Expression of Human Glycophorin A in Wild Type and Glycosylation-deficient Chinese Hamster Ovary Cells

ROLE OF N- AND O-LINKED GLYOSYLATION IN CELL SURFACE EXPRESSION*

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Glycophorin A, the most abundant sialoglycoprotein on human red blood cells, carries several medically important blood group antigens. To study the role of glycosylation in surface expression and antigenicity of this highly glycosylated protein (1 N-linked and 15 O-linked oligosaccharides), glycophorin A cDNA (M-allele) was expressed in Chinese hamster ovary (CHO) cells. Both wild type CHO cells and mutant CHO cells with well defined glycosylation defects were used. Glycophorin A was well expressed on the surface of transfected wild type CHO cells. On immunoblots, the CHO cells expressed monomer (~38 kDa) and dimer forms of glycophorin A which co-migrated with human red blood cell glycophorin A. The transfected cells specifically expressed the M blood group antigen when tested with mouse monoclonal antibodies. Tunicamycin treatment of these CHO cells did not block surface expression of glycophorin A, indicating that, in the presence of normal O-linked glycosylation, the N-linked oligosaccharide is not required for surface expression. To study O-linked glycosylation, glycophorin A cDNA was transfected into the Lec 2, Lec 8, and IdID glycosylation-deficient CHO cell lines. Glycophorin A with truncated O-linked oligosaccharides was well expressed on the surface of IdID cells (cultured in the presence of N-acetylgalactosamine alone), Lec 2 cells, and Lec 8 cells with monomers of ~25 kDa, ~33 kDa, and ~25 kDa, respectively. In contrast, non-O-glycosylated glycophorin A (~19-kDa monomers) poorly expressed on the surface of IdID cells cultured in the absence of both galactose and N-acetylgalactosamine. Thus, under these conditions, in the absence of O-linked glycosylation, the N-linked oligosaccharide itself is not able to support appropriate surface expression of glycophorin A in transfected CHO cells.

Glycophorin A, an important constituent of the human red blood cell membrane (for reviews, see Refs. 1 and 2), is the most abundant sialoglycoprotein on the surface of these cells, numbering approximately 500,000 copies per cell. It is 131 amino acids in length, contains one transmembrane domain, is oriented with an extracytoplasmic amino terminus (3), and is highly homologous to another abundant glycoprotein on the red cell surface, glycophorin B (1, 2). Glycophorin A is highly glycosylated, containing 1 N-linked and 16 O-linked oligosaccharides (1-9). Most of the O-linked oligosaccharides have the classical structure, NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-R, found on many proteins (4); the N-linked oligosaccharide is predominantly a diacylated biantennary complex type structure (5). Due to this high degree of glycosylation, glycophorin A carries approximately 70% of the red blood cell sialic acid, although it comprises just 2-4% of the total membrane protein (1, 2). Glycophorin A encodes several blood group antigens important in transfusion medicine, including peptide antigens such as Mi.1 (6, 7), carbohydrate antigens such as Prc (8), T (9, 10), and Tn (11), and complex glycopeptide antigens such as M and N (12-14). Glycophorin A is also involved in the pathogenesis of malaria since its presence on the red blood cell surface is required for invasion by Plasmodium falciparum merozoites (15-18).

Since glycophorin A is structurally well characterized, it should provide an excellent model for studying the role that oligosaccharides play in glycoprotein biosynthesis, translocation, and surface expression. However, glycophorin A is predominantly found on mature red blood cells. These anuclear, biosynthetically inactive cells are unsuitable for examining biological and biochemical processes important in the expression of this glycoprotein. The K562 human erythroleukemia cell line (19) was used to study glycophorin A biosynthesis (20-23), but these studies yielded little information regarding the role of glycosylation in translocation and surface expression of glycophorin A. These cells synthesize small amounts of glycophorin A, and the oligosaccharide structures have not been completely defined. In addition, few options are available for perturbing the glycosylation of proteins in K562 cells other than using soluble glycosylation inhibitors (23).

The hypothesis that N- and O-linked oligosaccharides are important in surface expression of glycophorin A was examined in the present study. To address this hypothesis, glycophorin A cDNA was transfected into various Chinese hamster
methanol-fixed Clone 26.1 cells were examined by indirect immunofluorescence as described in Fig. 2. Live cells were treated with neuraminidase and/or incubated with tunicamycin as described under "Experimental Procedures." Panel A, live cells analyzed with monoclonal antibody 6A7; panel B, live cells treated with neuraminidase and then immediately analyzed with antibody 6A7; panel C, live cells treated with neuraminidase, cultured for 24 h in complete medium, and then analyzed with antibody 6A7; panel D, live cells treated with neuraminidase, cultured for 24 h in complete medium containing 5 µg/ml tunicamycin, and then analyzed with antibody 6A7; panels E and F, cells prepared as in panel D and then examined live or after fixation, respectively, with monoclonal antibody Pep80.

The effect of tunicamycin on cell surface expression of glycophorin A in transfected wild type CHO cells. Live and methanol-fixed Clone 26.1 cells were examined by indirect immunofluorescence as described in Fig. 2. Live cells were treated with neuraminidase and/or incubated with tunicamycin as described under "Experimental Procedures." Panel A, live cells analyzed with monoclonal antibody 6A7; panel B, live cells treated with neuraminidase and then immediately analyzed with antibody 6A7; panel C, live cells treated with neuraminidase, cultured for 24 h in complete medium, and then analyzed with antibody 6A7; panel D, live cells treated with neuraminidase, cultured for 24 h in complete medium containing 5 µg/ml tunicamycin, and then analyzed with antibody 6A7; panels E and F, cells prepared as in panel D and then examined live or after fixation, respectively, with monoclonal antibody Pep80.

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To exclude the possibility that the results in Fig. 5 were due to recycling of desialylated cell surface glycophorin A by intracellular vesicles through the Golgi and back to the plasma membrane, allowing resialylation (44), the experiment was repeated by culturing the cells with and without tunicamycin (5 µg/ml) in the presence of cycloheximide (10 µg/ml). Since cycloheximide blocks protein synthesis, this approach allows antibody 6A7 to distinguish between recycled and newly synthesized glycophorin A. The results (see Table I) demonstrate that there is no detectable recycling of desialylated cell surface glycophorin A with subsequent resialylation in this system.

The Role of O-linked Glycosylation in Surface Expression of Glycophorin A—To address the role of O-linked oligosaccharides in glycophorin A transport and expression, three glycoylation-deficient CHO cell lines were used: ldlD-5, Lec 8, and Lec 2. The O-linked oligosaccharides present on human red blood cell glycophorin A have the desialylated tetrasaccharide structure illustrated in Fig. 6 (1, 2, 4); the biosynthetic pathway leading to the formation of this structure is also illustrated (45).

The ldlD cells have a defect in the 4-epimerase enzyme (26, 27), blocking conversion of either glucose into galactose or N-acetylglucosamine into N-acetylgalactosamine. Thus, when these cells are cultured in medium lacking both galactose and N-acetylgalactosamine, no O-linked oligosaccharides are added to nascent glycoproteins. Similarly, when galactose alone is added to the medium, no O-linked oligosaccharides are added to nascent glycoproteins. When cultured in the presence of N-acetylgalactosamine alone, only this monosaccharide (or, possibly, the NeuAcα2-6GalNAc disaccharide) is found in an O-glycosidic linkage on glycoproteins. When both galactose and N-acetylgalactosamine are added to the medium, the mature tetrasaccharide can be synthesized. Since galactose and N-acetylgalactosamine are also constituents of glycosphingolipids and of N-linked oligosaccharides on glycoproteins, these are also modified in ldlD cells grown under restrictive conditions.

Following transfection of ldlD cells with pSG5gpa, several clonal cell lines were obtained which highly expressed glycophorin A when the cells were cultured in ldlD CM containing both galactose and N-acetylgalactosamine. One clone, Clone ldlD-5, was analyzed in detail by flow cytometry using glycophorin A-specific rabbit polyclonal antibody (Fig. 7). This antibody recognizes all forms of glycophorin A synthesized by ldlD cells under both permissive and restrictive conditions (see Fig. 8). If Clone ldlD-5 was cultured in ldlD CM containing galactose and N-acetylgalactosamine, high surface expression was seen (mean channel fluorescence 996), similar to...
**Table I**

**Cell surface glycoprotein A is not detectably recyled and resialylated in transfected CHO cells**

Clone 26.1 cells were treated with neuraminidase, cultured in the presence or absence of tunicamycin and cycloheximide, and analyzed by indirect immunofluorescence, as described under "Experimental Procedures." There was no effect of cycloheximide on cell viability when assayed by trypan blue exclusion. Monoclonal antibody 6A7 recognizes a sialic acid-dependent epitope on glycoprotein. "Positive" results indicate bright fluorescence identical with that seen in Fig. 5, panel A. "Negative" results indicate background fluorescence identical with that seen in Fig. 5, panels B and E. No intermediate results were seen.

| Neuraminidase Treatment | Incubate overnight | Indirect immunofluorescence with 6A7 |
|-------------------------|--------------------|-----------------------------------|
| Complete medium         | Nc                 | No                                | Positive |
| Complete medium         | Yes                | No                                | Negative |
| Complete medium         | Yes                | Yes in complete medium            | Positive |
| Complete medium         | Yes                | Yes in complete medium and         | Positive |
|                        |                    | tunicamycin                       |
| Complete medium and     | Yes                | Yes in complete medium and         | Negative |
| cytoheximide            |                    | tunicamycin and                   |
| Complete medium,        | Yes                | Yes in complete medium,           | Negative |
| tunicamycin, cycloheximide |      | tunicamycin, and                  |
| Cytoheximide            |                    | cytoheximide                      |

**FIG. 6.** CHO cell mutants defective in O-linked glycosylation. This represents the major biochemical pathway leading to O-linked protein glycosylation. The Lec 2, Lec 8, and ldlD cell lines have defects which interrupt the pathway at the biosynthetic steps indicated.

**FIG. 7.** Flow cytometric analysis of pSG5gpa-transfected ldlD cells cultured under various conditions. A clone of transfected ldlD cells (Clone ldlD-5) was cultured in ldlD CM (complete medium) without galactose or N-acetylgalactosamine (---,---), in ldlD CM with galactose alone (--,Gal), ldlD CM with N-acetylgalactosamine alone (GalNAc,---), or in ldlD CM supplemented with both sugars (GalNAc,Gal), as described under "Experimental Procedures." The cells were incubated with rabbit polyclonal anti-glycoprotein A antibody followed by fluorescein-conjugated goat anti-rabbit Ig. Non-specific background fluorescence was determined using normal rabbit serum followed by fluorescein-conjugated goat anti-rabbit Ig.

that found with Clone 26.1 cells probed with rabbit polyclonal antibody (mean channel fluorescence 896; data not shown). Somewhat diminished fluorescence intensity was observed when Clone ldlD-5 cells were cultured with N-acetylgalactosamine alone (mean channel fluorescence 434). This suggests that truncated O-linked oligosaccharides are sufficient for ensuring adequate surface expression. In contrast, when cultured in ldlD CM lacking galactose and N-acetylgalactosamine, these cells exhibited dim fluorescence (mean channel fluorescence of 11), suggesting that the presence of O-linked oligosaccharides is necessary for optimal surface expression of glycoprotein A. Diminished, but not absent fluorescence was seen when cells were cultured with N-acetylgalactosamine alone (mean channel fluorescence 247), although glycoprotein A synthesized by these cells should lack O-linked oligosaccharides. Untransfected wild type CHO cells (Pro-5) exhibited dim fluorescence by this method (mean channel fluorescence 37; data not shown). When permeabilized Clone ldlD-5 cells were probed with monoclonal antibody Pep80, equivalent binding was seen by classical indirect immunofluorescence regardless of whether the cells were cultured in the presence or absence of galactose and N-acetylgalactosamine (data not shown).
To biochemically examine the glycophorin A variants synthesized by transfected ldlD cells under various culture conditions, detergent lysates of Clone ldlD-5 cells were separated by SDS-PAGE and analyzed by Western blotting (Fig. 8). Using glycophorin A-specific rabbit polyclonal antibody, human red blood cells (Fig. 8, lane 7), transfected wild type CHO cells (Fig. 8, lane 6), and Clone ldlD-5 cells cultured in the presence of both galactose and N-acetylgalactosamine (Fig. 8, lane 5) all express glycophorin A monomers and dimers with virtually identical electrophoretic mobility. In contrast, Clone ldlD-5 cells grown under restrictive conditions express glycophorin A monomers and dimers of progressively faster electrophoretic mobility. This most likely results from the presence of truncated O-linked oligosaccharides (Fig. 8, lanes 2 and 3) on glycophorin A. Interestingly, glycophorin A in lane 3 migrates slower than that in lane 2. There is also increased heterogeneity in the banding pattern. It is possible that in the absence of galactose and N-acetylgalactosamine, the N-linked oligosaccharide on glycophorin A is restricted to be only a high mannose or hybrid form (46). In contrast, galactose allows further processing to occur leading to larger and more heterogeneous complex type N-linked oligosaccharides (46). Alternatively, small amounts of residual N-acetylgalactosamine in the culture medium or in the cells may allow sufficient O-linked glycosylation to permit surface expression.

To further confirm the results obtained with ldlD cells, glycophorin A cDNA was transfected into Lec 2 and Lec 8 cells. Lec 2 and Lec 8 cells have nonreversible defects in sequences of O-linked oligosaccharides on glycoproteins synthesized in both situations has similar O-linked oligosaccharides.

**DISCUSSION**

These results show stable expression of the M-allele of human glycophorin A in wild type CHO cells; the transfected glycoprotein exhibits a sialic acid-containing M antigenic epitope (Figs. 1–3). This represents one of the first examples of stable expression of a human blood group antigen in heterologous cells (49–51).

To examine the role in cell surface expression of glycophorin A of the N-linked oligosaccharide at Asn29, transfected wild type CHO cells were cultured with tunicamycin. Surface expression of the glycoprotein was not affected (Fig. 5). Analogous results were obtained by inactivating the N-linked glycosylation acceptor sequence by site-directed mutagenesis (Thr29→Met). These results agree well with the finding that individuals carrying the mutant allele Mi.1 (Thr29→Met) express the mutant protein on the surface of their red blood cells (7). Similarly, tunicamycin does not affect glycophorin A surface expression in K562 cells (21, 23). In addition, the homologous glycoprotein, glycophorin B, lacks an N-linked oligosaccharide but is expressed on human red blood cells (1, 2).

The role of N-linked glycosylation in glycoprotein surface expression has been previously described (37, 52–57). The oligosaccharide(s) may encourage proper folding of the nascent glycoprotein preventing aggregation or proteolysis in the endoplasmic reticulum (53, 58). However, N-linked glycosylation is not required for surface expression or secretion of all proteins (i.e. Ref. 59). In the current case, the O-linked oligosaccharides on glycophorin A may be sufficient to ensure its translocation in the absence of N-linked glycosylation.

To study the role of the O-linked oligosaccharides in glycophorin A surface expression, the cDNA was transfected into

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the Lec 2, Lec 8, and ldlD glycosylation-deficient CHO cell lines. Surface expression was evaluated by indirect immunofluorescence using rabbit polyclonal antibody. Transfected glycophorin A was highly expressed on the plasma membrane of Lec 2 and Lec 8 cells (data not shown) and also on the surface of ldlD cells cultured in the presence of N-acetylgalactosamine alone (Fig. 7). This suggests that even truncated, nonasialylated O-linked oligosaccharides promote high cell surface expression of glycophorin A. The results with Lec 2 and ldlD cells are analogous to those found with another heavily O-glycosylated glycoprotein, leukosialin, synthesized by the human T-cell Jurkat where the major O-linked oligosaccharide consisted of N-acetylgalactosamine alone (60). In contrast, when transfected ldlD cells were cultured in the absence of both galactose and N-acetylgalactosamine, conditions which prevent O-linked glycosylation, glycophorin A was poorly expressed at the cell surface (Fig. 7). Interestingly, intermediate levels of glycophorin A surface expression were seen when ldlD cells were cultured with galactose alone, although under these conditions O-linked glycosylation should also be prevented (Fig. 7). In addition, the protein synthesized by ldlD cells cultured with galactose alone showed greater heterogeneity on Western blots (Fig. 8). These latter two findings together suggest that the presence of galactose alone may allow extensive processing of the N-linked oligosaccharide permitting some surface expression of non-O-glycosylated glycophorin A. Alternatively, due to variations in the experimental conditions, the presence of small amounts of residual N-acetylgalactosamine in the culture medium or in the cells may allow sufficient O-linked glycosylation to permit surface expression. To distinguish between these two possibilities, it will be interesting to examine surface expression of the Mi.I mutant (described above) in transfected ldlD cells. When cultured with galactose alone or in the absence of both galactose and N-acetylgalactosamine, the protein should not be glycosylated at all. Biochemical analysis of the O-linked oligosaccharides on purified glycophorin A obtained from these cells should also be revealing. In a similar study, marked reduction in cell surface expression of the attachment glycoprotein of human respiratory syncytial virus has been found in cells in which both N- and O-linked glycosylation is blocked (61).

Several mechanisms may lead to poor surface expression or secretion of abnormally non-O-glycosylated glycoproteins. The protein may be retained or aggregated in the endoplasmic reticulum (62). Alternatively, as with the IL-2 receptor, it may be missorted to the wrong cellular compartment and not appear on the plasma membrane (63). In contrast, it may be expressed on the surface but then undergo rapid proteolysis, as with low density lipoprotein receptor (26, 64), decay accelerating factor (65), and the Epstein-Barr virus major envelope antigen (66). Similarly, the abnormal protein may reach the surface but then be rapidly internalized and degraded. Interestingly, secretion of the O-glycosylated secretory glycoproteins human chorionic gonadotropin (66) and apolipoprotein E (67) is not affected by blocking their acquisition of O-linked oligosaccharides. The mechanism leading to diminished surface expression of non-O-glycosylated glycophorin A is under investigation.

By extending the approach outlined here, it will be possible to investigate the structural correlates of carbohydrate, glycopptide, and peptide blood group antigens on glycophorin A. This is possible because transfected glycophorin A was highly expressed with or without N-linked oligosaccharides and with truncated, nonasialylated O-linked oligosaccharides. For example, it is predicted that transfected Lee 8 cells will express the T antigen (9, 10) and ldlD cells cultured with N-acetylgalactosamine alone will express the Tn antigen (11) on glycophorin A. In addition, since human anti-M alloantibodies recognize complex glycopeptide epitopes (13, 14, 68), cell lines expressing variant oligosaccharides will permit a detailed analysis of this specificity. Finally, site-directed mutagenesis will enable study of the amino acid sequences important in glycopeptide and peptide epitopes such as M and Mi.I, respectively (6, 7, 12-14, 68). Similarly, a detailed analysis of the interaction between P. falciparum merozoites and glycophorin A can be undertaken, particularly since the parasite recognizes signals encoded by both the amino acid sequence and oligosaccharides on glycophorin A (69-72). Thus, the availability of these cell lines will allow detailed investigations regarding the antigenicity, role in host-parasite interactions, and intracellular biosynthesis and trafficking of this interesting glycoprotein.

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Cell lines and tissue culture - Wild type CHO cells (Clone Pro 5; 25) were obtained from the American Type Culture Collection (Rockville, MD). Lec 2 (8) and Lec 8 (8), and BALB/3T3 (35) cell lines were obtained from the American Type Culture Collection. Guinea pig epithelial cells (11D CM) were maintained as described (43). Cultures were grown in alpha-Minimal Essential Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 20 μg/ml of amphotericin B (11D CM). In some experiments 11D CM was further supplemented with 20 mM ascorbate. 

Transfection - CHO cells were co-transfected with 25 μg of p50sia and 1 μg of pVIII (p155) by the calcium phosphate precipitation method (35) with a transfection stock (see Figure 4.3). 

Membrane immunofluorescence - To block N-linked glycosylation of cellular fibronectins, CHO cells were cultured in complete medium containing 5 μg/ml of tunicamycin for 24 h. Sialic acid residues were removed from the surface of red blood cells or live CHO cells by incubation with 1 unit of Disialyl-lectin per million cells for 30 min at 37°C. After washing, fluorescein-conjugated secondary antibodies diluted in 5% BSA/PBS were added and incubated for 30 min at 4°C. After washing, cells were fixed overnight in 1% paraformaldehyde and then analyzed for fluorescence intensity by an IHC microscopy (Beckton-Dickinson, Mountain View, CA). Ten thousand cells were analyzed for each condition. Dead cells were detected by low forward and right angle scatter and excluded from analysis.

Western blot - Ghosts were prepared from human red blood cells (38) and the proteins solubilized in an equal volume of 2X Laemmli sample buffer (125 mM Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol, and 0.002% bromophenol blue). CHO cells were solubilized in lysate buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0 with 0.5% NP40 and 1 mM PMSF), and the solubilized proteins (38) were fractionated by vertical SDS-PAGE. Horizontal immunoblotting was performed with secondary antibodies for horseradish peroxidase (Pierce) (4). The blots were then incubated with primary rabbit antibodies for horseradish peroxidase in PBS-BSA at room temperature. The membranes were washed with PBS-BSA and incubated with PPO (Pierce) for 1 h at 37°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat antiserum against rabbit IgG (Sigma) for 1 h at room temperature. The membranes were washed and incubated with PPO for 30 min at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat antiserum against rabbit IgG (Sigma) for 1 h at room temperature. After washing, the membranes were incubated with PPO for 30 min at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat antiserum against rabbit IgG (Sigma) for 1 h at room temperature. After washing, the membranes were incubated with PPO for 30 min at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated goat antiserum against rabbit IgG (Sigma) for 1 h at room temperature.
Failure: The neuraminidase on V, which leads to human monoclonal antibody 6A7. Lanes 1, 4: Clone 26.1 cells transfected with pSG5gpa (lanes 4, 6) or buffer alone (lanes 1, 3), and then incubated with mouse monoclonal antibody 6A7, as described in Experimental Procedures. The migration position of the glycoprotein A homodimer is indicated by the closed circle, the position of the monomer is indicated by the open circle. The three panels represent experiments performed on separate occasions.