Structures of Novel Sialylated O-Linked Oligosaccharides Isolated from Human Erythrocyte Glycophorins*

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The O-linked oligosaccharides attached to human erythrocyte glycophorins were extensively characterized. In addition to the previously described disialylated tetrasaccharide, NeuNacα2→3Galβ1→3( NeuNacα2→6)GalNAcOH and monosialylated trisaccharide, NeuNacα2→3Galβ1→3GalNAcOH, novel trisialylated oligosaccharides were isolated. Methylation analysis, fast atom bombardment-mass spectrometry, and enzymatic degradation were used to elucidate the following novel structures:

NeuNacα2→8NeuNacα2

6

NeuNacα2→3Galβ1→4GalNAcOH

NeuNacα2

6

NeuNacα2→8NeuNacα2→3Galβ1→3GalNAcOH

These results suggest that O-linked oligosaccharides with a disialosyl group, NeuNacα2→8NeuNacα2→, may be present in various tissues.

The O-linked oligosaccharides of human glycophorins were the first to be characterized among O-linked oligosaccharides isolated from membrane glycoproteins. These structures were elucidated by Thomas and Winzler (1), as being NeuNacα2→3Galβ1→3( NeuNacα2→6)GalNAc and NeuNacα2→3Galβ1→3GalNAc. Following this finding, the same tetrasaccharide and trisaccharide were found to be widely distributed, and shown to be present in fetuin (2), epiglycanin (3), AH66 hepatoma cells (4), IgD (5), human fibronectin (6), leukosialin (7), swine mucine (8), Swann rat chondrosarcoma (9), human mammary epithelial protoglycan (10), and others (11).

O-linked oligosaccharides present in human glycophorins apparently contribute to the expression of blood group antigens. In particular, sialic acid residues have been shown to be an integral part of MN blood group antigens (12, 13). In addition, one of the blood group MN variants carried different oligosaccharides in the glycophorin and NeuNacα2→3Galβ1→3Galβ1→3/4 GlcNAcβ1→6)GalNAc was found in Mill (14). In another rare blood group, Cad, the antigen was shown to be carried by NeuNacα2→3(GalNAcβ1→4)Galβ1→3( NeuNacα2→6)GalNAc (15). This structure is unique since it contains β-linked N-acetylgalactosamine. These results strongly suggest that full characterization of O-linked oligosaccharides in human glycophorins is important to understand the antigenic expression of human glycophorins.

In this report, we have extensively characterized sialylated O-linked oligosaccharides isolated from human glycophorins. Structures of novel trisialylated oligosaccharides are elucidated.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

This paper establishes the presence of novel sialylated O-linked oligosaccharides which contain the NeuNacα2→8NeuNacα2→ structure (Table II). As far as we are aware, this is the first report of such oligosaccharide structures in mammalian cells, although the presence of trisialylated O-linked oligosaccharides was suggested in rabbit brain glycoproteins (25, 26). Glycophorins contain about one-third the amount of trisialylated oligosaccharides compared to monosialylated oligosaccharides. Nevertheless, the detection of tri- sialylated oligosaccharides has not been previously reported. This is probably because disialylated oligosaccharides have been routinely isolated by elution with 400 mM pyridine formate buffer from a Dowex 1 column (for example, see Ref. 14), which also elutes trisialylated oligosaccharides; the large amounts of disialosyl oligosaccharides overshadow the detection of trisialosyl oligosaccharides using traditional techniques. Our results clearly demonstrate the efficacy of FAB-MS for detecting the presence of minor components in a mixture.

It is noteworthy that more disialosyl groups are found in the sialosyl residues attached to N-acetylgalactosamine than in those attached to galactose in the trisialosyl oligosaccharides. It appears, therefore, that NeuNacα2→8 sialyltransferase acts preferentially on a sialic acid residue attached to N-acetylgalactosamine by a 2→6 linkage. The absence of Galβ1→3( NeuNacα2→8NeuNacα2→6)GalNAcOH in the disialosyl oligosaccharides may be due to the absence, in glycophorins, of the presumptive precursor oligosaccharide,

1 Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–6, Table I, and Footnote 3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-0083, cite the authors, and include a check or money order for $8.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: FAB-MS, fast atom bombardment-mass spectrometry; HPLC, high performance liquid chromatography.
Novel Trisialosyl O-Linked Oligosaccharides

Table II

<table>
<thead>
<tr>
<th>Proposed structures of O-linked oligosaccharides isolated from human glycoporins</th>
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<tbody>
<tr>
<td>Structures</td>
</tr>
<tr>
<td>NeuNAc2→3Galβ1→3GalNAcOH</td>
</tr>
<tr>
<td>NeuNAc2</td>
</tr>
<tr>
<td>NeuNAc2→3Galβ1→3GalNAcOH</td>
</tr>
<tr>
<td>NeuNAc2→8 NeuNAc2</td>
</tr>
<tr>
<td>NeuNAc2</td>
</tr>
</tbody>
</table>

*This saccharide is a minor component in the trisialosyl saccharides.

Galβ1→3 NeuNAc2→6 GalNAc (see Table II). This latter finding is consistent with the report by Bergh et al. (27); they found that N-acetylgalactosamine $\alpha$2→6 sialyltransferase acts with very low efficiency on GalNAc or Galβ1→3GalNAc in liver cells. It will be interesting to determine the biosynthetic pathway of the trisialosyl oligosaccharides.

In the present study, oligosaccharides were permethylated and separated by reverse phase HPLC. The separation by HPLC allowed us to isolate fully methylated oligosaccharides from undermethylated ones, and to enrich the minor components. This approach was taken because the recovery of oligosaccharides after HPLC was relatively low when intact oligosaccharides were applied to an amino-bonded column, as reported recently (28). In addition, the direct and sensitive analysis of the effluent was possible by FAB-MS since the oligosaccharides were already methylated. During the analyses of sialosyl linkages, we used trimethylsilylation for derivatization of sialic acids after methanolation. The method is rapid and probably much less destructive compared to acetylation procedures which involve heating (29).

Inoue and co-workers (30) isolated polysialoglycoproteins from rainbow trout eggs and found that O-linked oligosaccharides attached to the proteins have polysialosyl groups. Therefore, their structures are rather unusual and not from adult erythrocytes (36). Furthermore, unique $\alpha2$→9 linked disialosyl groups were found in polylactosaminoglycans from PA1 human embryocarcinoma cells (22). These results suggest that 1) disialosyl groups may be widely present in glycoproteins from various cell types and 2) their amount may be regulated by development and differentiation programs. We have shown recently that structures of O-linked oligosaccharides are characteristic of each cell lineage and differentiation stage (7, 18). It will be interesting to see if any particular tissue or cell type contains a significant amount of the trisialylated O-linked oligosaccharides elucidated in the present study.

Acknowledgments—We thank Dr. Friedrich Piller for useful discussion, Brian Bothner for assistance in the analysis of gas chromatography-mass spectrometry, and Candy Farmer for secretarial assistance.

REFERENCES

27. Bergh, M. E., Hooghwinkel, G. J. M., and van den Eijnden, D.
null
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These results clearly indicate that the two steric acid residues are separately linked to galactosyl and N-acetylgalactosaminyl.

Methylation analyses using two hydrolysis conditions indicate the presence of terminal steric acid, disialosyl galactosyl and 2,3-disialosyl N-acetylgalactosaminyl (Table II). The disialosyl fraction (Fractions 46-52 after TSK-DEAE chromatography) was applied to a Biogel P-4 column. As shown in Fig. 3B, this oligosaccharide fraction provided a major peak (Peak 1) which yielded NeuNAcZ-6)GalNAcOH. This fraction was converted to NeuNAc-Z-GalNAcOH after neuraminidase treatment (Fig. 5E). The disialosyl fraction (Fractions 31-35 after the TSK-DEAE column) was applied to the Bio-Gel P-4 column. This fraction yielded a major peak (Peak 2) which corresponded to NeuNAc-Z-GalNAcOH. These results confirm the structure to be that of the disialosyl fraction eluted in Fractions 31-35 after the reverse phase HPLC were mainly composed of unmodified forms of the above component and were not analyzed further.

Trisialosyl Oligosaccharides - Oligosaccharides, which eluted between Fractions 36 and 42 in TSK-DEAE column chromatography (Fig. 20), were judged to be trisialosyl oligosaccharides. These were further purified as their permethylated derivatives by reverse phase HPLC, as shown in Fig. 2C. FAHMS of each fraction indicated that the trisialosaccharide, which eluted between Fractions 38 and 41, was the same as the disialosyl trisaccharide described above, and further analysis was not made.

As for the trisaccharide, which eluted between Fractions 41 and 42, it represents the trimethylated trisaccharide and the analysis of Fraction 44 is described below. FAHMS of Fraction 44 provided a fragment ion at m/z 1335 and 1621, which respectively corresponded to the [M-H] of NeuNAc-Z-GalNAcOH (Fig. 21). The same spectrum provided a major fragment ion at m/z 300 for NeuNAc-Z-GalNAcOH and 341 for NeuNAc-Z-GalNAcOH and 341 for NeuNAc-Z-GalNAcOH. In addition, an ion at m/z 500 was produced by selenation of NeuNAc-Z-GalNAcOH from the parent molecule. These results can be interpreted as follows:

Methylation analyses using two hydrolysis conditions indicated the presence of terminal steric acid and steric acid (Mr - 33,000), which was probably the disialosyl trisaccharide described above (Table II). These results were obtained by alkaline borohydride treatment. The disialosaccharide fractions obtained after Sephadex-50 gel filtration (a and b) were subjected to further analysis. The tetrasaccharide (leukotrienn) was detected by tritium gas chromatography-mass spectrometry. The component in the major oligosaccharide in the trisialosyl oligosaccharides since only this component was detected when a small amount of the sample was analyzed.

Fig. 1. Sephadex G-50 gel filtration of glycopeptides and oligosaccharides from glycoproteins. A. Oligosaccharides from glycoproteins were subjected to gel filtration on Sephacryl S-50 in 0.2 M NaCl as described in “Experimental Procedures”. Each fraction contained 1.0 M NaCl and aliquots were taken for determining hexoses by phenol-HCl reaction. The glycosaccharide fractions indicated by the horizontal bars were subjected to alkaline borohydride treatment. The vertical arrows indicate the elution positions of N-4-deoxygalactosyl glycoproteins (leukotrienn) (Split 4, 5, 7, 8, 9) and NeuNAc-Z-GalNAcOH in Table II. The trisialosaccharide was isolated from Fig. 2C. The oligosaccharide obtained after alkaline borohydride treatment of glycoproteins C (II) and glycoprotein II (II) were subjected to Sephadex G-50 gel filtration under the same conditions as A. Oligosaccharide fractions indicated by the horizontal bars were subjected to further analysis.
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![Image](image-url)

**Fig. 3.** Reverse-phase HPLC of permethylated monosialyl (A), disialyl (B) and trisialyl (C) oligosaccharides. Each oligosaccharide fraction obtained after ion-exchange HPLC (Fig. 2A-B) was methylated and applied to a column of Spherisorb ODS equilibrated with water-acetonitrile (9:1, v/v) over 300 min. The column was then immediately eluted with acetonitrile (9:1, v/v) over 10 min. The vertical arrow indicates the fractions for which the spectra of FAB-MS are reproduced.

**Fig. 4.** Fast atom bombardment mass spectra of permethylated oligosaccharides fractionated by reverse-phase HPLC. The positive spectra of Fraction 30 (A) and Fraction 35 (B) in Fig. 3, Fraction 39 (C) in Fig. 3, and Fraction 44 (D) in Fig. 3, are reproduced.

A. The molecular ion for the fully methylated Neu5Ac-HexNAc gave the prominent signal at m/z 849. This is accompanied by peaks 132 and 105 which correspond to the methyl glycosides of Neu5Ac and Neu5Ac-HexNAc, respectively. Major fragment ions are present at m/z 376 and 344. The major signal at m/z 849 corresponds to the methyl glycoside of Neu5Ac-HexNAc which is accompanied by the signal at m/z 580 corresponding to the fragment ion of Neu5Ac-HexNAc. The minor signals at m/z 552 corresponds to the Neu5Ac-Hex fragment ion in its di-unmethylated form.

B. The molecular ion for the fully methylated Neu5Ac-HexNAc-HexNAc is present at m/z 873. Fragment ions at m/z 580, 376, and 344 correspond to Neu5Ac-HexNAc and (mono and disialic acid) respectively. Note that this fraction did not provide an ion at m/z 941.

C. The molecular ion for the fully methylated Neu5Ac-HexNAc-HexNAc-Hex is the predominant signal at m/z 849 which is accompanied by a signal at m/z 817. This signal is accompanied by fragment ions at m/z 580, 376, and 376. This oligosaccharide was well methylated and only a very small amount of unmethylated forms were detected. Note that this fraction did not produce a fragment at m/z 941 for the Neu5Ac-HexNAc-HexNAc-Hex structure.

D. The major signals at m/z 1595 and 1617 corresponding to the fully methylated Neu5Ac-HexNAc-HexNAc-Hex and its Na salt, respectively. These molecular ions were accompanied by peaks at m/z 376, 580, and 849. This oligosaccharide was well methylated and only a very small amount of unmethylated forms were detected. However, an ion at m/z 637, which would be produced by de-adduction of Neu5Ac-HexNAc from the parent molecule, was not detected. The signal at 1220 and 989 correspond to glycosidic products lacking one and two sialic acid residues, respectively.
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Table I. Relative Proportions of Methylated Sugars from O-Linked Oligosaccharides Isolated from Human Erythrocyte Glycophorin

<table>
<thead>
<tr>
<th>Methylated Sugars</th>
<th>Neutralglycoproteins</th>
<th>Fucose 30</th>
<th>Fucose 37</th>
<th>Fucose 44</th>
<th>Trisialosyl 30</th>
<th>Trisialosyl 44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td></td>
<td>0.4a</td>
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<tr>
<td>N-acylated methyl-galactose</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
<td>N-acylated methyl-galactose</td>
<td></td>
<td>0.4a</td>
<td>0.6</td>
<td>0.2</td>
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<tr>
<td>N-acylated sialyl-galactose</td>
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<tr>
<td>N-acylated sialyl-galactose</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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</tbody>
</table>

*The analysis of these components was made after hydrolysis with 0.5 M HCl at 100°C for 30 min. The yield of neuraminic acid was very low after 6 h hydrolysis.