Gal I is likely to be responsible for the formation of the ST2H antigen in human colon cancer tissue.

Experimental procedures

Materials

Lacto-*N*-*neo*tetraose (LNnT, Galβ1-4GlcNAcβ1-3Galβ1-4Glc), Lacto-*N*-fucopentaose I (LNFP I, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc), Lewis-Y hexasaccharide (Le^y H, Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc) and LS-tetrasaccharide c (LST-c, NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc) were purchased from Dextra Laboratories Ltd (Reading, UK). Cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), Guanosine 5′-diphospho-β-L-fucose (GDP-Fuc), the anti-FLAG M1 antibody and FLAG peptide were from Sigma-Aldrich Co. (St Louis, MO, USA). Glycosyltransferase affinity Gel-cytidine 5′-diphosphate (CDP-Gel, ligand concentration: 15 µmol/ml) was acquired from Calbiochem-Novabiochem International Inc. (La Jolla, CA, USA). PNGase F came from Roche (Basel, Switzerland). Other common chemicals were obtained from Wako pure chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise specified.

Colon cancer tissue

The patients involved in this study had undergone simultaneous resections of primary colon tumours and liver metastases at Osaka Medical Center for Cancer and Cardiovascular Diseases (Osaka, Japan). The cancerous tissues were rapidly frozen with liquid nitrogen and stored at −80°C until use. All the studies that used clinical human samples in this study were carried out at Osaka Medical Center for Cancer and Cardiovascular Diseases. This study was approved by the Local Ethics Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases. Informed consent was obtained from the patients.

Preparation of crude enzyme extract and partially purified glycosyltransferases

Frozen human colon cancer tissue was cut to a thickness of 20 µm with a cryostat microtome (CM 1900, Leica, Milton Keynes, UK). Twenty sections were collected and homogenized in 150 µl of 10 mM Tris–HCl (pH 7.4), and 0.25 M sucrose, supplemented with a complete protease inhibitor cocktail (EDTA-free, Roche). After centrifugation at 600*g* for 5 min at 4°C, the resulting supernatant was used as a crude enzyme extract for the assay of the activity making the ST2H antigen structure. When full-length human ST6Gal I and ST6Gal II were transiently transfected into COS-1 cells, the majority of the enzyme activities expressed were found to be secreted out of the cells, as previously reported in the case of ST6Gal I transfected into COS-7 cells (*24*). Therefore, a conditioned medium of COS-1 cells transfected with each enzyme cDNA was used as an enzyme source for partial purification. Briefly, COS-1 cells were transiently transfected with pcDNA3.1/neo-hST6Gal I or pcDNA3.1/neo-hST6Gal II by electroporation (950 V, 99 µs, two times, Electro Cell Manipulator ECM 200, BTX, Holliston, MA, USA) in DMEM. Three days after transfection, the conditioned medium was collected and the protein portion was concentrated by ammonium sulphate precipitation (70% saturation). The resulting precipitate was dissolved in a small volume of 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)-NaOH buffer (pH 7.0) containing 1 mM $MnCl_2$, 0.1% CHAPS and 20% glycerol, followed by desalting with a HiTrap desalting column (5 ml, GE Healthcare UK Ltd, Buckinghamshire, UK) that had been equilibrated with the same buffer. The desalted fraction was further concentrated with an Amicon Ultra-4 centrifugal filter device (Millipore Co., Billerica, MA, USA) and then incubated with CDP-Gel (50 µl) with gentle rotation at 4°C for 4 h. The gel was thoroughly washed with the buffer and the bound proteins were eluted with a buffer containing 1 M NaCl. The eluted fraction was desalted and concentrated with a Microcon YM-10 centrifugal filter device (Millipore Co.) and then used for the activity assay. For the preparation of FUT1 and FUT2 fractions, the conditioned medium of HEK293 cells stably transfected with pFLAG-CMV-3-Dest-human FUT1 or human FUT2 was collected and the protein portion was fractionated by ammonium sulphate precipitation (50–70% saturation). The fraction was dissolved in a small volume of phosphate-buffered saline (PBS) (–) followed by desalting with a HiTrap desalting column (5 ml), pre-equilibrated with PBS (–). The desalted fraction was further concentrated with an Amicon Ultra-4 centrifugal filter device and the resulting concentrate was used for the activity assay. As a control fraction for each assay, a protein fraction was prepared from corresponding parental cells for each transfectant using the same procedure described earlier. The protein concentration was determined with a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin (BSA) as standard.

Glycosyltransferase activity assays

Glycosyltransferase activities were assayed using PA-oligosaccharide substrates. The enzyme fractions were incubated in an appropriate reaction mixture for the determination of each enzyme activity. In the assay using a crude extract of human colon cancer tissue, the activity making the ST2H antigen was assayed in a final volume of 20 µl consisting of 50 mM MES-NaOH buffer (pH 6.0), 1 mM $MnCl_2$, 100 mM L-Fuc, 1% Triton X-100, 1 mM CMP-NeuAc, 10 µM PA-type 2H and 10 µl of the tissue extract. When the partially purified soluble FLAG-tagged FUT1 and FUT2 fractions were assayed, the reaction was carried out in a final volume of 10 µl using a reaction mixture consisting of 50 mM MES-NaOH buffer (pH 6.0), 10 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/ml BSA, 5 µM PA-LNnT and 1 mM GDP-Fuc. The soluble ST6Gal I and ST6Gal II fractions were assayed in a final volume of 10 µl using a reaction mixture consisting of 50 mM MES-NaOH buffer (pH 6.0), 1 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/ml BSA, 5 µM PA-LNnT and 1 mM CMP-NeuAc. Screening of these glycosyltransferases for their activities forming the ST2H antigen was carried out under the same conditions as above for each enzyme except that the acceptor substrates used were PA-type 2H for ST6Gal I and ST6Gal II, and PA-LST-c for FUT1 and FUT2. After incubation at 37°C for 16 h, the reaction was stopped by boiling for 3 min, followed by centrifugation at 20,000*g* for 5 min. The resulting supernatant was injected into a TSKgel ODS-80TM column (0.46 × 15 cm, Tosoh) to separate and quantitate the products. The elution was performed isocratically at 55°C at a flow rate of 1 ml/min using either of two of the following mobile phase systems. Mobile phase I consisting of 50 mM acetic acid adjusted to pH 6.0 with trietylamine, containing 0.25% 1-butanol and mobile phase II consisting of 50 mM ammonium acetate to pH 6.0 containing 0.25% 1-butanol.

Electorospray ionization MS^n analysis

Mass spectra of PA-oligosaccharides were obtained on a Finnigan LCQ Deca XP ion-trap mass spectrometer (ThermoFischer Scientific, Waltham, MA, USA) equipped with a nanoESI device (AMR, Inc., Tokyo, Japan) connected to a Paradigm MS4 µHPLC system (Michrom BioResources, Inc., Auburn, CA, USA) equipped with a Magic C18 column (0.2 × 5 cm, Michrom BioResources, Inc.) as described earlier (*17*). MS^2 and MS^3 of fucose containing PA-oligosaccharides were performed using sodiated ions instead of protonated ions because intramolecular fucose rearrangements have been found in the CID spectra of protonated ions, but not in sodiated ions produced from oligosaccharides derivatized at their reducing termini with aromatic amines, such as 2-aminobenzamide, that may lead to erroneous conclusions about the oligosaccharide sequence (*25*).

Purification of soluble recombinant human ST6Gal I

COS-1 cells were transfected with the pFLAG-CMV-3-Dest-human ST6Gal I and selected for clones stably express the gene, based on resistance to 300 µg/ml of geneticine. The conditioned medium (300 ml) containing the soluble FLAG-tagged human ST6Gal I (FLAG-hST6Gal I) secreted from the clones was collected and the protein portion was concentrated by ammonium sulphate precipitation (70% saturation). The precipitate was dissolved in a small volume of 10 mM MES-NaOH buffer (pH 7.0), 0.1% CHAPS, 0.15 M NaCl, 10 mM EDTA and 20% glycerol, and then desalted with a HiTrap desalting column that had been equilibrated with the same buffer. The desalted protein fraction was concentrated with a Centriprep YM-30 centrifugal filter device (Millipore), and the resulting concentrate (7 ml) was incubated with CDP-gel (0.8 ml) with gentle rotation at 4°C for 16 h. After washing the gel with the buffer, the bound material was eluted with the buffer containing 1 M NaCl. The eluted fraction was concentrated and desalted with an Amicon Ultra-4 centrifugal filter device (0.6 ml) and the FLAG-hST6Gal I protein in the fraction was subsequently pulled down with an anti-FLAG M1 antibody-conjugated protein G Sepharose 4B (20 µl), which had been equilibrated with 10 mM MES-NaOH buffer (pH 7.0), 0.1% CHAPS, 0.15 M NaCl and 20% glycerol at 4°C for 4 h. The gel was thoroughly washed with the buffer, and the bound proteins were eluted with a buffer containing 0.1 mM FLAG peptide. The buffer of the purified FLAG-hST6Gal I fraction was replaced by 5 mM MES-NaOH (pH 7.0), 0.1% CHAPS and 20% glycerol using a Microcon YM-10, and the resulting fraction was used as an enzyme source for kinetic analyses.

Kinetic analysis of the purified FLAG-hST6Gal I

For kinetic analysis, the purified recombinant ST6Gal I (FLAG-hST6Gal I) was incubated in a reaction mixture of 10 µl consisting of 50 mM MES-NaOH buffer (pH 6.0), 0.1% CHAPS, 1 mM $MnCl_2$, 1 mg/ml BSA and various concentrations of PA-LNnT, PA-type 2H and CMP-NeuAc, at 37°C for 4 h. In all assays, the consumption of substrates was kept below 20% to ensure accurate initial rate measurements.

Expression of ST2H antigen in human colon cancer cell line, DLD-1 cells, by ST6Gal I

The human colon cancer cell line, DLD-1 cells, was maintained at 37°C in an RPMI-1640 medium supplemented with 10% foetal bovine serum, 100 U/ml of penicillin G and 0.1 mg/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO_2. The DLD-1 cells (three 10-cm-diameter dishes) were transiently transfected with pME-hST6Gal I (16 µg/10-cm-diameter dish) using a Lipofectamine 2000 reagent (Invitrogen, CA, USA) and after a 1 day culture, the cells were harvested. The transfected ST6Gal I activity in the DLD-1 cell lysate was assayed in a final volume of 10 µl consisting of 50 mM MES-NaOH buffer (pH 6.0), 1 mM $MnCl_2$, 0.5% Triton X-100, 1 mM CMP-NeuAc, 10 µM PA-LNnT and 3 µl of the cell lysate. The detection of ST2H antigen expression was performed by structural analysis of oligosaccharide moieties of acidic GSLs from the transfected cells according to a previously established method (*17*). In short, the acidic GSLs were extracted from the cell pellets (2×10^6 cells), and the oligosaccharide moieties of the extracted acidic GSLs were released by digestion with a recombinant endoglycoceramidase II from *Rhodococcus* sp. (Takara). The reducing ends of the released oligosaccharides were pyridylaminated, and the resulting PA-oligosaccharides were analysed by

NP- and RP–HPLC. The structures of the PA-oligosaccharides were assessed by 2D sugar chain mapping, and the retention time of each PA-oligosaccharide was given in glucose units (GU) based on the elution times of PA-isomaltooligosaccharides. The PA-oligosaccharides were also analysed by LC-ESI-IT-MS.

Protein expression system in cultured cells, preparation of fluorescent oligosaccharide substrates and authentic products and exoglycosidase digestion and structural analysis of enzymatic product

These sections are described in '*Supplementary Experimental Procedures*'.

Results

ST6Gal I exhibits the activity making the ST2H antigen structure

Two α2,6-sialyltrasnsferases, ST6Gal I and ST6Gal II, and two α1,2-fucoyltransferases, FUT1 and FUT2, were the candidate enzymes for ST2H antigen biosynthesis (Fig. 1). To elucidate an enzyme producing this antigen, we carried out a screening of these candidate enzymes for their activities in making the ST2H antigen *in vitro*. We partially purified the four enzymes, soluble ST6Gal I and ST6Gal II, and soluble FLAG-tagged FUT1 and FUT2, from the conditioned medium of COS-1 or HEK293 cells transfected with each enzyme cDNA, as described in the 'Experimental Procedures' section. The partially purified enzymes showed complete activities under standard assay conditions by using their common acceptor substrate, PA-lacto-*N*-neotetraose (LNnT), whereas the ST6Gal II fraction showed a slightly lower activity than the others (Supplementary Fig. S1). Using these enzyme fractions, the activity making the ST2H antigen was evaluated. It was found that when ST6Gal I was incubated with 5 μM PA-type 2H (Fucα1-2Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA) and 1 mM CMP-NeuAc at 37°C for 16 h, a small but distinct product peak estimated at 0.6% conversion of the acceptor substrate used was detected in its reaction mixture (Fig. 2A), though the others did not show any positive product peak (Fig. 2B–D). To prepare a sufficient amount of the product of the ST6Gal I reaction for structural analysis, the reaction was carried out using an increased concentration (50 μM) of the acceptor substrate, PA-type 2H, and finally 3 pmol of the product was fractionated and collected by NP- and RP–HPLC. A portion of 200 fmol of the collected product was next analysed by ESI-IT-MS. As shown in Fig. 3A–C, the MS^{1-3} spectra revealed that the product had one extra NeuAc residue linked to the acceptor, PA-type 2H. In order to confirm the type of sialyl linkage, the product was digested with linkage-specific exoglycosidases and the digested products were analysed by RP–HPLC (Fig. 3D–F). The product was sensitive to digestion with *Corynebacterium* sp. α1,2-fucosidase and the digested product was eluted at the same position as PA-LST-c (NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA), but not PA-SPG (NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA) (Fig. 3D). Moreover, the α1,2-fucosidase-digested product was resistant to an additional digestion with bovine testis β-galactosidase (Fig. 3E), indicating that the incorporated NeuAc binds to the terminal Gal residue of PA-type 2H in an α2,6-linkage. In addition, the

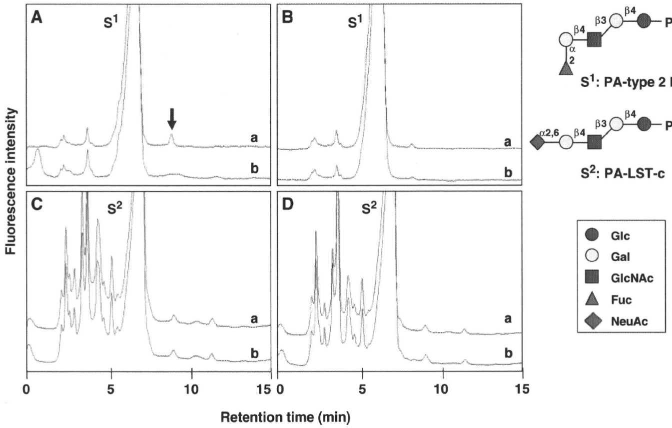

Fig. 2 The screening of candidate glycosyltransferases, ST6Gal I, ST6Gal II, FUT1 and FUT2, for their activities in forming the ST2H antigen. Reversed phase HPLC profiles of the reaction mixtures of partially purified soluble ST6Gal I (A), soluble ST6Gal II (B), soluble FLAG-tagged FUT1 (C) and soluble FLAG-tagged FUT2 (D) are shown. The elution was performed isocratically using mobile phase I, as described in the 'Experimental Procedures' section. The reactions were performed using the acceptor substrates, PA-type 2H (A and B) and PA-LST-c (C and D), together with appropriate donor substrates for each enzyme (CMP-NeuAc for ST6Gal I and ST6Gal II, GDP-Fuc for FUT1 and FUT2). The results obtained in the reaction of the enzyme fraction (a) and of the control protein fraction (b), which were prepared as in the 'Experimental Procedures' section, are shown. The arrow in (A) indicates the potential product of the ST6Gal I reaction.

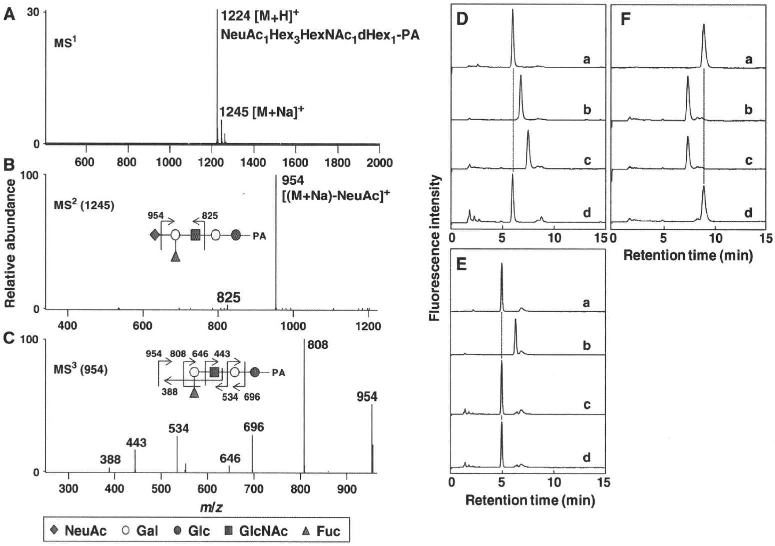

Fig. 3 Structural analysis of the product of the ST6Gal I reaction in Fig. 2A. (A) The MS^1 spectrum of the product of the ST6Gal I reaction. (B) The MS^2 spectrum of [M+Na]$^+$ precursor ion at *m/z* 1245 detected in MS^1. (C) MS^3 spectrum of [(M+Na)-NeuAc]$^+$ precursor ion at *m/z* 954 detected in MS^2. The MS$^{2-3}$ fragment ions were assigned as shown schematically. (D) Exoglycosidase digestion of the product with α1,2-fucosidase. Reversed phase HPLC profiles of (a) authentic PA-LST-c, (b) authentic PA-SPG, (c) the product without exoglycosidase digestion and (d) the product after digestion with *Corynebacterium* sp. α1,2-fucosidase are shown. (E) Sequential exoglycosidase digestions of the product with α1,2-fucosidase and β-galactosidase. Reversed phase HPLC profiles of (a) authentic LST-c, (b) the product without exoglycosidase digestion, (c) the product after digestion with α1,2-fucosidase and (d) the product subjected to digestions with α1,2-fucosidase and with following bovine testis β-galactosidase are shown. (F) Linkage analysis of the incorporated NeuAc residue in the product with site-specific sialidases. Reversed phase HPLC profiles of (a) authentic PA-type 2H, (b) the product without exoglycosidase digestion, (c) the product after digestion with *Salmonella typhimurium* LT2 α2,3-sialidase and (d) the product after digestion with *Arthrobacter ureafaciens* α2,6(3/8)-sialidase. Elution was performed isocratically using mobile phase II, as described in the 'Experimental Procedures' section.

transferred NeuAc residue was resistant to digestion with *Salmonella typhimurium* LT2 α2,3-sialidase, but was sensitive to digestion with *Arthrobacter ureafaciens* α2,6(3/8)-sialidase (Fig. 3F), indicating again that the transferred NeuAc residue has an α2,6-linkage. Taken together, these findings suggest that human ST6Gal I possesses the activity to make the ST2H antigen. ST6Gal I could not transfer a NeuAc residue to PA-LNFP I, a type 1 chain derivative of the blood group H antigen (Supplementary Fig. S2), suggesting that the *N*-acetyllactosamine unit presenting an α1,2-fucosyl residue needs to be a type 2 chain for α2,6-sialylated blood group H (SH) antigen production.

Kinetic analysis of the ST6Gal I reaction related to ST2H antigen formation

The elucidation of a kinetic factor associated with the transfer rates is important for understanding the ST6Gal I reaction regarding ST2H antigen formation. For kinetic analysis, we prepared a soluble FLAG-tagged human ST6Gal I recombinant protein (Fig. 4A), as described in the 'Experimental Procedures' section. SDS–PAGE analysis of the purified recombinant ST6Gal I showed two bands with molecular weights of 55 K and 57 K (Fig. 4B, lane 2), both of which were found to be recombinant ST6Gal I, as indicated by the immunoblot analysis using an anti-FLAG M1 antibody (Fig. 4B, lane 3). In addition, PNGase F treatment revealed that both species were *N*-glycosylated to a similar extent (Fig. 4B, lanes 4 and 5). The recombinant ST6Gal I protein was found to keep its activity that transfers a NeuAc from the donor CMP-NeuAc to two types of acceptors, PA-LNnT and PA-type 2H, and the relative activity towards PA-type 2H was estimated to be ~2% when the activity against PA-LNnT is 100% (Fig. 4C). Using this recombinant ST6Gal I, the reaction in relation to the formation of the ST2H antigen was kinetically analysed. The recombinant ST6Gal I was incubated with various concentrations of acceptors, PA-LNnT or PA-type 2H, in the presence of a fixed concentration (1 mM) of the donor substrate CMP-NeuAc (Fig. 5). As indicated by the [S]-v and double reciprocal plots

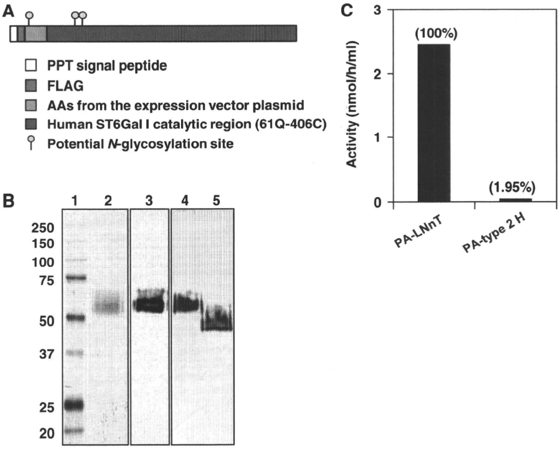

Fig. 4 Purification of the soluble *N*-terminally FLAG-tagged human ST6Gal I recombinant protein. (A) Schematic representation of the construct of the recombinant human ST6Gal I. The preprotrypsin (PPT) leader sequence directs secretion of a fusion protein into the culture medium; a FLAG-tag and some additional residues were fused at the Gln-61 position of human ST6Gal I. (B) SDS–PAGE analysis of the purified recombinant ST6Gal I. Proteins were separated on 10% SDS-gel under reducing conditions and visualized by silver staining; lanes 1 and 2 indicate a molecular size marker and the purified recombinant ST6Gal I, respectively. The recombinant ST6Gal I proteins were detected by immunoblotting using an anti-FLAG M1 antibody (lanes 3–5). In lane 5, the proteins were deglycosylated by digestion with PNGase F. (C) Acceptor substrate specificity of the purified recombinant ST6Gal I. The enzyme reaction was performed using the 5 μM acceptor substrates shown and 1 mM CMP-NeuAc for 4 h at 37°C. The number in parenthesis indicates the relative activity when the activity towards the acceptor oligosaccharide PA-LNnT is set at 100.

(Fig. 5A, B, E and F), it appeared that the enzyme was inhibited by the acceptor PA-oligosaccharide itself in a range of high concentration (>0.8 mM), and thus the down- and up-curvatures observed in the [S]-v and the double reciprocal plots, respectively, were found to be due to 'substrate inhibition' kinetics. Therefore, the concentrations of the acceptor in the linear portion of the double reciprocal plots were used to determine the kinetic parameters. With respect to the donor substrate, on the other hand, the reactions followed Michaelis–Menten-type kinetics (Fig. 5C, D, G and H). Table I summarizes the kinetic parameters for the reactions with two types of acceptor substrates. While the apparent K_m values for the donor, which were determined using two types of acceptors, were essentially the same, the K_m values for the acceptors were slightly different. The K_m value for PA-type 2H was 3.9 times higher than that for PA-LNnT, suggesting that the α1,2-fucosyl residue linked to the terminal position of Gal in the LNnT structure affects the binding of the acceptor substrate that accounts in part for the different reaction rate. On the other hand, the V_{max} value found in the analysis using PA-type 2H as the acceptor was 11.1 times lower than that for PA-LNnT. These results suggest that the lower rate of the reaction for ST2H antigen synthesis compared to that for LST-c synthesis can be ascribed to both the binding and the catalytic steps. On the basis of the catalytic efficiency (V_{max}/K_m for the acceptor), the specificity of ST6Gal I with respect to the type 2H structure was estimated to be 42 times lower than that for the LNnT structure.

Overexpression of ST6Gal I directs the expression of the ST2H antigen in DLD-1 cells

To examine whether or not the biosynthetic pathway for the ST2H antigen by ST6Gal I occurs also *in vivo*, we performed a study on overexpressing ST6Gal I in a human colon cancer cell line, DLD-1 cells. The DLD-1 cells were selected because this cell line was found to substantially express the type 2H structure on its GSLs (Supplementary Fig. S3), which is an essential precursor oligosaccharide substrate for ST2H antigen formation by ST6Gal I (Fig. 1) and, moreover, because β-galactoside α2,6-sialyltransferase activity was not detected at all in the DLD-1 cell lysate (data not shown), suggesting that ST6Gal I gene expression may be down-regulated in this cell line. The ST6Gal I cDNA or control empty vector plasmid were transfected to the DLD-1 cells, and after a 1-day culture, PA-oligosaccharides were prepared from acidic GSLs in mock- and ST6Gal I-transfected DLD-1 cells, as described in the 'Experimental Procedures' section. The expression of the transfected ST6Gal I in the DLD-1 cells was confirmed by the enzyme activity assay (ST6Gal I transfectants: 61.9 pmol/h/mg of protein; mock transfectants: not detected), and also by the detection of the expression of an LST-c structure

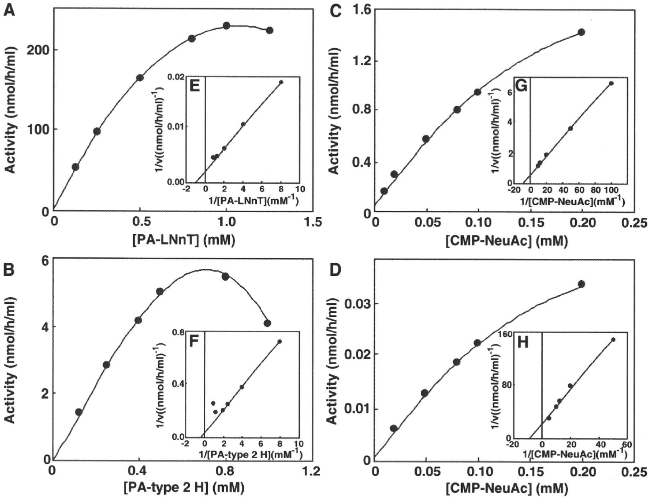

Fig. 5 Kinetic analysis of the ST6Gal I reactions involving PA-LNnT and PA-type 2H. The purified recombinant ST6Gal I activity was measured with various concentrations of acceptor substrates, PA-LNnT (A and E) and PA-type 2H (B and F), in the presence of a fixed concentration (1 mM) of donor substrate, CMP-NeuAc. The purified recombinant ST6Gal I activity was also measured with various concentrations of CMP-NeuAc in the presence of a fixed concentration (5 μM) of acceptor substrates, PA-LNnT (C and G) and PA-type 2H (D and H). The results are shown in the form of [S]-v (A–D) and double reciprocal plots (E–H).

Table I. Kinetic parameters for the ST6Gal I reactions with PA-LNnT and PA-type 2H.

	Acceptor			Donor[a]
Acceptor substrate	V_{max} (nmol/h/ml)	K_m (mM)	V_{max}/K_m (%)	app. K_m (mM)
PA-LNnT	455	0.9	500 (100)	0.11
PA-type 2H	41	3.5	12 (2.4)	0.10

[a] CMP-NeuAc, parameters were determined in the presence of 5 μM of the acceptor substrates.

in the acidic GSLs (Supplementary Fig. S4). The prepared PA-oligosaccharides from both mock- and ST6Gal I-transfected cells were first fractionated by NP-HPLC (Fig. S4). In our previous studies (*16*, *17*), the retention times, given in GUs with respect to the PA-ST2H antigen in NP- and RP-HPLC, namely, a 2D map, have already been proven to be 4.74 and 5.35, respectively. Thus, we collected a broad peak detected around GU 4.74 by NP-HPLC (Supplementary Fig. S4), and further separated it by RP–HPLC. As shown in Fig. 6, three major PA-oligosaccharide peaks were detected in both mock and ST6Gal I transfectant-derived fractions by RP–HPLC. The peaks G2 and G2′ from mock- and ST6Gal I-transfected cells, respectively, were eluted at the position of GU 5.35, which was the same value as the PA-ST2H antigen, and the relative expression level of G2′ was found to be significantly increased compared to the level of G2. To confirm ST2H antigen formation in the ST6Gal I-transfected DLD-1 cells, the peaks G2 and G2′ were next analysed by LC-ESI-IT-MS (Fig. 7). G2 was confirmed to be a single oligosaccharide species by mass chromatography (Fig. 7A), and to have an *m*/*z* value of 1369 $[M+H]^+$, $NeuAc_2Hex_3HexNAc_1$-PA (Fig. 7B), indicating that G2 was not an ST2H antigen, and that the DLD-1 cells exhibited a basal expression of this type of sugar chain in their acidic GSLs. The peak G2′, on the other hand, was found to be a mixture of two oligosaccharide species (Fig. 7C) having *m*/*z* values of 1369 $[M+H]^+$, which was estimated to be the same species as G2, and 1223 $[M+H]^+$, $NeuAc_1Hex_3HexNAc_1dHex_1$-PA, which was a PA-ST2H antigen (Fig. 7D), indicating that the ST2H antigen was surely biosynthesized in the ST6Gal I-transfected DLD-1 cells. The structure of the PA-ST2H antigen in G2′ was further confirmed by MS^2 analysis (Fig. 7E). The observed increase in the level of G2′ as compared to the level of G2 (Fig. 6) can therefore be ascribed to the increase in the newly synthesized ST2H antigen in the ST6Gal I transfectants. Collectively, these findings demonstrated that the biosynthetic pathway of the ST2H antigen by ST6Gal I certainly occurs *in vivo*.

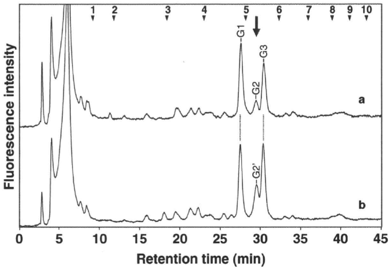

Fig. 6 Comparison of the RP-HPLC profiles of the acidic PA-oligosaccharides detected at around NP-GU 4.74 in Supplementary Fig. S4 from mock- (a) and ST6Gal I-transfected (b) DLD-1 cells. Three kinds of major PA-oligosaccharide peaks were detected and termed G1–G3. G2′ is so named to distinguish it from G2. The numbered arrowheads indicate the elution positions of PA-isomaltooligosaccharides with corresponding degrees of polymerization. The elution position of the PA-ST2H antigen (GU 5.35) is indicated by an arrow. G1 was found to have an *m*/*z* value of 1332 $[M+\text{triethylamine}]^+$ by the LC-ESI-IT-MS analysis, and its composition was estimated to be $(HSO_3)_1Hex_4HexNAc_2$-PA. G3 was found to possess an *m*/*z* value of 1369 $[M+H]^+$, and its composition was estimated to be $NeuAc_2Hex_3HexNAc_1$-PA. G3 in the ST6Gal I-transfected cells was found to contain a component of G2′ by the LC-ESI-IT-MS analysis. Thus, the increase in the level of G3 in the ST6Gal I transfectants, compared to the mock transfectants, can be thought to be ascribed to the overlap of the peaks G2′and G3. The peaks appeared at 2–25 min due to contaminating materials.

The ST2H antigen-producing activity can be detected in human colon cancer tissue homogenate

In order to examine whether the activity in making the ST2H antigen is actually detectable in human colon cancer tissue (which is the only tissue so far known to express the ST2H antigen), we carried out an exploration of the activity in the human colon cancer tissue homogenate. It was found that the ST2H antigen-producing activity was actually detected in the human colon cancer tissue homogenate when the homogenate was incubated with 10 μM PA-type 2H and 1 mM CMP-NeuAc at 37°C for 16 h (Fig. 8A, label P). MS^{1-3} spectra of the product further confirmed ST2H antigen formation by the tissue extract (Fig. 8B–D).

Discussion

It has been reported that glycosphingolipids (GSLs) having type 2 lactosamine chain derivatives, i.e. those with Le^x, Le^y, dimeric Le^x and their sialylated derivatives are accumulated in human colon cancer (*9*, *11*, *13*). In addition, we previously reported that a unique and a novel fucoganglioside structure, the ST2H antigen was also accumulated in human colon cancer cells (*17*). However, until now, little is known about the enzymatic basis for ST2H antigen biosynthesis and the biological function(s) of this newly identified fucoganglioside structure. In this study, as the first trial to elucidate an enzymatic basis for ST2H antigen biosynthesis, we have explored the enzyme responsible for the formation of the ST2H antigen. We first planned to purify the activity forming the ST2H antigen from human colon cancer tissues, but we could not obtain substantial amount of the cancer tissues as an enzyme source. Next, we tried to explore the human colon cancer cell lines expressing the ST2H antigen by screening the cell lines, such as WiDr, HCT-116, Colo-320, HT-29 and DLD-1 cells, but none of them were found to express this antigen (the data for DLD-1 cells are shown in Supplementary Fig. S3, and the others are not shown). Therefore, we decided to screen candidate known glycosyltransferases, two α2,6-sialyltrasnsferases, ST6Gal I and ST6Gal II, and two α1,2-fucoyltransferases, FUT1 and FUT2, for their activities in making the ST2H antigen (Fig. 1), and have revealed that among the four candidate enzymes, ST6Gal I really exhibits the activity that makes the ST2H antigen by transferring a NeuAc from the donor CMP-NeuAc to the terminal position of Gal in the acceptor type 2H structure via an α2,6-linkage (Figs 2A and 3). To our knowledge, this is a novel acceptor substrate specificity of the mammalian ST6Gal I that is directly associated with ST2H antigen formation and has not previously been reported by others. Contrary to this result, neither FUT1 nor FUT2 exhibited the activity that transfers an α1,2-fucosyl residue onto the terminal Gal of the LST-c structure in this experiment, which is another possible pathway for ST2H antigen biosynthesis (Fig. 2C and D). These findings suggest that the pathway for ST2H antigen formation proceeds via α2,6-sialylation of the type 2H structure by ST6Gal I, rather than via α1,2-fucosylation of the LST-c structure by FUT1 or FUT2. The reaction order of these enzymes with respect to ST2H antigen biosynthesis is accordingly predicted as follows. FUT1 or FUT2 initially act on the nLc_4 structure and the essential precursor oligosaccharide of the ST2H antigen, that is, the type 2H structure, is synthesized. ST6Gal I subsequently acts on the type 2H structure, resulting in the formation of the ST2H antigen. As for ST6Gal II, we could not prepare an enzyme fraction with an activity comparable to the other candidate enzymes in the standard assay (SupplementaryFig. S1B) because of the difficulty in purifying it in high yield. Although Takashima *et al.* (*19*) previously reported that ST6Gal II gene expression was not detected in human colon cancer tissues by semiquantitative RT–PCR on commercially available human multiple-tissue cDNA panels, and that ST6Gal II could not transfer a sialic acid to ceramide-conjugated oligosaccharide substrates such as paragloboside (Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), re-evaluation of ST6Gal II regarding its ability to form the ST2H antigen using an enzyme preparation having higher activity than that used in this study might be necessary for obtaining a more definite result.

It appears that the blood group H antigen structure needs to be a type 2 lactosamine chain derivative for SH antigen formation because of the inability of ST6Gal I to transfer an α2,6-sialyl residue onto a type 1 chain derivative of the blood group H antigen, LNFP I (Supplementary Fig. S2B). The result is

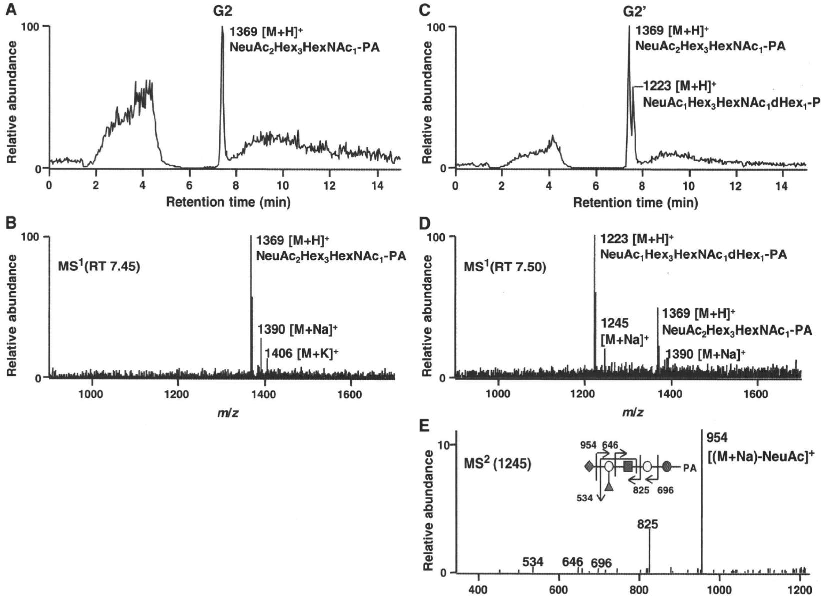

Fig. 7 LC-ESI-IT-MS analyses of peaks G2 and G2′ in Fig. 6. (A) Mass chromatograms of G2 and (C) G2′. The peaks appeared at 2–5 min due to contaminating materials. (B) Representative MS^1 spectra of G2 at retention time (RT) 7.45 min and (D) G2′ at RT 7.50 min. (E) MS^2 spectra of $[M+Na]^+$ precursor ion at *m/z* 1245 detected in MS^1 of D. The MS^2 fragment ions were assigned as shown schematically. The fragment ions with numbered mass values in panel E are sodium adduct ions.

compatible with our present finding that a type 1 chain derivative of the SH antigen has not been discovered in colon cancer tissues and with previous reports on the acceptor substrate specificity of human ST6Gal I that the enzyme hardly transfers a sialyl residue onto a type 1 lactosamine chain compared to that for the type 2 chain (*18*, *26*).

In this study, we tried to explore human colon cancer cell lines expressing the ST2H antigen by screening several colon cancer cell lines, such as WiDr, HCT-116, Colo-320, HT-29 and DLD-1 cells, but none of them were found to express the ST2H antigen on their GSLs. Thus, the human colon cancer cells in human colon cancer specimens are the only examples of the cells so far known to exhibit ST2H antigen expression. Although the cell lines expressing the ST2H antigen have not yet been identified, the DLD-1 cells, among the five examined cell lines, were found to exhibit a substantial expression of the type 2 lactosaminyl chain (*neo*lacto-series) and type 2H structures on their GSLs (Supplementary Fig. S3), whereas the others did not substantially express these structures (data not shown). Furthermore, β-galactoside α2,6-sialyltransferase activity and a representative ST6Gal I product, LST-c structure were not detected at all in the DLD-1 cells, and this may suggest a down-regulation of ST6Gal I gene expression in this cell line. We postulated that the transfection of ST6Gal I cDNA into the DLD-1 cells may induce ST2H antigen expression in the cells by the reaction of the transfected ST6Gal I with an endogenously expressed type 2H structure. As we had expected, the overexpression of ST6Gal I could induce ST2H antigen expression in the DLD-1 cells (Figs 6 and 7), directly demonstrating that the biosynthetic pathway for the ST2H antigen by the enzyme proceeds *in vivo*.

Kinetic analysis using purified recombinant ST6Gal I revealed that the presence of an α1,2-fucosyl residue on the terminal position of Gal in the LNnT structure affects both the binding and, more profoundly, the catalytic steps of the ST6Gal I reaction. The catalytic efficiency with respect to the production of the ST2H antigen has been found to be 42 times lower than that for the LST-c structure. This lower specificity of ST6Gal I towards type 2H structure might explain one of the reasons why the ST2H antigen was present only in quite small quantities in human colon cancer

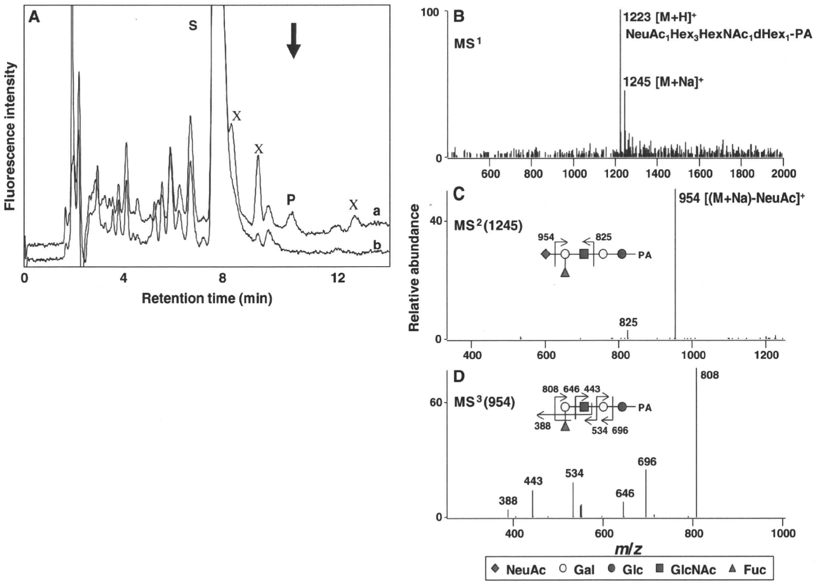

Fig. 8 The activity forming the ST2H antigen in human colon cancer tissue. (A) A reversed phase HPLC profile of the reaction mixture of the homogenate of human colon cancer tissue with the use of PA-type 2H and CMP-NeuAc as the acceptor and donor substrates, respectively, is shown. Elution was performed isocratically using mobile phase I, as described in the 'Experimental Procedures' section. The results in the reactions with (a) or without (b) the donor substrate are shown. Labels S and P indicate the peaks of acceptor substrate PA-type 2H and the product PA-ST2H antigen, respectively. The arrow indicates the elution position of the authentic PA-ST2H antigen. Xs indicate the unidentified peaks, which might not be PA-oligosaccharides, since these peaks did not exhibit typical saccharide spaced ladder signals in the MS^2 analysis (data not shown). (B) The MS^1 spectrum of the product P. (C) The MS^2 spectrum of $[M+Na]^+$ precursor ion at *m/z* 1245 detected in MS^1(D) MS^3 spectrum of $[(M+Na)\text{-NeuAc}]^+$ precursor ion at *m/z* 954 detected in MS^2. The MS$^{2-3}$ fragment ions were assigned as shown schematically.

tissues compared to other ST6Gal I products, such as LST-c (IV^{6}NeuAcα-nLc$_4$), IV^{6}NeuAcα-nLc$_6$, IV^{6}NeuAcα,III^{3}Fucα-nLc$_6$ and so on (*16*). Moreover, the finding might also suggest that a relatively higher level of ST6Gal I activity than that in normal cells is necessary for producing the ST2H antigen in colon cancer cells.

There are many lines of evidence that suggest the up-regulation of ST6Gal I in the malignant transformation of human colon epithelial cells. The increase in β-galactoside α2,6-sialyltransferase activity in human colon cancer specimens was first reported by Dall'Olio *et al.* (*27*) and thereafter confirmed by other groups and our group (*17*, *28*, *29*). The up-regulation of α2,6-saialyltransferase activity in human colon cancer seems to be consistent with its mRNA level. Several groups have examined the transcript level of glycosyltransferase genes in human colon cancer by oligonucleotide arrays (*30*) or a semiquantitative RT–PCR (*31*, *32*) and have reported that ST6Gal I gene expression was markedly enhanced in malignant transformation. In addition, the accumulation of glycoconjugates having α2,6-sialylated lactosaminyl structures in human colon cancer, which was detected using specific monoclonal antibodies, lectins or 2D sugar chain mapping and mass spectrometry, has been reported (*17*, *33–36*). All the above findings equally suggest the up-regulation of ST6Gal I in human colon cancer, and a milieu preferable for ST2H antigen biosynthesis is likely to emerge in human colon cancer cells.

In summary, this study has demonstrated that human ST6Gal I possesses a novel acceptor substrate specificity that has not so far been reported by others, that is, α2,6-sialylation of the terminal position of Gal in the type 2H structure, directly related to ST2H antigen formation. The biosynthesis of the ST2H antigen by ST6Gal I has been demonstrated to surely proceed *in vivo*, and ST2H antigen expression can be certainly induced by the action of ST6Gal I in the human colon cancer cell line, DLD-1 cells. ST6Gal I would be responsible for the formation of the ST2H antigen in human colon cancer tissues through this novel acceptor substrate specificity. However, the functional aspect of this antigen in human colon cancer cells remains unclear. Further studies are needed to clarify the biological function(s) of the ST2H antigen in human colon cancer cells. We believe that the finding concerning the enzymatic basis for ST2H antigen formation elucidated in this study will be beneficial for performing functional analyses of the ST2H antigen using gene manipulation methods, such as overexpression and knock down of the related glycosyltransferases.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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