CHARACTERIZATION OF ACCEPTOR PROTEINS OF HUMAN ERYTHROCYTES FOR ENZYMATICALLY TRANSFERRED *N*-ACETYLGALACTOSAMINE

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1. Introduction

The blood group A-gene specified *N*-acetylgalactosaminyltransferase of human plasma transfers α-*N*-acetylgalactosamine (GalNAc) from UDP-GalNAc to glycoprotein and glycolipid acceptors of human erythrocytes creating new antigens. This enzyme has been claimed to be specific for H antigens and used for their identification and quantitation [1–4].

This study aimed to identify and characterize those erythrocyte glycoproteins which can act as acceptors for [^{14}C]GalNAc. I here report that [^{14}C]GalNAc was transferred to most, if not all, of the membrane glycoproteins. Band 3 was the most heavily labelled protein, but also the sialoglycoproteins were labelled. Band 3 and band 4.5 proteins but not the sialoglycoproteins bound to the blood group A-specific lectin from *Vicia cracca*. The products of the transferase-catalyzed reaction and the molecules recognized by the blood group A-specific lectin are not identical.

2. Materials and methods

Fresh normal human erythrocytes, the ABO types of which were determined by standard techniques, and human plasma pooled from several donors of blood group A were obtained from the Finnish Red Cross Blood Transfusion Service (Helsinki). *N*-Acetylgalactosaminyltransferase was isolated by affinity chromatography on Sepharose 4B [5] and concentrated by ultrafiltration to 0.5% of the original plasma volume (A-enzyme).

To 0.4 ml packed red cells were added 0.36 ml 0.05 M cacodylate buffer (pH 6.8), 0.2 ml A-enzyme preparation, and 40 μl UDP-[^{14}C]GalNAc (1 μCi), the Radiochemical Centre (Amersham). Incubation took place at 37°C with occasional stirring for 5 h unless otherwise indicated. The cells were washed twice with PBS by centrifugation at 4°C. The membranes were isolated by centrifugation in 5 mM Tris–HCl (pH 8.0) at 20 000 × *g* for 15 min.

Red cells were labelled with galactose oxidase/NaB^3H_4 as in [6] without neuraminidase treatment.

For endo-β-galactosidase treatment [7], 50 μl washed, packed, labelled cells were suspended in 0.2 ml 0.15 M NaCl adjusted to pH 5.8 with acetic acid, containing 2 mU endo-β-galactosidase (Seikagaku, Tokyo). The cells were then incubated at 37°C for 2 h with gentle shaking.

For immunoprecipitation, labelled erythrocyte membranes were solubilized in 1% Triton X-100–PBS and after centrifugation immunoprecipitation was performed using rabbit anti-human glycophorin A serum and protein A-containing staphylococci [8].

For affinity chromatography the blood group A-specific lectin from *V. cracca* [9] was coupled to Sepharose 4B (Pharmacia, Stockholm) at 5 mg protein/ml wet gel [10]. [^{14}C]GalNAc-labelled erythrocyte membranes were solubilized in 1% Triton X-100–PBS/2 mM phenylmethylsulfonyl fluoride and passed through the affinity column (1 × 19 cm) at room temperature. Adsorbed glycoconjugates were eluted with 10 mM *N*-acetyl-D-galactosamine in Triton X-100–PBS. After counting, the labelled proteins were precipitated with 67% ethanol at −20°C and solubilized in electrophoresis buffer.

SDS–Polyacrylamide slab gel electrophoresis was done according to Laemmli [11] on 8% acrylamide gels. The slab gels were fixed after completion of the electrophoresis in 20% sulfosalicylic acid overnight. The gels were treated for fluorography [12] and

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vacuum-dried. The dried gels were covered with Kodak RP X-Omat film, wrapped in aluminium foil, and stored at −70°C for 1–8 days until developed.

3. Results

Erythrocytes of blood group O were labelled with [^{14}C]GalNAc using the A-enzyme for different times. The incorporation of radioactivity was linear up to 5 h (not shown), after which haemolysis became apparent. The 5 h labelling time was chosen for subsequent experiments to avoid proteolysis.

Fig.1 shows labelled red cells analyzed on a slab gel. Fig.1B,D shows red cells labelled with galactose oxidase/NaB^{3}H$_4$, which labels most of the red cell glycoproteins [6]. It can be seen (fig.1F,H) that the A-enzyme also transfers label into most glycoproteins. Band 3 and band 4.5 proteins were clearly labelled. Part of these were aggregated (top of gels). Inclusion of 3 M urea in the electrophoresis samples diminished aggregation. Also the sialoglycoproteins, glycophorin A dimer and monomer (GPA-D and GPA-M) seemed to be labelled. Further evidence for this was obtained by treating labelled cells before electrophoresis with endo-β-galactosidase. This enzyme is known to hydrolyse the carbohydrate portions of band 3, band 4.5 and macroglycolipids, but to leave the sialoglycoproteins intact [7]. All major labelled glycoproteins but the sialoglycoproteins were sensitive to this enzyme (fig.1C,E,G,I). When cylindrical gels were used for quantitation of radioactivity it was found that ⩾80% of the ^{14}C-label was in the region of glycoproteins of $M_r \geqslant 35\,000$ (not shown).

As the electrophoretic mobility in SDS-gels cannot be taken as a definite proof for the identity of a molecule, the incorporation of [^{14}C]GalNAc into glycophorin A was confirmed by precipitation with specific anti-glycophorin A antiserum. Fig.2 shows the [^{14}C]GPA-D and [^{14}C]GPA-M precipitated from

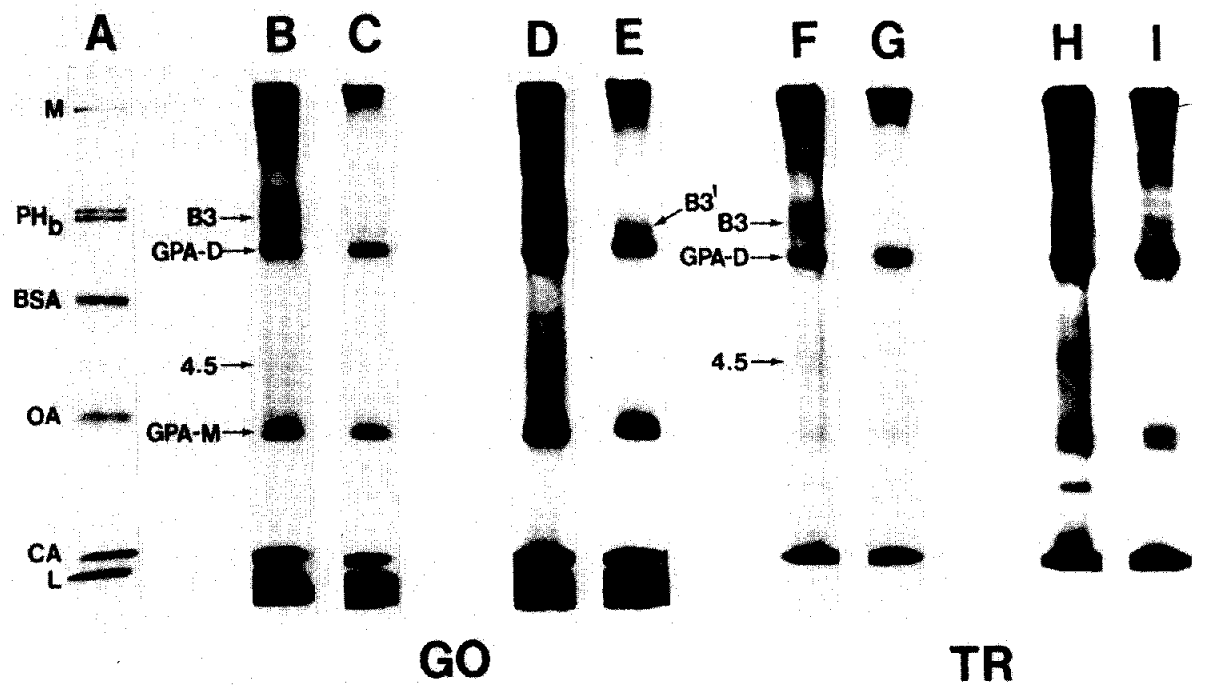

Fig.1. Fluorography of a slab gel of surface-labelled erythrocytes. O cells were labelled by use of the A-enzyme (TR) (F–I) or with the galactose oxidase/NaB^{3}H$_4$ (GO) method (B–E). Half of the cells (C,E,G,I) was treated with endo-β-galactosidase; the other half (B,D,F,H) was not. Lanes D and E correspond to lanes B and C, respectively, after a longer exposure to show the more weakly labelled components. Likewise, H and I correspond to F and G, respectively. ^{14}C-Labelled standard proteins (A) are: M, myosin; Ph$_b$, phosphorylase *b*; BSA, bovine serum albumin; OA, ovalbumin; CA, carbonic anhydrase; L, lysozyme; B3, band 3; B3′, fraction of band 3 resistant to endo-β-galactosidase; GPA-D, glycophorin A dimer; 4.5, glycoproteins in the band 4.5 region; GPA-M, glycophorin A monomer.

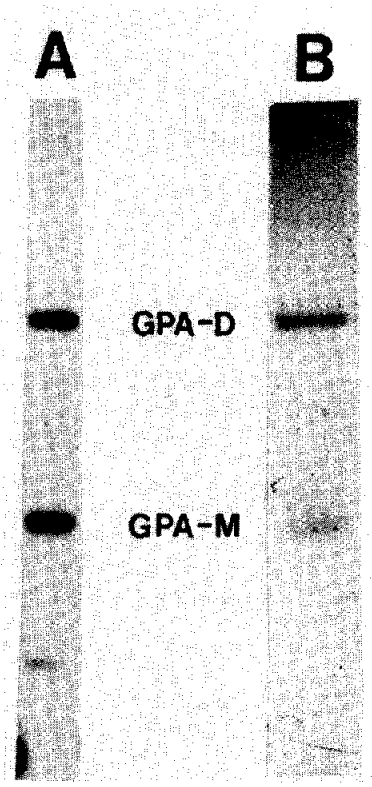

Fig.2. Immunoprecipitation of glycophorin A from O cells labelled by use of the A-enzyme: (A) Surface pattern of periodate/NaB^3H_4-labelled red cells showing the labelled glycophorin A; (B) glycophorin A precipitated with anti-glycophorin A antiserum from O red cells labelled using the A-enzyme; GPA-D, glycophorin A dimer; GPA-M, glycophorin A monomer.

labelled Triton X-100-solubilized membranes.

When erythrocytes of blood groups A_1, A_2 and B were labelled with [^{14}C]GalNAc in parallel with O cells, the amounts of ^{14}C-label incorporated were 27%, 75% and 63%, respectively, compared to that of O cells (100%). When these cells were analyzed on a slab gel, the labelling pattern was found to be similar to that of O cells in all cases (not shown).

Expression of A-activity in the [^{14}C]GalNAc-labelled glycoproteins of O cells was studied using the *V. cracca* lectin-affinity column. When the Triton X-100 solubilized membranes were fractionated on the column, 40% of the label passed through the column, while 60% of the label was eluted with 10 mM GalNAc. After concentration the samples were electrophoresed and it was found that the sialoglycoproteins did not bind to the column, while band 3 and band 4.5 proteins bound (fig.3).

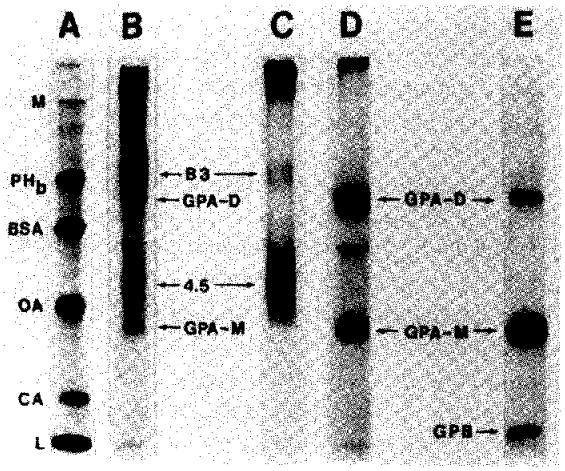

Fig.3. Fractionation of [^{14}C]GalNAc-labelled glycoproteins from O cells on the *Vicia cracca* lectin column: (A) ^{14}C-labelled standard proteins as in fig.1(A); (B) O red cells labelled by use of the A-enzyme (urea at 3 M was included in the sample buffer); (C) glycoproteins from [^{14}C]GalNAc-labelled O cells that bound to the *V. cracca* column; (D) glycoproteins from [^{14}C]GalNAc-labelled O cells not bound to the *V. cracca* column; (E) surface pattern of periodate/NaB^3H_4-labelled red cells showing the sialoglycoproteins. *Abbreviations:* as in fig.1; GPB, glycophorin B

4. Discussion

Although it has been firmly established that glycoproteins markedly contribute to ABH-activity [13–15], full agreement on identities of these glycoproteins has not been achieved. For identification of ABO blood group antigens, blood group-specific lectins [16,17] and *N*-acetylgalactosaminyltransferases (A-enzyme) from different sources [3,4,18,19] have been used. There is general agreement that the glycoproteins band 3 and 4.5 carry ABH-activity but the contribution of the major sialoglycoprotein, glycophorin A to this activity has remained controversial [16,17,19,20]. Purified glycophorin A from A erythrocytes was unable to inhibit agglutination of such cells by anti-A antiserum [20]. In agreement with this, surface-labelled glycophorin A from A cells did not bind to the blood group A-specific *V. cracca* lectin [16]. However, the AB-specific *Bandeiraea simplicifolia* lectin was reported to bind to the sialoglycoproteins [17]. Isolated sialoglycoproteins acted also as acceptors for [^{14}C]GalNAc [19].

I have here studied erythrocyte acceptors for [^{14}C]GalNAc using the A-enzyme preparation isolated

from human plasma, and the acquisition of blood group A activity with emphasis on glycophorin A. When cylindrical SDS-gels were used, ⩾80% of the ^{14}C-label was incorporated into glycoproteins. The labelling profile (not shown) was similar to that in [18] but the identity of proteins was not further studied in [18]. The use of slab gels made the identification of labelled glycoproteins unambiguously possible. Band 3 was the most heavily labelled glycoprotein but also the sialoglycoproteins were labelled (fig.1). Immunoprecipitation (fig.2) with rabbit anti-glycophorin A antiserum was used to confirm the labelling of glycophorin A. The labelled glycophorin A failed, however, to bind to the *V. cracca* lectin (fig.3). This shows that although glycophorin A can act as in vitro-acceptor for the enzymatically transferred *N*-acetyl-D-galactosamine, this does not result in glycophorin A molecules reacting with the blood group A-specific lectin. This may be due to A-determinants not reacting with the *V. cracca* lectin. Another possibility is that the A-enzyme is not strictly specific for H-determinants. Structural analyses of purified glycophorin A have not revealed ABH-determinants on either O- or N-linked oligosaccharides [21,22].

Glycoconjugates identified with the A-enzyme or blood group A-specific lectin may not exactly correspond to molecules reacting with blood group A isoantibodies.

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