FEBS Letters 406 (1997) 296–300 FEBS 18429

Blood group MN-dependent difference in degree of galactosylation of O-glycans of glycophorin A is restricted to the GalNAc residues located on amino acid residues 2–4 of the glycophorin polypeptide chain

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Received 3 February 1997; revised version received 26 February 1997

Abstract Glycophorin A (GPA) of human erythrocytes contains a minor number of unsubstituted GalNAc residues (Tn receptors) which are recognized by *Moluccella laevis* lectin (MLL). The lectin reacts better with blood group N- than M-type of GPA which suggests a higher number of Tn receptors in GPA-N than in GPA-M. To find out whether this difference is restricted to a defined domain of GPA, the N-terminal tryptic glycopeptides of GPA-M and GPA-N (a.a. residues 1–39) and their fragments obtained by degradation with CNBr (a.a. residues 1–8 and 9–39) were analyzed. The untreated and desialylated glycopeptides were tested as inhibitors of MLL in ELISA, and the content of GalNAc-ol was determined in the products of β-elimination of the asialoglycopeptides by gas-liquid chromatography/mass spectrometry. The asialoglycopeptides 1–39 and 1–8 derived from GPA-N showed about 2 and 4 times higher content of non-galactosylated GalNAc residues, respectively, and higher reactivity with MLL than their counterparts derived from GPA-M, while asialoglycopeptides 9–39 of GPA-M and GPA-N did not show such differences. These results demonstrate that higher expression of non-galactosylated GalNAc in GPA-N than in GPA-M is confined to GalNAc residues located in the amino-terminal portion of GPA polypeptide chain, between the blood group M- and N-specific amino acid residues 1 and 5.

Key words: Blood groups MN; Glycophorin; Lectin; *Moluccella laevis*; O-Glycan; Tn receptor

1. Introduction

Blood group M- and N-types of glycophorin A (GPA) of human erythrocytes differ in two amino acid residues (M: Ser^1/Gly^5, N: Leu^1/Glu^5). GPA carries one N-glycan on Asn^{26} and about 15 O-glycans. The major O-glycan is the tetrasaccharide NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-which is accompanied by a minor number of other structures, due to microheterogeneity of glycosylation (for review see [1]). We have found previously that *Moluccella laevis* lectin (MLL), highly specific for *N*-acetylgalactosamine [2–4], shows anti-Tn (GalNAcα1-Ser/Thr) specificity and reacts better with GPA-N than with GPA-M [5]. The interaction of GPA with MLL is increased and the difference between the reactivity of GPA-M and GPA-N is decreased after desialylation of GPA [5]. MLL is only weakly inhibited by galactose and the disaccharide Galβ1-3GalNAc (≈500 times less than by GalNAc) [3,5]. We have found that the contribution of Galβ1-3GalNAc- chains of asialoGPA to its interaction with MLL is negligible and the lectin recognizes unsubstituted GalNAc residues in GPA ([5], and unpublished results). Our results suggested that GPA contains a minor number of non-galactosylated GalNAc residues, which are partly substituted by α2-6-linked *N*-acetylneuraminic acid, and that number of these residues is higher in GPA-N than in GPA-M. This conclusion was confirmed by finding that *Vicia villosa* anti-Tn lectin (which does not react with native erythrocytes or GPA) also reacts more strongly with asialoGPA-N than asialoGPA-M [6]. Moreover, GalNAc-ol was identified in the products of β-elimination of GPA (1 residue per several GPA molecules), its content was approximately 2 times higher in asialoGPA and was also higher in GPA-N and asialoGPA-N than in GPA-M and asialoGPA-M, respectively [7].

The above results suggested that efficiency of in vivo galactosylation of GalNAc residues in GPA is modulated by amino acid residues at positions 1 and/or 5 which are different in GPA-M and GPA-N. That effect can be expected to be more pronounced in the proximity of these residues. The Ser/Thr residues of GPA are glycosylated at positions 2, 3 and 4, and at several positions within amino acid residues 10–50 of GPA polypeptide chain [8,9]. The aim of the experiments described in this report was to examine whether the difference in galactosylation of GalNAc residues is restricted to positions 2–4 of GPA. It was done by analysis of various glycosylated fragments of GPA-M and GPA-N for the content of non-galactosylated GalNAc residues and for reactivity with MLL.

2. Materials and methods

2.1. GPA and its fragments

GPA-M and GPA-N were purified from the membranes of outdated human blood group O, M and N erythrocytes, respectively [10]. Amino-terminal tryptic glycopeptides of GPA were obtained as described [11]. The glycopeptide fraction was a mixture of GPA fragments containing a.a. residues 1–39 and 1–30/31 [8] and for simplicity is further designated as glycopeptide 1–39. Two fractions, containing glycopeptides 1–8 and 9–39, were obtained by CNBr-degradation of the glycopeptides 1–39 at Met^8 [12]. The glycopeptides were desial-

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Abbreviations: GPA, glycophorin A; GPA-M and GPA-N, GPA of blood group M- and N-type, respectively; MLL, *Moluccella laevis* lectin; GLC, gas liquid chromatography; MS, mass spectrometry

Dedicated to Professor Hans Paulsen on the occasion of his 75th birthday.

PII S0014-5793(97)00284-6

ylated by mild acid hydrolysis in 0.025 M sulfuric acid at 60°C for 4 h, neutralized with NaOH and repurified by gel filtration on the Bio-Gel P-4 column.

2.2. Analysis of carbohydrate composition

The glycopeptides were hydrolyzed in 4 M trifluoroacetic acid at 100°C for 4 h and monosaccharides were quantitated as alditol acetates using a 5890 Hewlett-Packard gas chromatograph equipped with a mass spectrometer (GLC-MS). Xylose served as internal standard and an HP-1 capillary column (0.2 mm×12 m) at a temperature gradient 150–230°C (8°/min) was used.

2.3. The β-elimination and determination of GalNAc-ol

The asialoglycopeptides were incubated in 0.05 M NaOH/1 M $NaBH_4$ in the presence of Cd^{2+} ions, according to the method of Likhosherstov et al. [13]. The reaction mixture was neutralized with 50% acetic acid and supplemented with xylitol and perseitol as internal standards. The samples, dried in a rotary evaporator by co-evaporation with methanol and peracetylated with acetic anhydride in pyridine for 35 min at 100°C, were directly analyzed in a GLC-MS system [14], under the conditions described above.

2.4. Affinity-purification of M. laevis lectin

The PBS (20 mM phosphate buffer/0.15 M NaCl/0.02% sodium azide, pH 7.4) extract of ground and defatted *M. laevis* seeds (50 g) was obtained as described previously [3]. From 50 g seeds, 580 ml of the extract containing 4 g protein was obtained. CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) (10 ml) was conjugated with asialoGPA-M (9.6 mg). The extract was applied in three portions to the asialoGPA-Sepharose column at a rate of 12 ml/h, and the column was washed with PBS until the absorbance of the effluent at 280 nm was less than 0.1. The lectin, which was totally bound to the column, was eluted with 3 M KSCN and fractions (4 ml/15 min) giving absorption at 280 nm were pooled, dialyzed against PBS containing 0.1 M galactose, and then against PBS. The precipitate formed was removed by centrifugation at 12 000×*g* for 20 min and the supernatant was concentrated by ultrafiltration. The yield (23 mg of lectin per 50 g of seeds) was similar to that obtained by previous method [3]. Analysis by SDS-polyacrylamide gel electrophoresis showed the typical pattern of MLL bands and no contamination. The lectin agglutinated native and desialylated ON erythrocytes at concentrations of 15 μg/ml and 0.5 μg/ml, respectively.

2.5. Lectin microtiter plate assays

Inhibition of MLL with glycopeptides was tested using microtiter plates (Nunc, MaxiSorp, Vienna, Austria) coated with asialoGPA-N, and the bound lectin was revealed by rabbit anti-MLL antibodies and goat anti-rabbit Ig antibodies conjugated with alkaline phosphatase, as described earlier [5]. Inhibition of peanut agglutinin (PNA, Sigma, St. Louis, MO) was tested using the same method with biotinylated PNA and alkaline phosphatase-conjugated ExtrAvidin (Sigma). Lectin biotinylation and details of the test were as described [15].

3. Results

Glycopeptide 1–39 of PGA contains about 12 out of 15 GPA O-glycans. As reported earlier, when this glycopeptide is derived from GPA-N it shows higher affinity for MLL than the corresponding peptide from GPA-M, while the tryptic glycopeptides 40–61 of GPA-M and GPA-N, carrying the remaining three O-glycans, show similar reactivity with MLL [5]. Therefore, we analyzed now only the glycopeptides 1–39 and their fragments 1–8 and 9–39.

Molar concentrations of glycopeptides in solutions were calculated from their carbohydrate composition, assuming that glycopeptides 1–39, 1–8 and 9–39 contain 12, 3 and 9 O-glycans, respectively, and that glycopeptides 1–39 and 9–39 contain one N-glycan. The validity of this assumption was confirmed by the results of inhibition of PNA binding to asialoGPA-coated ELISA plates. The asialoglycopeptides obtained from GPA-M inhibited PNA almost identically as their counterparts from GPA-N (Fig. 1).

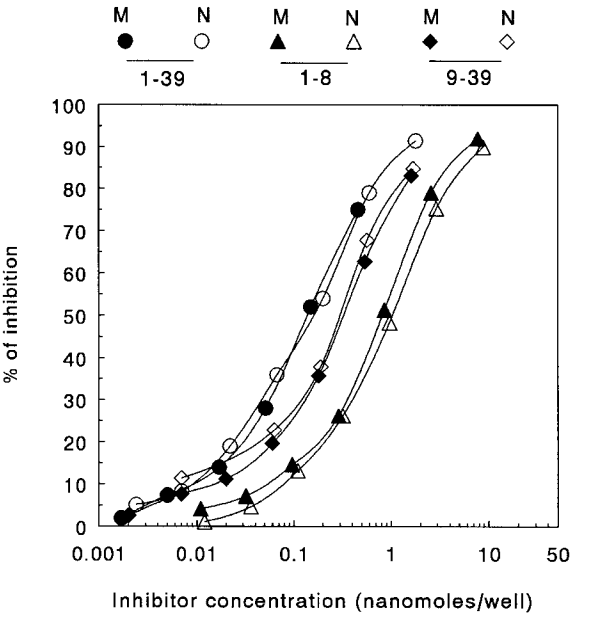

Fig. 1. Inhibition of PNA binding to asialoGPA-coated ELISA plates with serially diluted asialoglycopeptides 1–39 (circles), 1–8 (triangles) and 9–39 (diamonds), derived from GPA-M (full symbols) and GPA-N (empty symbols).

All the glycopeptides tested inhibited MLL binding to asialoGPA-N-coated ELISA plates and desialylation of the glycopeptides increased markedly their inhibitory activity (Fig. 2), in agreement with previous results [5]. Moreover, untreated glycopeptides 1–39 and 1–8 of GPA-N showed stronger inhibition (3 and 4 times, respectively) than the respective glycopeptides of GPA-M, and this difference was lower, but still distinct (≈2-fold) with the corresponding asialoglycopeptides (Fig. 2A,B). Glycopeptides 9–39 of GPA-M and GPA-N inhibited MLL similarly (Fig. 2C); the slightly higher activity of glycopeptide 9–39 from GPA-N most probably resulted from its contamination by undegraded glycopeptide 1–39, or glycopeptide 1–8. The concentrations of glycopeptides giving 50% inhibition of MLL and PNA are summarized in Table 1.

GLC-MS analysis of the products of β-elimination was performed with asialoglycopeptides only, due to the low content of unsubstituted GalNAc and scarcity of material. A new method of analysis [14] was used, which differed from the previous method [7] in that the products of β-elimination were not fractionated by gel filtration, but the sample was directly peracetylated and applied to the gas chromatograph. Under the conditions used only monosaccharide alditols are detectable, and their possible partial loss during the fractionation of β-elimination products is avoided. Two monosaccharide alditols were detected, GalNAc-ol and Gal-ol (example of analysis is shown in Fig. 3). Identification of these components was based on their retention times in GLC and on mass spectra which were typical for peracetylated alditols of these monosugars and were described elsewhere [16]. The content of GalNAc-ol was generally low in all asialoglycopeptides (< 1 residue per molecule), and was similar in GPA-M and GPA-N derived glycopeptides 9–39. On the other hand, the content of GalNAc-ol was more than 2 times higher in GPA-N asialoglycopeptide 1-39 and 4 times higher in asialo-

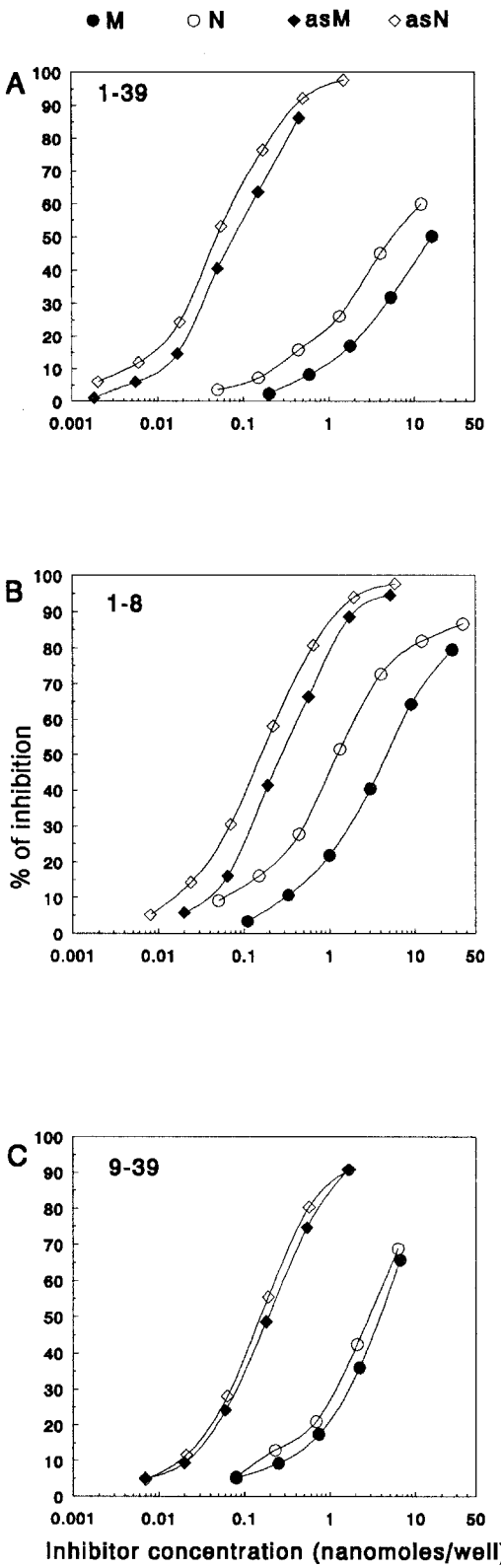

Fig. 2. Inhibition of MLL binding to asialoGPA-N-coated ELISA plates by serially diluted untreated (M, N, circles) and desialylated (asM, asN, diamonds) glycopeptides 1–39 (A), 1–8 (B) and 9–39 (C) obtained from GPA-M (full symbols) and GPA-N (empty symbols).

glycopeptide 1–8 than in the respective GPA-M fragments (Table 2). The content of Gal-ol was higher than that of GalNAc-ol and did not show blood group MN-dependent differences. Gal-ol was also detected earlier in β-elimination products of GPA [7,17] and its origin is not clear.

4. Discussion

In earlier studies free GalNAc-ol and NeuAcα2-6GalNAc-ol were either detected as minor components of β-elimination products of glycophorins [18,19], or were not detected at all [17,20]. They may have escaped detection due to low content or/and loss during the fractionation of released oligosaccharides. A minor amount of Tn receptors in asialoglycophorins was also detected by means of natural human anti-Tn antibodies [21]. Our previous results [7], together with the present ones, confirm the existence of non-galactosylated GalNAc residues in GPA and show that their level is significantly higher in GPA-N vs. GPA-M. Moreover, we report now that the difference in the content of non-galactosylated GalNAc residues between GPA-M and GPA-N is localized in the amino-terminal fragment of GPA, containing amino acid residues 1–8 (which include blood group MN-specific amino acid residues 1 and 5) and three O-glycosidic chains linked to Ser^2, Thr^3 and Thr^4. The differences in the content of GalNAc residues are well correlated with reactivity of glycopeptides with MLL. Only the glycopeptides 1–39 and 1–8 derived from GPA-N showed higher reactivity with MLL than respective GPA-M-derived glycopeptides.

The results of MLL inhibition assays show that the activity of untreated GPA [5] and GPA-derived glycopeptides (Table 1) is over 10 times lower than activity of their desialylated counterparts, despite our earlier finding [7] that only approximately half of the non-galactosylated GalNAc residues is sialylated. It shows that in addition to partial sialylation of GalNAc residues, sialic acid residues present on adjacent oligosaccharide chains may partly block access of unsubstituted GalNAc residues. On the other hand, the differences in the inhibitory activity of GPA-M vs. GPA-N are usually higher (over 15-fold) [5] than those found for blood group M and N glycopeptides (3–4-fold, Table 1). Most likely, in aggregated GPA only some most exposed Tn receptors (which include those dependent on blood group N) are available for MLL, while in non-aggregated M and N glycopeptides all GalNAc residues can react. This conclusion is supported by our present results which show that blood group N-dependent decrease of GalNAc galactosylation is localized in the amino-terminal portion of GPA. Although the number of Tn receptors in GPA is low, MLL binds GalNAc strongly, with K_a of 10^5–10^6 M^{-1} [4]. It may explain why MLL, in contrast to other anti-Tn lectins, detects these receptors on GPA and

Table 1
Concentrations of untreated and desialylated GPA glycopeptides giving 50% inhibition of MLL and PNA binding to asialo-GPA-N-coated ELISA plates

	Native, MLL (nanomol/well)	Asialo, MLL (nanomol/well)	Asialo, PNA (nanomol/well)
M, 1–39	16.0	0.08	0.13
N, 1–39	5.3	0.04	0.14
M, 1–8	4.5	0.28	0.83
N, 1–8	1.2	0.14	1.00
M, 9–39	2.4	0.20	0.31
N, 9–39	1.9	0.17	0.30

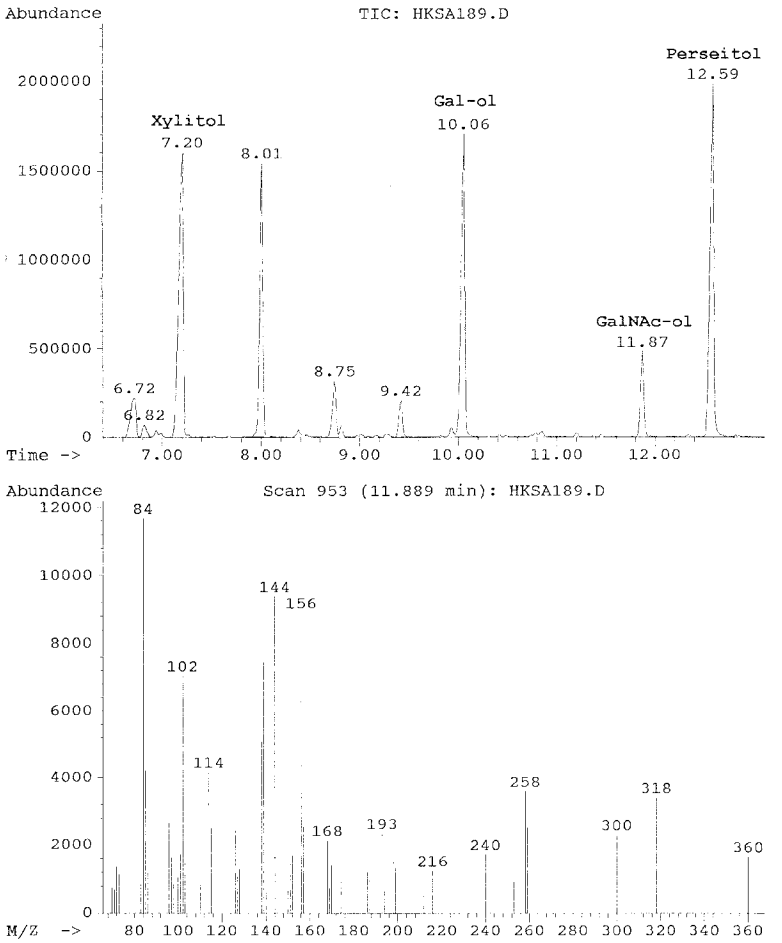

Fig. 3. GLC-MS analysis of unhydrolyzed peracetylated β-elimination products of the glycopeptide 1-39 of GPA-N, containing internal standards (xylitol and perseitol). The upper part shows pattern of elution of xylitol, Gal-ol, GalNAc-ol and perseitol from GLC column. Peak with retention time 8.01 and other small peaks are non-carbohydrate components. In the lower part a mass spectrum of 11.87 peak (GalNAc-ol) is shown.

why a small difference in the content of Tn receptors in GPA-N vs. GPA-M is also distinctly detectable by MLL.

In conclusion, our results demonstrate directly that difference in reactivity of GPA-M and GPA-N with MLL is due to the different content of non-galactosylated GalNAc residues (Tn receptors) in N-terminal fragments of GPA. The only reason for this difference is different amino acid residues in GPA-M and GPA-N. Modulation of GalNAc enzymatic galactosylation by amino acid sequence of GalNAc-peptides has been demonstrated in model studies in vitro [22]. Our results show that such modulation occurs also in vivo and affects mainly GalNAc residues located in proximity of 'modulating' amino acid residues. A specific agglutination of blood group N erythrocytes by MLL is an example illustrating that even a subtle alteration of glycosylation resulting from amino acid replacements may distinctly influence glycosylation-dependent cellular interactions, e.g. by passing the threshold of cell surface receptor density required for agglutination.

Table 2
Content of GalNAc-ol and Gal-ol in the products of β-elimination of GPA-M and GPA-N glycopeptides

	GalNAc-ol	Gal-ol
	mol/mol glycopeptide	
M, 1–39	0.28	3.8
N, 1–39	0.62	3.2
M, 1–8	0.10	0.9
N, 1–8	0.38	0.7
M, 9–39	0.17	2.2
N, 9–39	0.22	2.8

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