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Biochemical characterization of the *O*-glycans on recombinant glycophorin A expressed in Chinese hamster ovary cells

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Alterations in *N*- and *O*-linked glycosylation affect cell surface expression and antigenicity of recombinant glycophorin A expressed in transfected Chinese hamster ovary (CHO) cells. To understand these effects further, glycophorin A was purified by immunoaffinity chromatography from transfected wild type and glycosylation deficient CHO cells. The *O*-glycans were characterized both biochemically, using gel filtration and high performance anion exchange chromatography, and immunologically, using carbohydrate specific monoclonal antibodies to probe Western blots. The *O*-glycans of human erythrocyte glycophorin A consist mainly of short oligosaccharides with one, two, or three sialic acid residues linked to a common disaccharide core, Galβ1-3GalNAcα1-Ser/Thr, with the disialylated structure being the most abundant. With the exception of the trisialylated derivative, the same structures were found on recombinant glycophorin A expressed by wild type CHO cells. However, in contrast to human erythrocyte glycophorin A, the monosialylated oligosaccharide was the most abundant structure on the recombinant protein. Furthermore, recombinant glycophorin A was shown to express a small amount of the Tn antigen (GalNAcα1-Ser/Thr). Recombinant glycophorin A had the same *O*-glycan composition, whether purified from clones expressing high or moderate levels of the recombinant glycoprotein. This indicates that the level of expression of the transfected glycoprotein did not affect its *O*-glycan composition. Deletion of the *N*-linked glycosylation site at Asn_{26}, by introducing the Mi.I mutation ($Thr_{28} \rightarrow$ Met) by site-directed mutagenesis, did not markedly affect the *O*-glycan composition of the resulting recombinant glycoprotein expressed in wild type CHO cells. This demonstrates that the presence or absence of the *N*-glycan did not influence *O*-linked glycosylation of the recombinant glycoprotein. Finally, the *O*-glycans on recombinant glycophorin A expressed in the Lec 2 and Lec 8 glycosylation deficient CHO cells were characterized. The *O*-glycans on Lec 2 cell glycophorin A were predominantly Galβ1-3GalNAcα1-Ser/Thr (T antigen), while those on Lec 8 glycophorin A were exclusively GalNAcα1-Ser/Thr (Tn antigen). These results will lead to a better understanding of the cell biology and immunology of this important human erythrocyte glycoprotein.

Keywords: Glycophorin, glycoproteins, *O*-glycans, blood group antigens

Introduction

Glycophorin A is the major sialoglycoprotein of human erythrocytes. It is highly glycosylated, containing one *N*-glycan and 15 *O*-glycans [1]. These *N*- and *O*-glycans have been biochemically characterized [2–4]. Glycophorin A is the carrier of several carbohydrate, glycopeptide and peptide blood group antigens, including those in the MN and Miltenberger (Mi) systems [5–7]. In addition to its importance in transfusion medicine [8–12], glycophorin A is involved in the pathogenesis of malaria, since its presence on the erythrocyte surface is required for invasion by *Plasmodium falciparum* merozoites [13].

We have used a molecular biological approach to study the cell biology and antigenicity of glycophorin A. Understanding the biochemical structures of the attached oligosaccharides is important for the appropriate interpretation of these results. For example, studies of the role of *N*- and *O*-linked glycosylation in intracellular transport of glycophorin A in transfected Chinese hamster ovary (CHO) cells demonstrated that appropriate *O*-linked glycosylation was necessary for optimal cell surface expression [14, 15]. In addition, by using site-directed mutagenesis to introduce mutations into the cDNA encoding glycophorin A, several transfected CHO cell lines expressing variant antigens of glycophorin A were constructed [15, 16]. These cell lines are a potential source of artificial antigens of use in blood

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group serology [17]. Since many mouse monoclonal [18–28] and human antibodies [12, 29] directed against glycophorin A recognize carbohydrate [12, 28, 30] or glycopeptide [18, 21–27, 29] blood group antigens, the nature of the oligosaccharides on the recombinant glycoprotein may modify recognition of these epitopes. Finally, for many strains of *Plasmodium falciparum*, merozoite binding to glycophorin A depends not only on the underlying polypeptide sequence, but also on the presence of sialylated oligosaccharides [31]. Thus, the use of recombinant glycophorin A as a model for analysing the fine specificity of this parasite-erythrocyte interaction will require characterization of the oligosaccharides attached to the glycoprotein.

The present study biochemically characterized the *O*-glycans on recombinant glycophorin A expressed by wild type CHO cells. The *O*-glycans on recombinant glycophorin A expressed by the glycosylation deficient Lec 2 and Lec 8 mutant CHO cell lines [14] were also analysed. Finally, the influence of *N*-linked glycosylation on *O*-linked glycosylation of glycophorin A was examined by analysing the *O*-glycans on recombinant Mi.I mutant glycophorin A expressed in wild type CHO cells. Mi.I mutant glycophorin A was constructed by site-directed mutagenesis [15]; the $Thr_{28} \rightarrow$ Met mutation deletes the only potential *N*-linked glycosylation site at Asn_{26} on glycophorin A [7, 32]. In each case, the biochemical results obtained with the recombinant glycoproteins were compared with those found for the *O*-glycans on native, human erythrocyte glycophorin A.

Materials and methods

Cell lines

Wild type CHO cells (Clone Pro-5) and Lec 2 and Lec 8 glycosylation deficient CHO cell lines [33, 34], were obtained from the American Type Culture Collection (Rockville, MD). Lec 2 cells have a defect in sialylation of glycoconjugates [35]; Lec 8 cells have a defect in galactosylation (and subsequent sialylation) of glycoconjugates [36]. Clone 26.1 and Clone 22.2, CHO Pro-5 derived cell lines which express the transfected M-allele of recombinant glycophorin A at high and moderate levels, respectively, were generated as described [14]. Clonal cell lines expressing the M-allele of glycophorin A in transfected Lec 2 and Lec 8 cells were similarly generated [14]. A CHO Pro-5 derived clonal cell line expressing transfected Mi.I. mutant glycophorin A was generated as described [15]; the Mi.I mutation ($Thr_{28} \rightarrow$ Met) was constructed by site directed mutagenesis [37]. Cells were cultured in alpha-Minimal Essential Media (Sigma, St Louis, MO) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B. Transfected cells were maintained in this medium containing, in addition, 0.5 mg ml^{-1} of active geneticin (G418; GIBCO, Grand Island, NY).

Immunoaffinity column

Ascites containing mouse monoclonal antibody 6A7 [23] was kindly provided by W. L. Bigbee. This antibody recognizes a sialic acid dependent epitope on the M-allele of glycophorin A [23, 24]. One ml of ascites was diluted 1:1 with 0.1 M sodium borate buffer, pH 8.2 and incubated with 1 ml of protein-G agarose (GIBCO-BRL, Gaithersburg, MD) with gentle rocking at 4 °C for 16 h. Unbound antibody was washed off with 0.1 M sodium borate buffer. The bound antibody was covalently cross-linked to protein G by incubating the beads with 25 mM dimethylpimelimidate dihydrochloride (Pierce, Rockford, IL) in 200 mM triethanolamine, pH 8.2 for 30 min at room temperature with gentle rocking. The reaction was terminated by the addition of 100 mM ethanolamine, pH 8.2 and incubation for 5 min at room temperature. The affinity column was stored at 4 °C in phosphate buffered saline (PBS; 10 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.4) containing 0.01% sodium azide.

Purification of recombinant glycophorin

Confluent CHO cells were harvested from ten 165 cm^2 tissue culture flasks. The cells were washed twice in ice cold PBS containing 200 μg ml^{-1} of phenylmethylsulfonyl fluoride and then solubilized in 2 ml of Lysis Buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40) containing 1 mM EDTA and 200 μg ml^{-1} of phenylmethylsulfonyl fluoride. After a 20 min incubation on ice, the detergent lysates were centrifuged at 14 000 × **g** for 10 min. The supernatants were removed and centrifuged again at 150 000 × **g** for 30 min. The resulting clarified lysate was incubated with 1 ml of protein-G agarose, previously equilibrated with Lysis Buffer, with gentle rocking for 16 h at 4 °C. The lysate was separated from the protein-G agarose, applied to the antibody 6A7-immunoaffinity column (1 ml bed volume), and incubated with gentle rocking at 4 °C for 16 h. The column was sequentially washed with 15 ml of Lysis Buffer, 5 ml of 100 mM glycine, pH 2.8, and 5 ml of Lysis Buffer. The bound glycophorin A was subsequently eluted with 5 ml of 100 mM triethylamine, pH 11.4 containing 0.5% NP-40. One ml fractions were collected and immediately neutralized by adding 50 μl of 1.0 M sodium phosphate buffer, pH 6.8. The fractions containing glycophorin A were vacuum-dialysed against water to remove the detergent and to concentrate the sample, using 10 000 molecular weight cut off collodion bags (Schleicher and Schuell, Keene, NH). The purity of the samples was analysed by SDS-PAGE separation and staining with Coomassie blue [38] and periodic acid Schiff reagent [39].

Purification of human erythrocyte glycophorin A

Human glycophorin A was purified from 450 ml of freshly drawn blood (type MN) as previously described [39]. Purified glycophorin A was further purified using the

antibody 6A7-immunoaffinity column, as described above. This separated glycophorin A from the other glycophorins (glycophorins B, C, and D) in the preparation.

Analysis of O-glycans

The *O*-glycans were released from affinity purified glycophorin A by β-elimination [40, 41]. The sample was incubated in 0.05 M NaOH and 1 M $NaBH_4$ containing 10 mCi of ^{3}H-$NaBH_4$ (specific activity >100 mCi mmol^{-1}, New England Nuclear, Boston, MA) at 50 °C for 18 h. The reaction was neutralized by adding 50% acetic acid. Borate salts were removed by repeated evaporation with methanol which was acidified with a drop of acetic acid. The sample was then passed through 1 ml of Dowex 50W-X8 (H^+-form, Bio-Rad, Richmond, CA) cation exchange resin in water. The resulting sample was applied to a Bio-Gel P-2 (<400 mesh, Bio-Rad) column (0.9 cm × 90 cm) and eluted with 0.05 M acetic acid at a flow rate of 5.4 ml h^{-1} [42]. Fractions (0.9 ml) were analysed for radioactivity by liquid scintillation counting. The fractions containing the radiolabelled *O*-glycans were pooled, lyophilized, and further analysed by high-performance anion exchange (HPAE) chromatography using a Dionex system [43]. The oligosaccharides were separated on a Carbo Pak PA1 column (Dionex Corporation, Sunnyvale, CA) using 0.2 M NaOH as Eluent 1 and 0.2 M NaOH containing 0.5 M sodium acetate as Eluent 2. The elution programme was: 99% Eluent 1 and 1% Eluent 2 for 5 min, followed by a linear gradient to 60% Eluent 1 and 40% Eluent 2 at 65 min. The column flow was 1 ml min^{-1} and 1 ml fractions were collected and counted in a scintillation counter.

Sialic acid was released from the oligosaccharides by mild acid hydrolysis in 2 M acetic acid at 80 °C for 3 h [44]. Periodate oxidation was performed with 8 mM sodium periodate in 0.1 m sodium acetate buffer, pH 5.5 at 4 °C for 48 h, as described [45]. After oxidation, the sample was neutralized and reduced with $NaBH_4$. Terminal non-reducing galactose residues were hydrolysed using bovine testis β-galactosidase (Sigma), as described [46].

Metabolically labelled *O*-glycans were isolated and analysed as follows. CHO cells were cultured in 10 cm diameter petri dishes to near confluence. The cells were metabolically labelled for 24 h with 10 μCi ml^{-1} of ^{3}H-glucosamine (Amersham Corp., Arlington Heights, IL). After labelling, the cells were lysed and glycophorin A was purified by immunoprecipitation [47] with a 1:250 dilution of ascites containing the Pep-80 mouse monoclonal antibody. This antibody was generously provided by E. Lisowska and is directed against the cytoplasmic domain of glycophorin A [48]. The immunoprecipitated protein was further purified by preparative SDS-PAGE on 10% gels [38]. After electrophoresis, the gel was fixed, incubated with Amplify (Amersham), dried, and autoradiographed for 48 h with Amersham Hyperfilm. The autoradiogram was used to localize the glycophorin A bands in the dried gel. The bands were excised, cut into 3 mm × 3 mm pieces, and incubated with Pronase (Calbiochem, San Diego, CA) to prepare glycopeptides [49]. The Pronase digest was lyophilized, dissolved in 0.1 ml of water, and desalted by gel filtration on a 0.5 cm × 20 cm column of Sephadex G-15 (Pharmacia, Piscataway, NJ). The fractions containing radioactive material were pooled and dried under a stream of nitrogen. The *O*-glycans were released from the desalted glycopeptides by β-elimination (see above). The oligosaccharides were analysed, as above, by gel filtration on a Bio-Gel P-2 (<400 mesh) column (0.9 cm × 90 cm). Fractions of 0.9 ml were collected and analysed by scintillation counting. The elution positions of β-glucan standards, prepared by partial hydrolysis of dextran [50], were detected using a refractive index monitor.

Western blotting

Glycophorin A was immunoprecipitated from CHO cell lysates with monoclonal antibody Pep-80, as above. The immunoprecipitated proteins were separated by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes [51]. The membranes were blocked for 2 h at 37 °C with PBS containing 5% bovine serum albumin and then incubated with the appropriate primary antibody. Monoclonal antibody 22.19 was generously provided by E. Lisowska [52]; this mouse IgM antibody is directed against the T antigen (Galβ1-3GalNAcα1-Ser/Thr) and was used as a 1:20 dilution of culture supernatant. Monoclonal antibody NCC-Lu-35 was generously provided by S. Hirohashi [53]; this mouse IgM antibody is directed against the Tn antigen (GalNAcα1-Ser/Thr) and was used as a 1:5 dilution of culture supernatant. Peroxidase conjugated goat anti-mouse IgM (Kirkegaard and Perry, Gaithersburg, MD) was used as secondary antibody at a 1:100 000 dilution. The bands were visualized using a chemiluminescence detection system (ECL, Amersham). In addition, detergent soluble CHO cell proteins were directly separated by SDS-PAGE, blotted on to nitrocellulose, probed with Pep-80 monoclonal antibody (a 1:500 dilution of ascites), and visualized with a 1:1000 dilution of peroxidase conjugated goat anti-mouse IgG (Kirkegaard and Perry) and 4-chloro-1-naphthol [14].

Results

Purification of recombinant glycophorin A

Glycophorin A was purified by immunoaffinity chromatography from Clone 26.1 and Clone 22.2, wild type CHO cells expressing high and moderate levels, respectively, of the human recombinant glycoprotein. These recombinant forms of glycophorin A are designated GPA-26.1 and GPA-22.2, respectively. Glycophorin A was also purified from wild type CHO cells expressing the Mi.I mutant

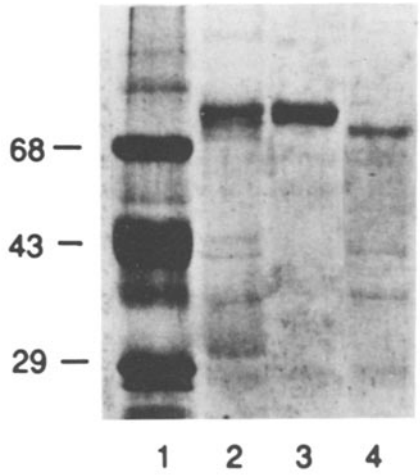

Figure 1. SDS-PAGE analysis of immunoaffinity purified glycophorin A. Glycophorin A was purified from human erythrocytes and transfected CHO cell lines, separated by SDS-PAGE, and stained with Coomassie blue, as described in the Materials and methods section. The molecular weights of the electrophoretic standards are indicated on the left: bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). Lane 1: molecular weight standards; lane 2: glycophorin A from human red blood cells (GPA-RBC); lane 3: glycophorin A from wild type CHO cell line Clone 26.1 (GPA-26.1); lane 4: glycophorin A from wild type CHO cell line expressing the Mi.I mutant (GPA-Mi.I).

(GPA-Mi.I). Approximately 50 μg of GPA-26.1 and GPA-Mi.I were isolated from 10 T-162 flasks of confluent transfected CHO cells. Approximately 20 μg of GPA-22.2 were isolated from 10 T-162 flasks of confluent cells. Glycophorin A, isolated from human red blood cells (GPA-RBC) by a classical method [39], was also further purified using the immunoaffinity column. Analysis by SDS-PAGE demonstrated that the glycoproteins were highly purified by this method and that they migrated almost solely as homodimers with an apparent molecular weight of approximately 80 kDa (Fig. 1). GPA-RBC (Fig. 1, lane 2), GPA-26.1 (Fig. 1, lane 3), and GPA-22.2 (data not shown) had similar electrophoretic mobilities, while GPA-Mi.I (Fig. 1, lane 4) migrated slightly faster due to the absence of the *N*-glycan [7]. In addition, the only glycosylated proteins detected in these preparations, as judged by periodic acid Schiff staining [39], were the glycophorin A proteins (data not shown). No glycoproteins were identified by SDS-PAGE analysis using this immunoaffinity purification procedure with untransfected CHO cells (data not shown).

Comparison of O*-glycans isolated from immunoaffinity purified GPA-RBC, GPA-26.1, GPA-22.2, and GPA-Mi.I*

The *O*-glycans were released by treatment with alkaline borohydride containing ^{3}H-$NaBH_4$; this specifically labels the reducing termini of the released oligosaccharides. The alkaline borohydride-treated samples were separated by gel filtration on Bio-Gel P-2. Polypeptides and glycopeptides eluted near the void volume, while the oligosaccharides were retained on the column and eluted just prior to the salt peak (data not shown).

The oligosaccharide fractions were pooled and further analysed by HPAE chromatography (Fig. 2). Under these chromatographic conditions, fractions with greater negative charge elute later. The *O*-glycans from GPA-RBC separated into three peaks with the approximate ratio of 2:5:0.1 (Fig. 2, panel A). This is consistent with the previously reported *O*-glycan composition of human erythrocyte glycophorin A [2, 4], with peak I corresponding to Neu5Acα2-3Galβ1-3GalNAc-ol, peak II corresponding to Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNAc-ol, and peak III corresponding to Neu5Acα2 - 8Neu5Acα2 - 3Galβ1 - 3-[Neu5Acα2-6]GalNAc-ol and/or Neu5Acα2-3Galβ1-3-[Neu5Acα2-8Neu5Acα2-6]GalNAc-ol. Residual radiolabelled salt eluted in the void volume (Fig. 2A). When the *O*-glycans prepared from transfected CHO cells were analysed by HPAE chromatography, the profiles shown in Fig. 2, panels B and C, were obtained for GPA-26.1 and GPA-Mi.I, respectively. The results with GPA-22.2 were virtually identical to those shown in panels B and C (data not shown). In each case, peaks comigrating with the monosialylated and the disialylated structures were found; no peak comigrating with the trisialylated structure(s) was detected. However, in contrast to GPA-RBC, the ratio of the monosialylated to the disialylated oligosaccharides was approximately 3:1 for GPA-26.1, GPA-22.2, and GPA-Mi.I. These results demonstrate that, by HPAE chromatography, the level of recombinant protein expression or the presence or absence of the *N*-glycan on recombinant glycophorin A did not have a major effect on the type or amount of the *O*-glycans.

When the GPA-26.1 oligosaccharides were treated with mild acid, peaks I and II disappeared and all the radiolabelled material was recovered in the void volume (Fig. 2, panel D). Since neutral oligosaccharides elute in the void volume, this confirmed that the oligosaccharides in peaks I and II were both sialylated. Identical results were found with GPA-22.2 and GPA-Mi.I (data not shown).

The sialylation of the GPA-26.1 *O*-glycans was further analysed using periodate oxidation. Since periodate treatment cleaves carbon-carbon bonds between vicinal hydroxyl groups, it will cleave the bond between carbons 4 and 5 of 3-substituted GalNAc-ol. If sialic acid is bound to carbon 6 of GalNAc-ol, as in Galβ1-3[Neu5Acα2-6]GalNAc-ol, it will be liberated from the labelled oligosaccharide. In contrast, if sialic acid is linked to the galactose residue in either Neu5Acα2-3Galβ1-3GalNAc-ol or Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNAc-ol, it will remain linked to the labelled oligosaccharide following periodate oxidation. When the GPA-26.1 *O*-glycans were analysed by HPAE chromatography after periodate treatment, the peak corresponding to the disialylated compound disappeared while

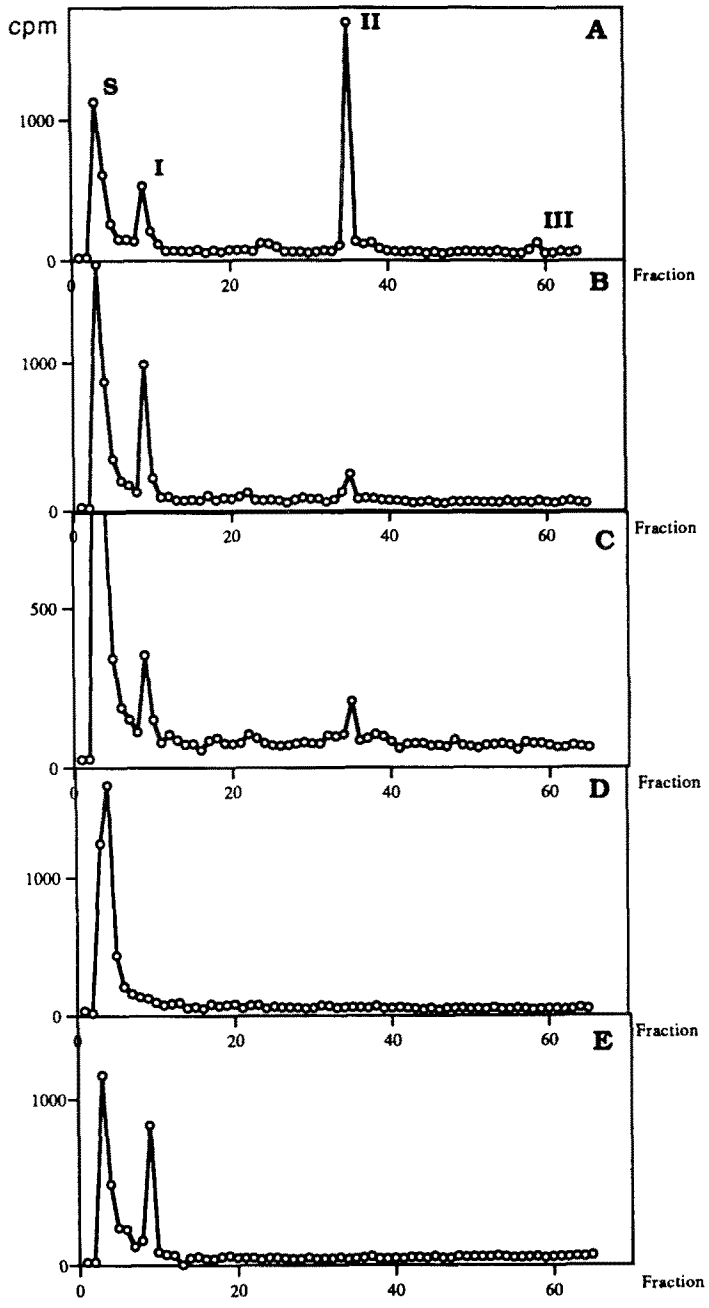

Figure 2. HPAE chromatography of *O*-glycans released by β-elimination from immunoaffinity purified glycophorin A. Glycophorin A was purified, the *O*-glycans were released by β-elimination, and the oligosaccharides treated with mild acid or periodate, as described in the Materials and methods section. Panel A: GPA-RBC *O*-glycans; panel B: GPA-26.1 *O*-glycans; panel C, GPA-Mi.I *O*-glycans; panel D: GPA-26.1 *O*-glycans after mild acid treatment; panel E: GPA-26.1 *O*-glycans after periodate oxidation. Peaks I, II, and III correspond to the *O*-glycans found on human erythrocyte glycophorin A [2, 4]. Peak I corresponds to Neu5Acα2-3Galβ1-3GalNAc-ol, peak II to Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNac-ol, and peak III to Neu5Acα2-8Neu5Acα2 - 3Galβ1[Neu5Acα2 - 6]GalNac-ol and/or Neu5Acα2 - 3Galβ1-3[Neu5Acα2-8Neu5Acα2-6]GalNAc-ol. The peak labelled 'S' corresponds to the residual salt peak in panels A–C.

that corresponding to a monosialylated compound was found (Fig. 2, panel E). This suggests that the disialylated oligosaccharide contains at least one sialic acid linked to the 6-position of GalNAc-ol and that the monosialylated oligosaccharide is sialylated on the galactose.

These conclusions were further confirmed by analysing metabolically labelled *O*-glycans isolated from recombinant glycophorin A. Clone 26.1 CHO cells were metabolically labelled with ^{3}H-glucosamine. This sugar will be incorporated into glycoconjugates as *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid [54]. The radiolabelled glycophorin A was immunoprecipitated with mouse monoclonal antibody Pep-80, an antibody directed against the cytoplasmic domain of glycophorin A. Therefore, the binding of this antibody is independent of glycosylation [48]. The immunoprecipitated protein was further purified by preparative SDS-PAGE and the *O*-glycans were released by β-elimination. The released oligosaccharides were analysed by gel filtration on Bio-Gel P-2. The chromatogram obtained for the *O*-glycans isolated from GPA-26.1 is shown in Fig. 3, panel A. Glycopeptides containing *N*-glycans eluted in the void volume (fractions 27–35). The doublet eluting in fractions 50–59 probably corresponds to the disialylated and monosialylated structures, respectively, seen in Fig. 2, panel B. A minor peak is also seen in Fig. 3, panel A (fraction 63–65) co-eluting with the GalNAc-ol standard. Fractions 50–59 were pooled, treated with mild acid, and analysed again by gel filtration (Fig. 3, panel B). This resulted in one peak eluting as a neutral disaccharide (fractions 55–62) and another peak (fractions 64–67) comigrating with the free sialic acid standard. Fractions 55–62 from Fig. 3, panel B were pooled, treated with β-galactosidase, and analysed by gel filtration; the radiolabelled material eluted as a single peak comigrating with the GalNAc-ol standard (Fig. 3, panel C). Taken together these results strongly suggest that the major *O*-glycan structures found on human erythrocyte glycophorin A are also found on recombinant glycophorin A expressed in wild type CHO cells, but with different ratios of the mono- and disialylated structures. The trisialylated structure found on human erythrocyte glycophorin A could not be detected on GPA-26.1, GPA22.2, or GPA-Mi.I.

Analysis of the glycophorin A O*-glycans isolated from transfected Lec 2 and Lec 8 CHO cells*

Both Lec 2 and Lec 8 cells are deficient in sialic acid expression owing to their inability to transport CMP-Neu5Ac and GDP-Gal, respectively [35, 36]. Therefore, the sequences of *O*-glycans on glycoproteins synthesized in Lec 2 and Lec 8 cell lines are thought to be restricted to Galβ1-3GalNAcα1-Ser/Thr and GalNAcα1-Ser/Thr, respectively.

Thus, transfected Lec 2 and Lec 8 CHO cells were metabolically labelled with ^{3}H-glucosamine. The recombinant proteins produced by Lec 2 and Lec 8 cells (GPA-Lec 2

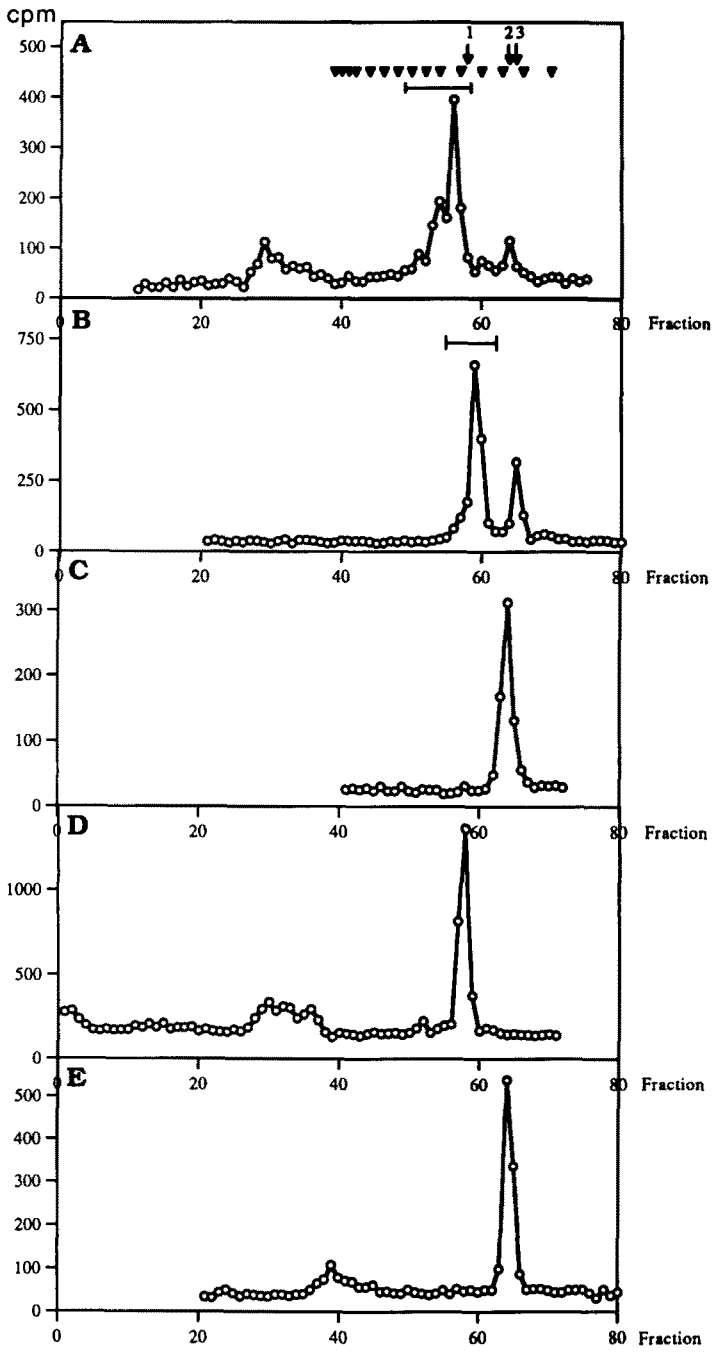

Figure 3. Bio-Gel P-2 gel filtration of *O*-glycans isolated from glycophorin A. Transfected CHO cells were metabolically labelled, glycophorin A was purified by immunoprecipitation, and the *O*-glycans were released by β-elimination and separated by gel filtration, as described in the Materials and methods section. Panel A: GPA-26.1, *O*-glycans; panel B: mild acid treated *O*-glycans from the pooled fractions indicated by the bar in panel A; panel C; β-galactosidase treated *O*-glycans from the pooled fractions indicated by the bar in panel B; panel D: GPA-Lec 2 *O*-glycans; panel E: GPA-Lec 8 *O*-glycans. The arrows indicate the elution positions of the following oligosaccharide standards: 1: Galβ1-4GlcNAc-ol; 2: GalNAc-ol; 3: Neu5Ac. Arrowheads indicate the elution positions of β-glucan oligomers (Glc_{15}-Glc_1) prepared by partial acid hydrolysis of dextran [50].

and GPA-Lec 8, respectively) were immunoprecipitated with mouse monoclonal antibody Pep-80, purified by preparative SDS-PAGE, and the released *O*-glycans analysed by gel filtration on Bio-Gel P-2, as above. Gel filtration analysis showed a single peak (fractions 56–60) for GPA-Lec 2 comigrating with a neutral disaccharide standard (Fig. 3, panel D), and a single peak (fractions 62–67) for GPA-Lec 8 comigrating with the GalNAc-ol standard (Fig. 3, panel E). In both cases, radiolabelled material corresponding to *N*-linked glycopeptides eluted later than in Fig. 3, panel A, consistent with the expected smaller size of the *N*-glycans in the glycosylation deficient cells. A minor peak was consistently seen at fraction 52 in the chromatogram of GPA-Lec 2 (Fig. 3, panel D), suggesting that less than 10% of the *O*-glycans on GPA-Lec 2 may exist as sialylated derivatives (Fig. 3D). The GPA-Lec 2 *O*-glycans were also separated by HPAE confirming the presence of a neutral disaccharide and a minor amount of monosialylated material (data not shown).

Western blot analysis of O*-glycans on recombinant glycophorin A*

The glycosylation of GPA-26.1, GPA-Lec 2, and GPA-Lec 8 was further analysed using monoclonal antibodies 22.19 and NCC-Lu-35, directed against the T and Tn antigens, respectively [52, 53]. The T and Tn antigens are composed of the Galβ1-3GalNAcα1-Ser/Thr and GalNAcα1-Ser/Thr structures, respectively. The recombinant glycoproteins were immunoprecipitated with antibody Pep-80, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with monoclonal antibodies. Western blots of the immunoprecipitated recombinant glycoproteins probed with antibody Pep-80 revealed the presence of the monomer, heterodimer, and homodimer forms of glycophorin A for each cell line (Fig. 4, lanes 1–3). GPA-Lec 8 and GPA-Lec 2 migrate faster than GPA-26.1 since they have smaller *O*-glycans. Antibody 22.19 bound only GPA-Lec 2 (Fig. 4, lane 6), confirming the results described above demonstrating the presence of Galβ1-3GalNAcα1-Ser/Thr on this glycoprotein. Antibody NCC-Lu-35 recognized the GalNAcα1-Ser/Thr antigen on GPA-Lec 8 (Fig. 4, lane 10), also confirming the results described above. In addition, a faint band was seen at the position of the GPA-26.1 monomer (Fig. 4, lane 8), implying that small amounts of the Tn antigen are present on recombinant glycophorin A expressed by wild type CHO cells. This is consistent with finding small amounts of GalNAc-ol on GPA-26.1 by gel filtration (Fig. 3, panel A).

Discussion

A model system was recently developed, using recombinant glycophorin A expressed in transfected CHO cells, to study the importance of *N*- and *O*-linked glycosylation in cell surface expression and antigenicity of this glycoprotein [14].

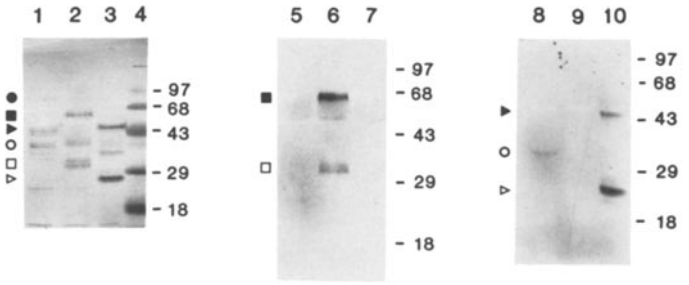

Figure 4. Western blot analysis of glycophorin A. Glycophorin A was immunoprecipitated with antibody Pep-80, separated by SDS-PAGE, and transferred to nitrocellulose. The blot was probed with monoclonal antibodies Pep-80 (lanes 1–3), 22.19 (lanes 5–7), and NCC-Lu-35 (lanes 8–10), and the bands detected colorimetrically (lanes 1–4) or by chemiluminescence (lanes 5–10), as described in the Materials and methods section. Lanes 1, 5, 8: GPA-26.1; lanes 2, 6, 9: GPA-Lec 2; lanes 3, 7, 10: GPA-Lec 8. The molecular weight markers are shown in lane 4 and are indicated on the right of each gel: phosphorylase B (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18 kDa). The positions of homodimer (closed symbols) and monomer (open symbols) forms of GPA-26.1 (circles), GPA-Lec 2 (squares), and GPA-Lec 8 (arrowheads) are indicated on the left of each panel.

Initial studies demonstrated that appropriate *O*-linked glycosylation was necessary for normal cell surface expression of glycophorin A, as the *N*-glycan by itself only supported weak surface expression [14]. In addition, site-directed mutagenesis was used to construct the Mi.I mutant of glycophorin A, deleting the one potential *N*-linked glycosylation site on this glycoprotein [15]. Studies with transfected Mi.I mutant glycophorin A demonstrated that, in the absence of both *N*- and *O*-glycans glycophorin A was not expressed at the cell surface [15].

To understand and exploit this system further it was necessary to characterize the *O*-glycans on the recombinant glycophorin A expressed in various types of CHO cells. The *O*-glycans on human erythrocyte glycophorin A have been previously characterized [2, 4]. They primarily consist of a neutral disaccharide core, Galβ1-3GalNAcα1-Ser/Thr, substituted with one, two, or three sialic acid residues; the disialylated compound is the most abundant structure. The current study found that recombinant glycophorin A expressed by transfected wild type CHO cells (GPA-26.1) contained the same mono- and disialylated oligosaccharides (Figs 2–4). The level of expression of the recombinant glycoprotein did not affect its overall *O*-linked glycosylation since virtually identical results were obtained with high and moderately expressing clones, Clones 26.1 and 22.2, respectively (data not shown). However, in contrast to human erythrocyte glycophorin A, the monosialylated oligosaccharide was the most abundant structure on the recombinant glycoprotein (Fig. 2), the trisialylated oligosaccharide was not detected (Fig. 2), and a small amount of the Tn antigen (GalNAcα1-Ser/Thr) was found (Figs 3, 4). Since the electrophoretic migration of recombinant glycophorin A was very similar to that of human glycophorin A (Fig. 1 and [14]), this difference in glycosylation probably reflects a change in the overall sialylation of the *O*-glycans and not a decrease in the number of *O*-glycans.

Since *N*-linked glycosylation is co-translational [55], while *O*-linked glycosylation occurs later in the secretory pathway (see [56–58]), it is possible that the presence or absence of an *N*-glycan could affect subsequent *O*-linked glycosylation at local or distant Ser and Thr residues. Nonetheless, the deletion of the *N*-linked glycosylation site on the Mi.I mutant of glycophorin A did not markedly affect overall *O*-linked glycosylation of this glycoprotein (Fig. 2). It was not determined whether *O*-linked glycosylation at individual sites was affected.

The *O*-glycans on glycophorin A expressed by the transfected glycosylation deficient cell lines Lec 2 and Lec 8 were studied both with chromatographic and immunological methods. The oligosaccharides found on GPA-Lec 2 (Figs 3 and 4) primarily consisted of the neutral disaccharide Galβ1-3GalNAcα1-Ser/Thr, the T antigen. A small fraction was identified which has a structure consistent with the monosialylated derivative (Fig. 3). This finding agrees with previous reports characterizing this cell line [35]. In contrast, GPA-Lec 8 contained only the GalNAcα1-Ser/Thr structure, the Tn-antigen.

Knowledge of the *O*-glycan structures on recombinant glycophorin A allows for the detailed study of the involvement of carbohydrate and peptide components of important human blood group antigens carried on this glycoprotein. A detailed study of the specificity of antibodies to these antigens can be performed in a controlled system in which it is possible to vary the peptide backbone by site-directed mutagenesis and the carbohydrate composition by transfection into CHO cell lines with defined glycosylation capabilities [14, 33, 34]. Indeed, some mouse monoclonal antibodies to erythrocyte glycophorin A do not bind to the recombinant protein expressed in wild type CHO cells [16]. This suggests that either these antibodies are sensitive to the slight differences in sialylation of the recombinant glycoprotein [59], or that recombinant glycophorin A is glycosylated at different Ser and Thr residues. Finally, since *Plasmodium falciparum* malaria merozoites bind to structures on glycophorin A consisting of both carbohydrate and peptide elements [13, 31], the use of recombinant variants of glycophorin A may also prove fruitful for studying this host-parasite interaction.

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