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The Structure of a Fucose-containing *O*-Glycosidic Carbohydrate Chain of Human Platelet Glycocalicin

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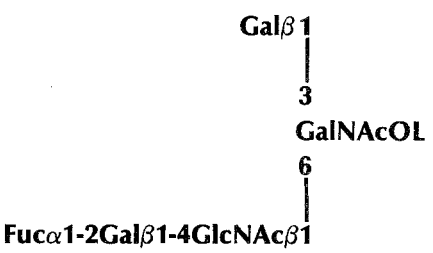
The hydrazinolysis procedure currently used for the release of *N*-glycosidic carbohydrate chains was applied to glycocalicin. The resulting mixture of oligosaccharide-alditols was fractionated by high-voltage paper electrophoresis into a neutral (5%) and several acidic fractions. The neutral compounds were passed over Bio-Gel P-4. Some *N*-glycosidic oligosaccharide-alditols, of the *N*-acetyllactosamine type as well as of the oligomannoside type, were found to be present. However, oligosaccharide-alditols derived from *O*-glycosidic carbohydrate chains were also found, indicating a partial cleavage of GalNAc1-*O*Ser/Thr linkages under the hydrazinolysis conditions applied. One of the neutral *O*-glycosidic components was characterized, by 500-MHz ^{1}H-NMR spectroscopy in combination with sugar analysis, as the following pentasaccharide-alditol:



In addition the afuco analogue of this compound was obtained.

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Glycoprotein Ib is one of the predominant glycoproteins of the human platelet membrane [1, 2]. It is involved in the platelet adhesion process [3]. Glycocalicin forms the main part of the α chain of glycoprotein Ib. Cleavage of glycocalicin from the platelet surface leads to loss of the platelet aggregation response to von Willebrand's factor [4]. Knowledge of the carbohydrate structures of these glycoproteins is necessary to gain insight into the possible role of these chains in the interaction processes.

Glycocalicin contains 40% carbohydrate by weight, comprising *O*- as well as *N*-glycosidic chains [5]. Treatment of glycocalicin with alkaline borohydride resulted in the release of mainly *O*-glycosidic carbohydrate chains. The major oligosaccharide-alditol was proved to be NeuAcα2-3Galβ1-3(NeuAcα2-3Galβ1-4GlcNAcβ1-6)GalNAcOL [5-7], and as minor constituents NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcOL and NeuAcα2-3Galβ1-3GalNAcOL were found [5]. Fucose, although present in relatively low amounts in the alkaline borohydride cleavage product, could not be characterized as an integral constituent of one of the acidic oligosaccharides. We describe here the characterization of two neutral *O*-glycosidic oligosaccharide-alditols, one of which contains fucose. These compounds were isolated from the mixture of *N*- and *O*-glycosidic carbohydrate chains obtained by subjecting glycocalicin to the hydrazinolysis procedure [8].

Materials and Methods

Glycocalicin was isolated from human platelet membranes as described before [5]. An aliquot (40 mg) was suspended in 0.5 ml anhydrous hydrazine and heated at 100°C for 8 h. After evaporation of hydrazine, the material was *N*-reacetylated and reduced [8]. For reduction with [^{3}H]-labelled $NaBH_4$, 20% of the sample was dissolved in 400 μl 0.08 M NaOH and treated with $NaBH_4$ containing 3.4 mCi NaB^3H_4 (sp. act. 341 mCi/mmol; New England Nuclear, Boston, USA) in 400 μl dimethylformamide. The remainder of the sample was reduced with NaB^2H_4. To facilitate the detection of oligosaccharide-alditols, 12 μCi (50%) of the tritium-labelled alditols were added. Paper electrophoresis (Whatman 3MM paper, 70 V/cm, 90 min) was carried out at pH 5.4 in a buffer consisting of pyridine/acetic acid/water, 3/1/387 by vol. The neutral fraction was applied to a column (100 $\times$ 2 cm) of Bio-Gel P-4 (Bio-Rad, Richmond, USA, 400 mesh) using water as eluent at a flow rate of 20 ml/h at 55°C.

For sugar analysis, trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation. Subsequently, they were analysed by GLC on a CPsil5 WCOT fused silica capillary column (25 m $\times$ 0.32 mm i.d.) using a Varian Aerograph 3700 gas chromatograph [9, 10].

Prior to ^{1}H-NMR spectroscopic analysis the samples were repeatedly treated with $^2H_2$ (99.996 atom % ^{2}H, Aldrich, Milwaukee, USA) at p^{2}H 7 and room temperature, with intermediate lyophilization. 500-MHz ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON Hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating under control of an Aspect-2000 computer. Experimental details have been described previously [5, 11].

Table 1. Molar carbohydrate composition of glycocalicin before and after hydrazinolysis, and of isolated fractions.

Monosaccharides	Molar ratios in			
	intact glycocalicin	hydrazinolysate	neutral fraction	*O*-glycosidic fraction
Fuc	1.7	1.4	1.4	0.6
Man	3.0[a]	3.0[a]	3.0[a]	0.4
Gal	10.2	8.1	4.7	2.2
GalOL	—	1.0	—	—
GalNAc	4.2	2.5	+[c]	—
GlcNAc	6.0	7.7	4.0	1.2
GalNAcOL	—	1.0	0.4	1.0[b]
GlcNAcOL	—	1.1	0.5	—
NeuAc	8.5	9.5	+	—

[a] Molar ratios relative to 3 mol mannose.
[b] Molar ratios relative to 1 mol 2-acetamido-2-deoxygalactosaminitol.
[c] +, Detectable, but less than 0.1.

Results and Discussion

The carbohydrate composition of the liberated oligosaccharides obtained by the hydrazinolysis procedure is compared with that of intact glycocalicin in Table 1. The hydrazinolysis procedure resulted in the formation of not only oligosaccharide-alditols ending in *N*-acetylglucosaminitol but also in *N*-acetylgalactosaminitol and galactitol. Since it has been found that alkaline borohydride treatment of glycocalicin leads to the conversion of the total content of *N*-acetylgalactosamine into *N*-acetylgalactosaminitol [5, 7], *N*-acetylgalactosamine is only involved in the linkage of the *O*-glycosidic chains to serine and/or threonine. Apparently, the oligosaccharide-alditols ending in *N*-acetylgalactosaminitol obtained during the hydrazinolysis procedure represent intact *O*-glycosidic chains, while those ending in galactitol must correspond to peeling products. As can be deduced from Table 1, a significant portion of the GalNAc1-*O*Ser/Thr linkages have been split under the applied hydrazinolysis conditions.

Paper electrophoresis of the mixture of released carbohydrates resulted in a neutral fraction (5%) and a series of acidic fractions. Sugar analysis of the neutral fraction (Table 1) shows the presence of both *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol, pointing to the occurrence of *N*- as well as *O*-glycosidic chains. This is confirmed by 500-MHz ^{1}H-NMR spectroscopic analysis of this fraction; the spectrum showed signals arising from *N*-acetyllactosamine- and oligomannoside-type *N*-glycosidic chains [11, 12], as well as signals indicating *O*-glycosidic chains [13-17].

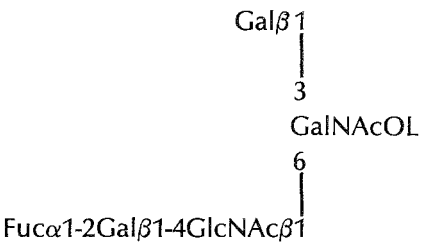
Table 2. ^{1}H Chemical shifts of structural-reporter groups of constituent monosaccharides of neutral *O*-glycosidic oligosaccharide-alditols obtained from glycocalicin by hydrazinolysis and subsequent purification, together with those of some reference compounds [13, 14]. The superscript following a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically bound fucose residues; it indicates the type of linkage of the adjacent monosaccharide. Chemical shifts are in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O at 27°C. Compounds are represented by a short-hand symbol notation [5, 11, 15]: ◇ = *N*-acetylgalactosaminitol; ■=galactose; ● = *N*-acetylglucosamine; □ = fucose.

Residue	Reporter group	Chemical shifts (ppm) in compound			
		A 	B 	C 	*O*-glycosidic fraction
GalNAcOL	H-2	4.399	4.400	4.398	4.386[a]
	H-3	4.061	4.083	4.060	4.056
	H-4	3.460	3.496	3.464	3.464
	H-5	4.285	4.265	4.287	4.290
	NAc	2.067	2.053	2.066	2.066
Gal^3	H-1	4.463	4.576	4.463	4.464
	H-4	3.898	3.92	3.90	3.901
GlcNAc^6	H-1	4.557	4.55	4.535	4.539[b]/4.559[c]
	H-6	3.996	3.999	3.99	3.996
	NAc	2.066	2.058	2.066	2.066
Gal^4	H-1	4.469	4.54	4.536	4.539[b]/4.469[c]
	H-4	3.923	3.92	3.98	3.986[b]/3.924[c]
Fuc^{2,3}	H-1		5.216		
	H-5		4.27		
	CH_3		1.244		
Fuc^{2,4}	H-1		5.306	5.309	5.306[b]
	H-5		4.226	4.227	4.223[b]
	CH_3		1.229	1.233	1.230[b]

[a] This chemical shift reflects GalNAc reduced by NaB^2H_4 (isotope shift effect); the signal is a doublet.
[b] Chemical shift values of structural-reporter groups of the fucose-containing *O*-glycosidic pentasaccharide, identical to C.
[c] Chemical shift values of the afuco *O*-glycosidic tetrasaccharide, identical to A.

The neutral fraction was subfractionated by filtration over Bio-Gel P-4. Sugar analysis indicates that one of the subfractions contains predominantly *O*-glycosidic material, i.e. the *N*-acetylgalactosaminitol/mannose ratio is 1 : 0.4 (Table 1). By consequence the considerable amount of fucose in this fraction suggests that it forms part of an *O*-glycosidic carbohydrate chain of glycocalicin. The ^{1}H-NMR chemical shifts of the structural reporter groups of this subfraction are compiled in Table 2, together with those of some reference compounds of related structure. The H-2 signal of *N*-acetylgalactosaminitol at δ

= 4.386 ppm points to substitution of *N*-acetylgalactosaminitol by a galactose residue in a β1-3 linkage [15]. This *N*-acetylgalactosaminitol is also substituted by *N*-acetylglucosamine in a β1-6 linkage, which is indicated by the position of the H-5 signal of *N*-acetylgalactosaminitol (δ = 4.290 ppm) [15]. The chemical shifts of H-1 and H-4 of Gal^3*, being δ = 4.464 and 3.901 ppm, respectively, show the terminal position of the Gal^3 residue [14, 15]. The fucose residue present in this fraction is characterized by the H-1 signal at δ = 5.306 ppm, together with the CH_3 doublet at δ = 1.230 ppm; the combination of these values points to the presence of fucose in α1-2 linkage to a Gal^4 residue [13-17]. Therefore, fucose is attached to the Galβ1-4GlcNAcβ1-6 branch. This conclusion is corroborated by the position of the H-1 signals of GlcNAc and Gal^4, which coincide at δ = 4.539 ppm. On the basis of the sugar composition (Table 1) and the ^{1}H-NMR data (Table 2), the fucose-containing *O*-glycosidic oligosaccharide-alditol could be identified as:



In addition, some signals in the ^{1}H-NMR spectrum indicate the presence of the afuco-analogue of this compound, as another component. In particular, the doublets at δ = 4.559 and 4.469 ppm represent the H-1 protons of GlcNAc^6 and Gal^4 respectively, for an afuco-α1-6- linked *N*-acetyllactosamine branch [14].

It seems most likely that the fucose-containing *O*-glycosidic pentasaccharide-alditol was also present in the mixture of oligosaccharide-alditols obtained previously [5] by the β-elimination procedure. However, owing to its low abundance, it could not be isolated and characterized.

The small amount of sugar present in the other neutral Bio-Gel P-4 fractions did not permit structural investigations. Structural analysis of the acidic fractions obtained from the hydrazinolysate is in progress.

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*The superscript following the abbreviation of the sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked.

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