ALKALI-STABLE BLOOD GROUP A- AND B-ACTIVE POLY(GLYCOSYL)-PEPTIDES FROM HUMAN ERYTHROCYTE MEMBRANE

Jukka FINNE, Tom KRUSIUS and Heikki RAUVALA
Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10 A, SF-00170 Helsinki 17

and

Riitta KEKOMÄKI and Gunnar MYLLYLÄ
Finnish Red Cross Blood Transfusion Service Kivihaantie 7, SF-00310 Helsinki 31, Finland

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1. Introduction

The study of the chemical nature of the ABH blood group antigens of the human erythrocyte membrane has been advanced in recent years by the isolation and characterization of several glycosphingolipids with blood group activity [1–3]. It has been reported that blood group ABH activity is present also in glycoproteins of the erythrocyte membrane [4–9]. However, since these observations have been mainly based on serological inhibition tests, the presence of blood group-active glycolipids in the glycoprotein preparations cannot be excluded. Owing to the water solubility of the large-molecular size blood group-active glycosphingolipids (the poly(glycosyl)ceramides), it has been suggested that these glycosphingolipids may have been regarded in some studies as glycoproteins [3]. The occurrence of protein-bound blood group ABH antigens in the erythrocyte membrane has therefore still been a matter of some controversy. In the present paper, we present direct chemical evidence for the occurrence of protein-bound blood group ABH antigens in the erythrocyte membrane. This was accomplished by the preparation of glycopeptides with pronase digestion from lipid-free membrane residues and the isolation of the blood group A- and B-active fractions using the α-galactosyl- and α-*N*-acetylgalactosaminyl-binding lectin [10,11] of *Bandeiraea simplicifolia* (BS I lectin). The glycopeptides are composed of about 50–60 sugar residues/molecule and contain an alkali-stable carbohydrate–peptide linkage.

2. Methods

Erythrocyte membranes were prepared from outdated blood group A or B blood as in [12]. The membranes were extracted with 83% ethanol and the dry residue was homogenized with 10 vol. 0.02 M phosphate buffer (pH 7.5) and 10 vol. butan-1-ol to solubilize the poly(glycosyl)ceramides [3]. The insoluble residue was further delipidated by homogenization in chloroform–methanol (2:1 and 1:2, v/v). Glycopeptides were prepared from the dry lipid-free residue by extensive digestion with pronase [13], and purified by gel filtration after precipitation of polyanionic material with cetyl pyridinium chloride [14].

The BS I lectin was isolated as in [10] and coupled to (150 mg protein/15 ml gel) CNBr-activated Sepharose 4B (Pharmacia) at pH 8.3 as described by the manufacturer. The column was washed and equilibrated at 4°C with 10 mM Na-phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.1 mM $CaCl_2$ (P_i/NaCl). The glycopeptides bound to the column were eluted with 5 mM galactose in P_i/NaCl. Fractionation of glycopeptides on concanavalin A–Sepharose was done as in [15].

Monosaccharides were determined after methano-

lysis as their trimethylsilyl derivatives by gas–liquid chromatography [16]. Long-chain (sphingosine) bases were determined by gas–liquid chromatography and mass spectrometry [17] and by mass fragmentography at *m/e* 174, which is a common fragment in the spectra of these substances. Amino acid analysis was performed by Dr Olli Simell, Children's Hospital, University Central Hospital, Helsinki.

The A and B blood group activities of glycopeptides were determined in the standard hemagglutination inhibition assay and the platelet ^{125}I-labelled protein A test (PIPA) [18]. In the latter assay the ability of glycopeptides to inhibit the binding of immune anti-A and anti-B antibodies on platelets of known AB groups was measured. Staphylococcal protein A labelled with ^{125}I was used as a marker of platelet-bound IgG. Human immune anti-A and anti-B sera with titers of 3200 and 64 by Coombs agglutination test were used in dilutions 1:32 and 1:4, respectively.

3. Results

3.1. *Isolation of α-galactose-containing glycopeptides from blood group B active erythrocytes*

The yield of dry lipid-free membrane residue from B erythrocyte was 58 mg/liter blood. The total glycopeptide fraction, which was prepared by pronase digestion of the lipid-free residue and purified by gel filtration, contained 17.7 μmol sugars/100 mg lipid-free residue. Most of the glycopeptides (fraction B/BS–) passed through the BS I–Sepharose column (fig.1). The glycopeptides bound to the column (fraction B/BS+) were eluted with 5 mM galactose; no radioactivity was observed in fractions eluted with 500 mM galactose. Fraction B/BS+ glycopeptides (1.48 μmol sugar/100 mg lipid-free membrane residue) was submitted to gel filtration on Sepharose G-50 (fig.2). The glycopeptides were eluted slightly after the void volume. On the basis of the elution volume an app. mol. wt 10 200 was calculated for the glycopeptides. The chemical composition of the glycopeptides is given in table 1. The main sugar components were galactose and *N*-acetylglucosamine. No *N*-galactosamine was observed.

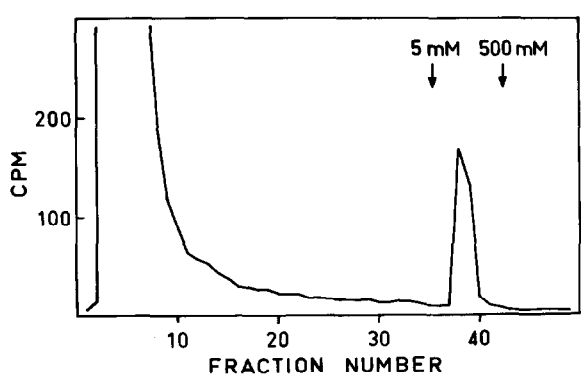

Fig.1. Fractionation of glycopeptides of blood group B-active erythrocytes on a column (1.4 × 10 cm) of BS I–Sepharose. The *N*-^{3}[H]acetylated glycopeptides were dissolved in P_i/NaCl and applied to the column equilibrated with the same buffer. Elution was carried out first with P_i/NaCl. At arrows, 5 mM and 500 mM galactose were included in the buffer. Fractions of 6.0 ml were collected.

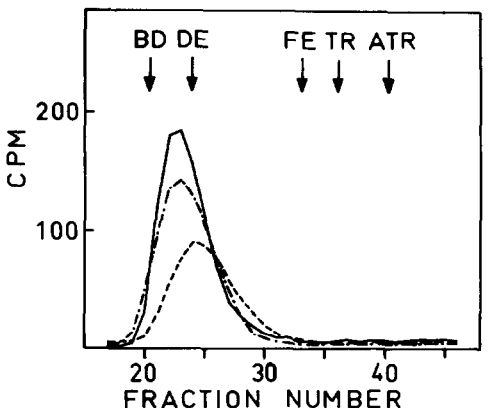

Fig.2. Gel filtration of glycopeptides bound to BS I lectin on a column (2 × 75 cm) of Sephadex G-50. The column was eluted with 0.1 M pyridine–acetic acid buffer (pH 5.0) and fractions of 4.6 ml were collected. For reference, the elution volumes (BD) Blue Dextran, (DE) Dextran T 10 (M_r 9400), (FE) fetuin glycopeptide (M_r 3400), (TR) transferrin glycopeptide (M_r 2400) and (ATR) asialotransferrin glycopeptide (M_r 1800) are shown. (——) Fraction B/BS+; (.-.-.) fraction A/ConA-A/BS+; (—) fraction A/ConA-B/BS+.

Since the glycopeptides contained mannose, their possible binding to concanavalin A was tested by subjecting them to chromatography on a column of concanavalin A–Sepharose. Most of the glycopeptides were not bound to the lectin. A small proportion of the material (5%) was obtained by

Table 1
Chemical composition of the blood group-active glycopeptides

Constituent	B/BS+	A/ConA-A/BS+ (mol/mol glycopeptide)	A/ConA-B/BS+
Fucose	7.0	5.5	4.6
Mannose	2.0	2.4	2.7
Galactose	24	20	18
Glucose	<0.2	<0.2	<0.2
N-Acetylglucosamine	21	22	18
N-Acetylgalactosamine	<0.2	2.8	2.9
Neuraminic acid	1.8	1.5	1.4
Total sugars	56	54	48
Aspartic acid	0.7	0.7	a
Threonine	0.1	0.1	a
Serine	0.2	0.1	a
Glutamic acid	0.2	0.2	a
Glycine	0.1	0.1	a
Isoleucine	0.1	0.1	a
Leucine	0.2	0.2	a
Long chain bases	<0.01	<0.01	<0.01

[a] not determined

In calculating the data, mol. wt 10 200, 10 000 and 8800 were used for fractions B/BS+, A/ConA-A/BS+ and A/ConA-B/BS+, respectively

elution of the column with 20 mM α-methyl-D-glucoside. Due to the scarcity of the material it was not subjected to further studies.

3.2. *Isolation of α-N-acetylgalactosamine-containing glycopeptides from blood group A-active erythrocyte*

The yield of dry lipid-free membrane residue from A erythrocyte was 98 mg/liter blood, and the purified glycopeptides contained 16.1 μmol sugars/100 mg lipid-free residue. Fractionation of glycopeptides by affinity chromatography with the two lectins was performed in a reversed order as compared to that described for the blood group B glycopeptides. Thus, the glycopeptides were first subjected to chromatography on concanavalin A–Sepharose. Three fractions were obtained: the glycopeptide fraction not bound to the lectin (fraction A/ConA-A) the fraction eluted with 20 mM α-methyl-D-glucoside (fraction A/ConA-B) and the fraction eluted with 200 mM α-methyl-D-glucoside (fraction A/ConA-C) [15]. Fractions A/ConA-A and A/ConA-B were subjected to chromatography on BS I–Sepharose. The glycopeptides bound to the column (fractions A/ConA-A/BS+ and A/ConA-B/BS+) were eluted with 5 mM galactose. The yields of the bound glycopeptide fractions were 1.41 and 0.28 μmol sugar/100 mg lipid-free residue, respectively.

The apparent molecular weights as determined by gel filtration (fig.2) were 10 000 and 8800 for the fraction A/ConA-A/BS+ and A/ConA-B/BS+ glycopeptides, respectively. The chemical compositions are given in table 1. As a difference to the blood group B glycopeptides, the blood group A glycopeptides contained *N*-acetylgalactosamine, and the proportion of galactose was lower.

In order to study the possible alkali-lability of the glycopeptides, fraction A/ConA-A/BS+ glycopeptides were treated with 0.05 M NaOH in 1 M $NaBH_4$ for 16 h at 45°C [19]. The sugar composition and elution volume in gel filtration were unaffected by this treatment. Also, no *N*-acetylgalactosaminitol was detected. These data indicate that the *N*-acetylgalactosamine residues were not involved in alkali-

labile *O*-glycosidic bonds to the peptide, and that the carbohydrate-peptide linkage was stable to mild alkali treatment.

3.3. *Blood group activity of the glycopeptides*

The antigen activity of the glycopeptide fractions was determined by their ability to inhibit the agglutination or red blood cells by natural antisera, and by their ability to inhibit the binding of class IgG anti-A antibodies to thrombocytes (table 2). The latter method was used because of its higher sensitivity. The data indicate that most of the blood group A-active glycopeptides from fractions A/ConA-A and A/ConA-B were bound to the BS I lectin, which is in agreement with the known specificity [10,11] of this lectin. Fraction ConA-C, which contains neutral mannose-rich glycopeptides [20], did not contain blood group activity. Respectively, glycopeptides isolated from blood group B erythrocytes exhibited blood group B activity. However, a proportion of the activity was not bound to the BS I lectin, possibly due to overloading of the column.

Table 2
Blood group A activity[a] of various glycopeptide fractions obtained from delipidated A erythrocyte membranes

Fraction	Blood group A activity	
	H I titer	PIPA % inhibition
Con A-A	+ 4	76
Con A-A/BS–[b]	–	51
Con A-A/BS+[c]	+ 8	100
Con A-B	–	19
Con A-B/BS–	–	0
Con A-B/BS+	+ 4	100
Con A-C	–	0

[a] The glycopeptide fractions showed no inhibitory activity when tested with B- or O-erythrocytes
[b] Diluted 1 : 4; no inhibition
[c] Diluted 1 : 6; 64% inhibition

The activities were determined by the ability of the glycopeptides to inhibit the hemagglutination of blood group A erythrocytes (H I) and their ability to inhibit the binding of anti-A antibodies to blood group A thrombocytes (PIPA). The amount of glycopeptides used corresponded to 25 mg lipid-free membranes residue except in Con-A-B which was obtained from 10 mg residue

4. Discussion

The results indicate that blood group A and B antigens of the erythrocyte membrane occur not only in the form of glycolipids but also in glycoproteins. This inference is supported by the following findings:

1. The blood group substances could be isolated after exhaustive delipidation of the membranes.

2. The blood group-active substances did not contain glucose or long-chain (sphingosine) bases, which are integral constituents of the glycosphingolipids.

3. The compounds isolated contained mannose and a proportion of the substances were also bound to concanavalin A.

4. The fractions isolated contained amino acids with aspartic acid as the main component.

5. From the fractions isolated with the aid of the BS I lectin, those from A but not from B erythrocytes contained *N*-acetylgalactosamine, and in an amount that cannot be explained by contamination of the glycopeptide fractions with glycosphingolipids.

The blood group-active glycopeptides were of significantly larger molecular size than several known glycopeptides (reviewed [21]). The glycopeptides differed also from the blood group substances of the ovarian mucins [22] in that they were stable to mild alkali treatment. The observation of aspartic acid as the main amino acid component suggests that the carbohydrate–peptide linkage is of the *N*-glycosidic type. Although alkali-labile A- or B-active carbohydrate units were not detected in the present study, the occurrence of such molecules in the glycopeptide fractions not bound to the BS I lectin cannot be be excluded. At least, a proportion of the erythrocyte membrane H activity has been ascribed to such chains [9].

The yield of the blood group-active glycopeptides was about 1.6 μmol or 0.3 mg/liter blood. Thus they are present in amounts comparable to those reported for poly(glycosyl)ceramides, which are major blood group active glycolipids of the human erythrocyte membrane (0.6 mg/liter blood) [3]. The sugar com-

position and molecular size of the glycopeptides were also very similar to these glycolipids. Since the glycopeptides contained a high proportion of galactose and *N*-acetylglucosamine it is possible that they contain a repeating unit composed of these two sugars. Studies of the chemical structure of these novel type of carbohydrate units of glycoproteins are in progress. Preliminary data indicate many structural similarities to the poly(glycosyl)-ceramides. On the basis of this similarity, we propose to designate these glycopeptides poly(glycosyl)peptides.

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