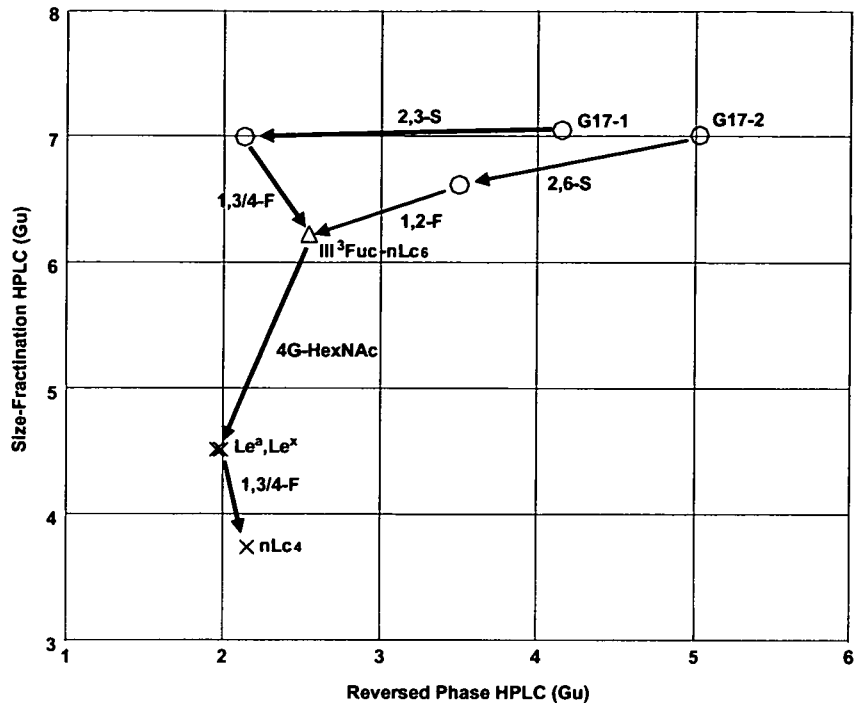

Fig. 5. Sequential digestion of PA-glycans G17-1 and G17-2. Circles indicate the positions of G17-1, G17-2, and their digests. Thick-line and thin-line arrows indicate the directions of the changes after glycosidase digestion of G17-1 and G17-2, respectively. Glycosidases are shown beside each line. Enzyme abbreviations and Xs are the same as in Fig. 3. The triangle represents the reference compound, III^{3}Fuc-nLc6, which was obtained in the analysis of the structure of G14-1 and G15.

of G15, as determined by α2,3-sialidase digestion as described above. After sialidase digestion, G14-1 and G15 had the same elution position (2.51, 6.23), whereas the coordinates of the desialylated product of G14-2 were (2.49, 6.12). These results indicate that the desialylated structures of G14-1 and G15 are the same but are different from desialylated G14-2. After sequential digestion with β1,4-galactosidase, β-*N*-acetylhexosaminidase and α1,3/4-fucosidase, the digestion products of G15 changed into the reference compound nLc_4. From these results, the structure of G15 is tentatively predicted to be NeuAcα2-6Galβ1-4HexNAc-Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc. After the fucosyl residue linked to the proximal GlcNAc of G15 was digested with bovine kidney fucosidase, the defucosylated product of G15 was cleaved by endo-β-galactosidase. The products of the digestion showed two peaks by HPLC, corresponding to Glc-PA and GlcNAcβ1-3Galβ1-4Glc-PA. The estimated structures of G15 and G14-1 are presented in Table 3. On α1,3/4-fucosidase digestion, the coordinates of desialylated G14-2 coincided with those of Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (nLc_6), as was determined in the above experiment, suggesting the structure of G14-2 to be NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (Table 3). These structures are consistent with MS/MS analysis data (data not shown).

Structure of G16-1, G16-2, and G16-3

Sialic acid is linked α2-3 to the terminal residue of G16-1, G16-2, and G16-3, as determined by α2,3-sialidase digestion. After sialidase digestion, G16-1 and G16-2 were converted to species with the same coordinates (3.66, 6.88) and G16-3 was converted to a species with coordinates (4.20, 6.88). These results indicate that G16-1 and G16-2 have the same backbone structure, which is different from that of G16-3. Desialylated G16-3 was sequentially digested with β1,4-galactosidase and β-*N*-acetylhexosaminidase twice. The first and second sequential digests gave products that corresponded to nLc_6 and nLc_4, respectively. This result indicated the structure of G16-3 to be NeuAcα2-3Galβ1-4HexNAc-Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc. Endo-β-galactosidase treatment of G16-3 produced two major peaks by HPLC, corresponding to GlcNAcβ1-3Galβ1-4Glc-PA and GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-PA. These results indicate that the subterminal HexNAc is GlcNAc linked β1-3 to galactose. On the basis of these results, the structure of G16-3 is estimated to be as listed in Table 3.

Sequential digestion of desialylated G16-2 with β1,4-galactosidase and then β-*N*-acetylhexosaminidase released two galactose and two HexNAc residues, respectively, as measured by MS (data not shown). The products of these digests coincided with the position of the reference com-

pound nLc_4 These results indicate that G16-1 and G16-2 contain two side chains, NeuAcα2-3Galβ1-4HexNAc and Galβ1-4HexNAc, which are linked together to the terminal galactose of nLc_4. When intact G16-2 was trimmed on a nonsialylated branch with β1,4-galactosidase and β-*N*-acetylhexosaminidase, the product was identical to G12. These

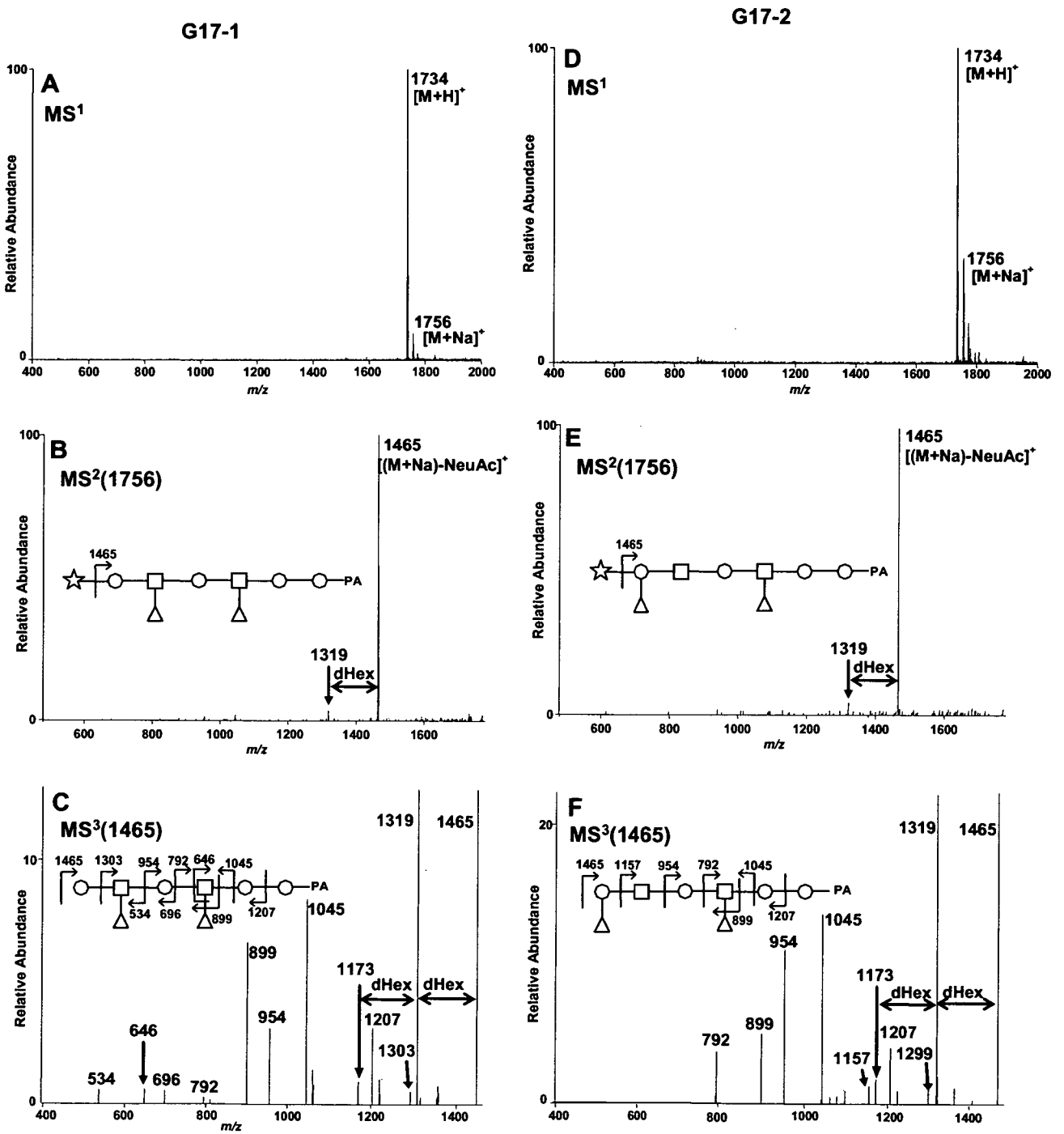

Fig. 6. MS^{1-3} spectra of G17-1 and G17-2. (A,D) MS^1 spectra of G17-1 (A) and G17-2 (D). (B,E) MS^2 spectra of $[M+Na]^+$ precursor ion at *m/z* 1756 detected in MS^1 of panel A (B) and panel D (E). (C,F) MS^3 spectra of $[(M+Na)\text{-NeuAc}]^+$ precursor ion at *m/z* 1465 detected in MS^2 of panel B (C) and panel E (F). Mass spectra in panels C and F are magnified to demonstrate clearly the minor product ions such as at *m/z* 534, 1157, and 1303. The *y* axes in panels C and F are labeled with the most abundant ions at *m/z* 1319 as 100%. Relative abundances of product ions at *m/z* 1465 in panels C and F are 38 and 45%, respectively, in these scans. Fragment ions numbered mass values in panels B, C, E, and F are sodium adduct ions. The MS/MS fragment ions were assigned as shown in Fig. 4.

results suggest the position of sialylation in G16-2 to be at the terminal galactose of the Galβ1-4GlcNAc chain on the side of the β1-3 linkage. In G16-1, the position of sialylation is at the terminal galactose of another Galβ1-4HexNAc chain (Table 3). We speculate another HexNAc to be GlcNAc and β1-6 linked to galactose; however, from the results of this study, we are unable to demonstrate this unambiguously. The structures of G16-1, G16-2, and G16-3 are consistent with MS/MS analysis data (data not shown).

Structure of G17-1 and G17-2

Sialic acid is linked α2-3 and α2-6 to the terminal residues of G17-1 and G17-2, respectively, as demonstrated by sialidase digestion as described above (Fig. 5). Desialylated G17-1 could be digested with α1,3/4-fucosidase. In contrast, desialylated G17-2 could be digested with α1,2-fucosidase but not with α1,3/4-fucosidase. The products of both digests were identical to Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-PA, which was determined as described above (Fig. 5). The digestion product from G17-1 was sequentially digested with β1,4-galactosidase, β-*N*-acetylhexosaminidase, and α1,3/4-fucosidase to yield a product identical to nLc_4 (Fig. 5). From these results, the structures of G17-1 and G17-2 are estimated to be as shown in Table 3. The structure of G17-2 has the same terminal structure as G10-2, NeuAcα2-6(Fucα1-2)Galβ1-4GlcNAcβ1, and has also not been reported previously. These structures were also confirmed by MS/MS analysis. Product ions in MS^3 spectra revealed the core structure of G17-1 and G17-2 to be Hex-HexNAc-Hex-HexNAc-Hex-Hex-PA (Fig. 6). Fucosylation of the inner HexNAc residue of G17-1 and G17-2 was suggested by the detection of a characteristic product ion at *m/z* 792, corresponding to $[dHex\text{-}HexNAc\text{-}Hex\text{-}Hex\text{-}PA+Na]^+$ in MS^3 spectra of G17-1 and G17-2. The position of attachment of the remaining fucose residue is also demonstrated by careful examination of product ions of MS^3 spectra. Product ions at *m/z* 1303, corresponding to $[dHex\text{-}HexNAc\text{-}Hex\text{-}(dHex\text{-})HexNAc\text{-}Hex\text{-}Hex\text{-}PA+Na]^+$, and at *m/z* 534, corresponding to $[Hex\text{-}HexNAc\text{-}dHex+Na]^+$, were detected in MS^3 spectra of G17-1, suggesting that the remaining fucose is linked to the subterminal HexNAc in G17-1. In contrast, in MS^3 spectra of G17-2, the product ion at *m/z* 1303 was not detected; instead, a product ion at *m/z* 1157, corresponding to [HexNAc-Hex-(dHex)HexNAc-Hex-Hex-PA], was detected, suggesting the attachment of the remaining fucose is to the outermost Hex in G17-2.

Discussion

Enzymatic release of carbohydrate moieties and fluorescent labeling, followed by 2-D mapping, MS/MS, and glycosidase digestion, revealed the structures of major acidic GSLs in colon adenocarcinoma. We were able to identify 22 kinds of acidic GSLs from approximately 20 mg of tissue. One of those identified is sulfated (G1), and the others are sialylated, including ganglio-series (G2, G3, G4, G5, G7, and G9), lacto-series (G11), and neolacto-series (G6, G8, G10-1,2, G12, G13, G14-1,2, G15, G16-1,2,3, and G17-1,2) GSLs. The structures of acidic GSLs in colon adenocarcinoma have been widely studied using a number of conventional methods, such as TLC and methylation analysis, and various kinds of acidic GSLs have been reported to be present in the tissues [8,11,13,15]. The acidic GSLs identified in this study include most of the acidic GSLs previously reported to be present in the tissue. For example, as major components, GM3 (G2), SPG (G6), $IV^6NeuAc\alpha\text{-}nLc_4$ (G8), SLe^x (G10-1), SLe^a (G11), $VI^3NeuAc\alpha\text{-}nLc_6$ (G12), and $VI^6NeuAc\alpha\text{-}nLc_6$ (G13) were identified, whereas $VI^3NeuAc\alpha III^3Fuc\alpha\text{-}nLc_6$ (G14-1), $VI^6NeuAc\alpha III^3Fuc\alpha\text{-}nLc_6$ (G15), and $VI^3NeuAc\alpha,V^3Fuc\alpha III^3Fuc\alpha\text{-}nLc_6$ (G17-1) were present as minor components. SM3 (G1) and GD1b (G9) have not been detected in the tissues in the previous studies. Furthermore, it is noteworthy that 2 novel fucogangliosides, G10-2 and G17-2, having a common carbohydrate moiety at their termini, namely NeuAcα2-6(Fucα1-2)Galβ1-4GlcNAc, were identified. To our knowledge, this unique structure has not been found in glycoproteins as well as in GSLs. In addition to G10-2 and G17-2, the structure of G16-1 has not been reported previously. Although the structures of G16-1 and G16-2 were not unambiguously determined in the current study, it is most likely that the undetermined HexNAc residue is GlcNAc linked β1-6 to terminal galactose of nLc_4 Assuming the above to be correct, G16-2 has been previously isolated from human erythrocytes [21], but G16-1 has not been isolated from any source. Several acidic GSLs, such as sulfatide and disialosyl lacto-series gangliosides (reported to be present in colon cancer tissues [11,13,15]), were not detected in this study. Sulfatide could not be detected in this study because of its high level of resistance to endoglycoceramidase II [16]. The reason for the lack of detection of the other gangliosides is not clear but may arise from sample variation.

There are two possible reasons why the newly identified acidic GSLs, G10-2, G16-1, and G17-2, have been undiscovered previously. One is that the quantities of these gangliosides are quite small. However, this is unlikely given that G17-1, which is present in this tissue at nearly equivalent levels to the newly characterized acidic GSLs seen in this analysis, previously have been isolated and characterized from adenocarcinoma of colon metastases to liver, that is, the same sample used in this study. The other possibility is the potential error in identifying acidic GSLs by TLC. These acidic GSLs might not be separated and purified to homogeneity by HPLC using organic solvents and TLC, probably due to the existence of one or more other components, such as their isomers, with identical chromatographic behavior. Even in our study, G10-2, G16-1, and G17-2 coeluted on the size fractionation column with their isomers, G10-1, G16-2, and G17-1, respectively.

It was only through the use of another chromatography method (an ODS column) that the species could be separated from each other. These results show that the high-resolution techniques employed here, consisting of pyridylamination of carbohydrate moieties of acidic GSLs followed by separation on two different kinds of chromatography column (amide and ODS), enabled the isolation and characterization of novel GSLs even in a comprehensively studied tissue. Therefore, the results encourage us to look for unique structures that may be present in other types of tumor tissues using this assay. However, it should be noted that of the 22 kinds of GSLs identified in this analysis, 12 did not match to standard oligosaccharides previously prepared and analyzed on the 2-D map. To generally apply the method used in this study to a variety of cancerous tissues, the number of prepared standard oligosaccharides would seem to be insufficient, and it is essential to obtain many more standard PA-oligosaccharide libraries for 2-D mapping. Furthermore, libraries of multistage MS^n spectra of *N*-linked oligosaccharides have been constructed and have served to identify structures [22,23]. A similar approach might also be necessary for the glycomic analysis of GSLs.

Two α2-6-sialyltransferases, ST6Gal-I [24] and ST6Gal-II [25], and two α-2-fucosyltranserases, FUT I and FUT II [26], are the candidate enzymes for the biosynthesis of the unique terminal structures of G10-2 and G17-2. It is necessary to clarify the specificities of the α2-6-sialyltransferases and the α-2-fucosyltranserases to elucidate the involvement of these glycosyltransferases in forming these carbohydrate structures. Furthermore, it is also important to determine whether or not the newly characterized G10-2 and G17-2 are tumor-associated antigens. Preparation of antibodies recognizing the unique structure common to their termini, NeuAcα2-6(Fucα1-2)Galβ1-4GlcNAc, may be useful for immunohistochemical analysis of cancerous and normal tissues.

The relative quantities of each acidic GSL were revealed in detail, showing ranges from 0.1 to 58.0% of the total. However, in the previous studies, it was impossible to analyze the quantity precisely because they were analyzed by densitometry or estimated from the density of orcinol-stained bands. We believe that the accumulation of more precise quantitative data of the kind presented here from various other tumors and normal tissues will help in understanding the characteristic features of the carbohydrate structures in individual tumors.

In summary, this study has demonstrated that the techniques used are sensitive enough and have enough resolving power to find novel acidic GSL structures from small quantities of already well-studied cancerous tissues The structural analysis of acidic GSLs in malignant tissues has been performed mainly in adenocarcinoma, whereas other types of malignant tumor, such as squamous cell carcinoma and sarcoma, have not been studied in detail. Application of the methods used in this study to these tumors will be important and will lead to the discovery of novel carbohydrate structures and reveal characteristic features of individual tumors.

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