Recombinant Miltenberger I and II Human Blood Group Antigens: The Role of Glycosylation in Cell Surface Expression and Antigenicity of Glycophorin A

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Glycophorin A is a heavily glycosylated glycoprotein (1 N-linked and 15 O-linked oligosaccharides) and is highly expressed on the surface of human red blood cells. It is important in transfusion medicine because it carries several clinically relevant human blood group antigens. To study further the role of glycosylation in surface expression of this protein, four mutations were separately introduced into glycophorin A cDNA by site-directed mutagenesis. Each of these mutations blocks N-linked glycosylation at Asn92 of this glycoprotein by affecting the Asn-X-Ser/Thr acceptor sequence. Two of these mutations are identical to the amino acid polymorphisms found at position 28 in the Mi.1 and Mi.11 Miltenberger blood group antigens. The mutated recombinant glycoproteins were expressed in transfected wild-type and glycosylation-deficient Chinese hamster ovary (CHO) cells. When expressed in wild-type CHO cells and analyzed on Western blots, each of the four mutants had a faster electrophoretic mobility than wild-type glycophorin A, corresponding to a difference of approximately 4 Kd. This change is consistent with the absence of the N-linked oligosaccharide at Asn92. Each of the four mutants was highly expressed on the surface of CHO cells, confirming that, in the presence of normal O-linked glycosylation, the N-linked oligosaccharide is not necessary for cell surface expression of this glycoprotein. To examine the role of O-linked glycosylation in this process, the Mi.1 mutant cDNA was transfected into the IdID glycosylation-deficient CHO cell line. When the transfected IdID cells were cultured in the presence of N-acetylgalactosamine alone, only intermediate levels of cell surface expression were seen for Mi.1 mutant glycophorin A containing truncated O-linked oligosaccharides. In contrast, when cultured in the presence of galactose alone, or in the absence of both galactose and N-acetylgalactosamine, Mi.1 mutant glycophorin A lacking both N-linked and O-linked oligosaccharides was not expressed at the cell surface. This extends previous results (Remaley et al., J Biol Chem 266:24176, 1991) showing that, in the absence of O-linked glycosylation, some types of N-linked glycosylation can support cell surface expression of glycophorin A. The glycophorin A mutants were also used for serologic testing with defined human antisera. These studies showed that the recombinant Mi.1 and Mi.11 glycoproteins appropriately bound anti-Vw and anti-Hut, respectively. They also demonstrated that these antibodies recognized the amino acid polymorphisms encoded by Mi.1 and Mi.11 rather than cryptic peptide antigens uncovered by the lack of N-linked glycosylation.

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The Miltenberger antigens Mi.1 and Mi.11 are peptide blood group antigens on glycophorin A; the corresponding antibodies are termed anti-Vw and anti-Hut, respectively. Less than 0.1% of individuals in a general population have red blood cells that carry the Mi.1 or Mi.11 antigens. Nevertheless, these antigens are clinically relevant because antibodies such as anti-Vw can cause hemolytic disease of the newborn. Interestingly, the Mi.1 and Mi.11 antigens provide naturally occurring amino acid polymorphisms affecting the Asn-X-Ser/Thr tripeptide sequon directing N-linked glycosylation. Both polymorphisms change the amino acid residue at Thr28, thus blocking the addition of the N-linked oligosaccharide at Asn92 (Fig 1).

In the present study, site-directed mutagenesis was used to construct the Mi.1 and Mi.11 mutants of glycophorin A.

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and these were expressed in wild-type and glycosylation-deficient CHO cells. These studies clarified the role that N- and O-linked glycosylation play in cell surface expression of glycophorin A. The recombinant glycoproteins were also serologically active with human anti-Vw and anti-Hut, suggesting a genetic engineering approach for constructing rare human blood group antigens useful in blood typing.

**MATERIALS AND METHODS**

**Cells, cell lines, and tissue culture.** Wild-type CHO cells (Clone Pro-5) were obtained from the American Type Culture Collection (Rockville, MD). Clone 26.1, expressing recombinant wild-type glycophorin A in transfected Pro-5 cells, was obtained as described previously. The glycosylation-deficient CHO cell line, lldD, was obtained from M. Krieger (Massachusetts Institute of Technology, Cambridge, MA). Pro-5 and Clone 26.1 cells were cultured in α-minimal essential media supplemented with 10% fetal calf serum (FCS), 2 mmol/L glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (αMEM CM). The lldD cells were maintained in either αMEM CM or αMEM supplemented with Ham's F12 and αMEM supplemented with 10% fetal calf serum, 2 mmol/L glutamine, IU/mL of penicillin, and 1% dialyzed FCS; insulin, transferrin, and selenium (ITS+ Premix; Collaborative Research Inc, Bedford, MA); and 2 mmol/L glutamine, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (lldD CM). In some cases, lldD CM was supplemented with 20 μmol/L galactose, 200 μmol/L N-acetylgalactos-amine, or both. Transfected cells were maintained in complete medium containing 0.5 mg/mL of active genetin (G418; GIBCO, Grand Island, NY). Human MM and NN red blood cells were collected in EDTA (0.2%) and obtained from the Blood Bank of the Hospital of the University of Pennsylvania; human Mi.I and Mi.II red blood cells were provided by F. Fisher (Immucor, Inc, Norcross, GA) and J. Moulds (Gamma Biologicals, Inc, Houston, TX), respectively.

**Antibodies.** Hybridoma cells secreting monoclonal antibody (MoAb) 6A7, which recognizes the M-allele of glycophorin A, were obtained from the American Type Culture Collection. Hybridoma culture supernatant was used as a source of this antibody. Rabbit serum containing polyoncylonal antibodies directed against the whole glycophorin A molecule was the generous gift of O.O. Blumenfeld (Albert Einstein College of Medicine, New York, NY). Human anti-M, -N, and -Vw were obtained from the Blood Bank of the Hospital of the University of Pennsylvania. Human anti-Hut was generously provided by J. Moulds.

**Construction of glycophorin A cDNA mutants.** The full-length cDNA of the M-allele of glycophorin A, a gift from M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA), was previously inserted into the unique EcoRI site of the pSG5 eukaryotic expression vector (Stratagene, La Jolla, CA) to produce pSG5gpa. Mutants of pSG5gpa were constructed using a polymerase chain reaction (PCR) based method of site-directed mutagenesis by overlap extension. Four different mutations were constructed as shown in Fig 1: Mi.I, Thr → Met; Mi.II, Thr → Lys; M3, Thr → Ala; M4, and Asn → Gln. The Mi.I and Mi.II mutants mimic the amino acid polymorphisms found on these variant human glycophorin A blood group antigens, both of these polymorphisms result in deletion of the Asn-Ser-Thr sequence for N-linked glycosylation at Asn26. The M3 and M4 mutants contain conservative amino acid changes that also delete the sequence for N-linked glycosylation at Asn26. The following DNA primers used for mutagenesis were obtained from the Wistar Institute Oligonucleotide Synthesis Facility (Philadelphia, PA). The external primers, A and D, complementary to the pSG5 vector, were used for all four mutants. The internal primers B(Mi.I) and C(Mi.I) were used to generate Mi.I. C(Mi.I) contains both a base change that creates a new restriction site for EcoR I to facilitate screening of recombinants. The internal primers B(Mi.II), B(M3), and B(M4), used to generate Mi.II, M3, and M4, respectively, contain base changes that mutate the following codons: B(Mi.II), ACG (Thr28) to GCG (Ala); B(M3), and B(M4), used to generate Mi.II, M3, and M4, respectively, contain base changes that mutate the following codons: B(Mi.II), ACG (Thr28) to AAG (Lys); B(M3), ACG (Thr28) to GCC (Ala); B(M4), AAT (Asn26) to CAA (Gln). The internal primer C(Mi.II-M4) used to generate Mi.II, M3, and M4, contains a conservative base change that deletes an NdeI restriction site to facilitate screening of recombinants. The sequence and region of complementarity of each primer is shown; bases that deviate from the sequence of native glycophorin A are underlined. A and C primers are in the sense orientation, whereas B and D primers are antisense. Base no. 16 in glycophorin A corresponds to A of the AUG initiation codon. Glycophorin A cDNA is inserted into the EcoRI site of pSG5 at base no. 1043. The sequences and complementary regions of the primers are shown in Table 1.

The plasmids containing the mutations were used to transform _Escherichia coli_ HB101 and constructs were isolated, characterized, and amplified using standard techniques. Regions in the mutant cDNAs that were generated by PCR amplification during mutagenesis were sequenced by the dideoxy method to verify both the presence of the desired mutations and the absence of undesired PCR-induced mutations.

**Transfection.** CHO cells were cotransfected by calcium phos-
phate precipitation with 10 μg of plasmid containing wild-type or mutant glycophorin A cDNA and 1 μg of a plasmid containing a selectable marker, pSV2neo, as described.21

Indirect immunofluorescence: Indirect immunofluorescence using mouse MoAb 6A7 was performed on cells grown in 8-well tissue culture chamber slides (Nunc Inc, Naperville, IL), as described.6

Western blots. CHO cells were solubilized in 50 mmol/L Tris/HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, pH 8.0, with 0.5% NP40 and 1 mmol/L phenylmethysulfonyl fluoride (PMSF). The soluble proteins were quantified, separated by vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% or 12% gels) using the Laemmli buffer system, and electrophoretically transferred to nitrocellulose. Proteins were detected in the gel with Coomassie blue and on the nitrocellulose with an immunoselectable marker, pSV2ne0, as described.21

Flow cytometry. Cells were detached from tissue culture flasks with 0.2% EDTA in Hanks' Balanced Salt Solution, pH 7.4, and washed in complete medium. The detached cells were resuspended in primary rabbit polyclonal antisera (diluted in 10 mmol/L Na,HPO4/NaH2PO4, 150 mmol/L NaCl, pH 7.4, containing 1% bovine serum albumin and 0.1% Na3I) at 1 × 106 cells/mL and incubated for 1 hour at 4°C. After washing, fluorescein-conjugated secondary antibody was added and the cells were incubated for 30 minutes at 4°C. Cells were washed and fixed overnight in 1% paraformaldehyde and then analyzed for fluorescence intensity on a FACScan analyzer (Becton Dickinson, Mountain View, CA). Ten thousand events were analyzed for each condition. Dead cells, detected by low forward and right angle scatter, were excluded, resulting in approximately 8,000 cells analyzed for each condition. The FACScan analyzer separated the amount of fluorescence into 1,024 channels.

Serologic studies. Intact human red blood cells were typed for the M, N, Mi.I, and Mi.II antigens using standard serologic techniques.24 Acetone powders were prepared from human red blood cell membranes and intact, recombinant CHO cells.25 These powders were used as insoluble immunoadsorbents to remove glycophorin A-specific antibodies from human antisera. Antisera were incubated with the powders for 30 minutes at room temperature or 37°C. The supernatants, obtained after centrifugation at 3,400g for 2 minutes, were then tested by standard serologic techniques.

RESULTS

Construction of glycophorin A cDNA mutants. The four mutants Mi.I, Mi.II, M3, and M4 (Fig 1) were constructed as described in Materials and Methods. Each of these mutations deletes the sequon for N-linked glycosylation at Asn26.

The results of sequencing the relevant nucleotides in the mutated cDNAs are also shown in Fig 1; the entire insert constructed by PCR was sequenced to rule out the presence of undesirable mutations artifically introduced by PCR.

Expression of glycophorin A mutants in wild-type CHO cells. Plasmids containing mutant or wild-type glycophorin A cDNA inserted in the pSG5 expression vector were transfected into wild-type CHO cells (Clone Pro-5) and protein expression was monitored by indirect immunofluorescence. Clonal stably transfected cell lines exhibiting high expression of each recombinant protein were isolated by limiting dilution. The biochemical characteristics of the glycophorin A synthesized by these cells were analyzed by Western blotting. Detergent soluble proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mouse MoAb 6A7 (Fig 2). Two major bands corresponding to the monomer and homodimer forms of the protein were identified with each cell line. Minor bands were also seen that probably represent heterodimers between recombinant glycophorin A and endogenous CHO cell protein(s).6 These major and minor bands had the same electrophoretic mobility in each of the four cell lines expressing mutant glycophorin A molecules (Fig 2, lanes 4 through 7). In contrast, the corresponding bands of wild-type glycophorin A (Fig 2, lane 3) migrated more slowly. This difference in mobility, corresponding to a difference of approximately 4 Kd per glycophorin A monomer, is consistent with the expected change due to the presence or absence of the N-linked oligosaccharide at Asn26. The role of the N-glycan in this change in migration was confirmed by studies involving metabolic labeling of native and Mi.I mutant glycophorin, followed by immunoprecipitation, SDS-PAGE, and autoradiography. When glycophorin A was immunoprecipitated from labeled Clone 26.1 cells and then treated with peptide-N-glycanase F, an enzyme that specifically removes N-glycans, it comigrated with Mi.1 mutant glycophorin, followed by immunoprecipitation, SDS-PAGE, and autoradiography. When glycophorin A was immunoprecipitated from labeled Clone 26.1 cells and then treated with peptide-N-glycanase F, an enzyme that specifically removes N-glycans, it comigrated with Mi.II mutant glycophorin A (data not shown).

Expression of Mi.II glycophorin A in ldlD CHO cells. The ldlD cells have a defect in 4-epimerase, blocking conversion of glucose into galactose and N-acetylglucosamine into N-acetylgalactosamine.14 Thus, when cells are cultured in medium lacking both galactose and N-acetylglucosamine, no O-linked oligosaccharides are added to nascent glycoproteins (Fig 3). Similarly, when galactose alone is added to the medium, no O-linked oligosaccharides are synthesized. When cells are cultured in the presence of N-acetylgalactosamine alone, only this monosaccharide (or,

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### Table 1. Primer Sequences and Complementary Regions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Complementary Region (base no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5'GGTAGAAACACATACATTCTG</td>
<td>pSG5 821-841</td>
</tr>
<tr>
<td>D</td>
<td>5'GCTATGGCATTATTTGTAACCC</td>
<td>pSG5 1096-1116</td>
</tr>
<tr>
<td>B(MI)</td>
<td>5'GGAGTGGCGATATGTCGCCGG</td>
<td>Glycophorin A 165-185</td>
</tr>
<tr>
<td>C(MI)</td>
<td>5'AGCATAAACGGGCACTATGCAAC</td>
<td>Glycophorin A 159-190</td>
</tr>
<tr>
<td>B(MII)</td>
<td>5'TAGTGCTCCCGTTTGTCTATTTG</td>
<td>Glycophorin A 148-175</td>
</tr>
<tr>
<td>B(MIII)</td>
<td>5'TAGTGCTCCCGTTTGTCTTGGCTATTTG</td>
<td>Glycophorin A 144-175</td>
</tr>
<tr>
<td>C(MII-M4)</td>
<td>5'ACACAGGGACACATACGGACC</td>
<td>Glycophorin A 159-182</td>
</tr>
</tbody>
</table>

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Fig 2. Western blot analysis of wild-type CHO cells transfected with plasmids containing wild-type and mutant glycophorin A cDNA. Proteins were isolated from transfected CHO cells, separated by SDS-PAGE, blotted onto nitrocellulose, and detected with mouse MoAb 6A7 followed by peroxidase-conjugated goat antiamouse IgG and 4-chloro-1-naphthol. The apparent molecular weights of the electrophoretic standards are indicated on the left. The migration position of the mutated glycophorin A homodimer is indicated by the solid circle; the positions of the corresponding heterodimers and monomers are indicated by the arrowheads and open circle, respectively. Lane 1, molecular weight standards; lane 2, untransfected CHO cells (Clone Pro-5); lane 3, CHO cells expressing wild-type glycophorin A (Clone 26.1); lane 4, CHO cells expressing Mi.I mutant glycophorin A; lane 5, CHO cells expressing Mi.II mutant glycophorin A; lane 6, CHO cells expressing M3 mutant glycophorin A; lane 7, CHO cells expressing M4 mutant glycophorin A.

Possibly, the NeuAcα2-6GalNAc disaccharide is found in O-glycosidic linkage on glycoproteins. When both galactose and N-acetylgalactosamine are added to the medium, the mature tetrascarhide can be synthesized. Because galactose and N-acetylgalactosamine are also found in both glycosphingolipids and N-linked glycoprotein oligosaccharides, these macromolecules are also modified in ldlD cells grown under restrictive conditions.

Because Mi.I glycophorin A lacks the appropriate amino acid sequence for N-linked glycosylation, expression of this glycoprotein in ldlD cells allows a direct examination of the role of O-linked glycosylation in the cell surface expression of glycophorin A. After transfection of ldlD cells with plasmid containing Mi.I cDNA, several clonal cell lines were obtained that expressed high levels of glycophorin A when the cells were cultured in ldlD CM supplemented with both galactose and N-acetylgalactosamine. One of these clones, Mi.I-ldlD, was examined in detail.

To examine the glycosylation of the Mi.I variant of glycophorin A in transfected ldlD cells under various culture conditions, detergent lysates were separated by SDS-PAGE and analyzed by Western blotting (Fig 4). Glycophorin A monomers and dimers with identical electrophoretic mobilities were found with Mi.I-ldlD cells cultured in the presence of both galactose and N-acetylgalactosamine (Fig 4, lane 4) and with Mi.I-transfected wild-type CHO cells (Fig 4, lane 3). This result was expected because the addition to the media of these two monosaccharides circumvents the 4-epimerase deficiency in ldlD cells. In contrast, Mi.I-ldlD cells grown under progressively restrictive conditions expressed glycophorin A monomers and dimers of progressively faster electrophoretic mobility. This most likely results from the synthesis of truncated O-linked oligosaccharides (Fig 4, lane 5) or the absence of O-linked oligosaccharides (Fig 4, lanes 6 and 7) on glycophorin A. Interestingly, monomers and dimers of Mi.I synthesized in ldlD cells under these conditions have identical electrophoretic mobilities when synthesized in the presence of galactose alone (Fig 4, lane 6) or in the absence of galactose and N-acetylgalactosamine (Fig 4, lane 7). These results (Fig 4, lanes 6 and 7) with Mi.I mutant glycophorin A differ from previous results obtained with wild-type glycophorin A cDNA synthesized in ldlD cells (Fig 8, lanes 2 and 3 in Remaley et al8). In the previous case, there was significant heterogeneity in the bands seen when the transfected cells were cultured with galactose alone. Taken together, these results suggest that altered processing of the N-linked oligosaccharide on wild-type glycophorin A in ldlD cells contributes to the heterogeneity of the banding pattern seen previously.

Role of glycosylation in cell surface expression of Mi.I glycophorin A. To examine the effect of changes in O-linked glycosylation on cell surface expression of glycophorin A, Mi.I-ldlD cells were analyzed by flow cytometry using glycophorin A-specific rabbit polyclonal antibody (Fig 5). This antibody recognizes all forms of glycophorin A synthesized by ldlD cells under both permissive and restrictive conditions (Fig 4). When Mi.I-ldlD cells were cultured in ldlD CM containing both galactose and N-acetylgalactosamine, high surface expression was seen (mean channel fluorescence, 621). This level of expression is similar to that found on the surface of wild-type CHO cells transfected with wild-type glycophorin A cDNA (data not shown). In
To determine whether Mi.I mutant glycophorin A was expressed intracellularly in ldlD cells, Mi.I-ldlD cells were permeabilized and analyzed by indirect immunofluorescence using an MoAb that recognizes a cytoplasmic epitope of glycophorin A. Using this approach, equivalent binding was seen regardless of whether the cells were cultured in the presence or absence of galactose and N-acetylgalactosamine (data not shown). This finding shows that Mi.I mutant glycophorin A molecules can function as authentic blood red blood cell antigens are defined by human anti-Vw and anti-Hut antisera, respectively. The recombinant Mi.I and Mi.II glycophorin A molecules were tested for their binding to these antisera for two reasons: (1) to determine whether the recombinant proteins can function as authentic blood group antigens; and (2) to determine whether these antisera recognize the amino acid polymorphisms at position 28, or whether they recognize a cryptic peptide antigen uncovered in the absence of N-linked oligosaccharide at Asn26.

The two site-directed mutants, Mi.I and Mi.II, red blood cell antigens are defined by human anti-Vw and anti-Hut antisera, respectively. The recombinant Mi.I and Mi.II glycophorin A molecules were tested for their binding to these antisera for two reasons: (1) to determine whether the recombinant proteins can function as authentic blood group antigens; and (2) to determine whether these antisera recognize the amino acid polymorphisms at position 28, or whether they recognize a cryptic peptide antigen uncovered in the absence of N-linked oligosaccharide at Asn26. The two site-directed mutants, Mi.I and Mi.II, both contain amino acid polymorphisms at the same position, amino acid 28, as observed with the corresponding naturally occurring antigens on human red blood cells. In contrast, the two mutants, M3 and M4, contain alternative conservative amino acid substitutions that delete the N-linked glycosylation sequon.

To address these questions, various human antisera were incubated with acetone powders prepared from CHO cell clones expressing either wild-type or mutant glycophorin A. After incubation, the resulting supernatants were tested for their ability to agglutinate antigen-typed red blood cells. The results shown in Table 2 show that both the Mi.I and Mi.II recombinant glycophorin A molecules function appropriately as blood group antigens and are specifically recog-
Our previous studies using wild-type glycophorin A expressed in wild-type CHO cells suggested that N-linked glycosylation was not required for cell surface expression of glycophorin A. In those studies, equal levels of cell surface expression of glycophorin A were seen in the presence and absence of the N-linked glycosylation inhibitor, tunicamycin. These results were obtained when expression of glycophorin A was examined in K562 cells cultured in the presence of tunicamycin. These results are not surprising because the Mi.I and Mi.II variants of glycophorin A, which lack the N-linked oligosaccharide, are well expressed on the surface of red blood cells obtained from individuals with these polymorphisms.

In the current study, site-directed mutagenesis was used to delete directly the sequon for N-linked glycosylation at Asn. Construction of these mutants by site-directed mutagenesis allowed a more detailed investigation of the respective roles played by the N- and O-linked oligosaccharides in cell surface expression of glycophorin A. These studies exploited the use of the CHO IdlD cell line, which has a reversible defect, primarily in O-linked, but also in N-linked glycosylation. Because there is no N-linked oligosaccharide on the Mi.I mutant protein, any changes in cell surface expression of glycophorin A, seen when Mi.I-IdlD cells are cultured under restrictive conditions, are due to changes in O-linked glycosylation.

These studies showed markedly decreased cell surface expression of Mi.I mutant glycophorin A when Mi.I-IdlD cells were cultured in the absence of galactose and N-acetylgalactosamine or in the presence of galactose alone. This finding shows that O-linked oligosaccharides are necessary for appropriate cell surface expression of glycophorin A.

Analyzing the results obtained when Mi.I-IdlD cells were cultured in the presence of galactose alone as compared with N-acetylgalactosamine alone allows one to determine the effect on cell surface expression of a complete block in O-linked glycosylation as compared with the synthesis of truncated O-linked oligosaccharides. Interestingly, the results of this study with Mi.I mutant glycophorin A (Fig 5) differed from those seen previously with wild-type glycophorin A. In the previous study, the transfected IdlD cells showed intermediate cell surface expression of wild-type glycophorin A when cultured in the presence of galactose alone and near normal expression when cultured with N-acetylgalactosamine alone. Therefore, the dependence on O-linked glycosylation of cell surface expression of glycophorin A is not as stringent when the N-linked oligosaccharide is present at Asn. This suggests that both classes of

**Table 2. Binding of Human Antisera to Recombinant Wild-Type and Mutant Glycophorin A**

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Acetone Powder Prepared From Transfected CHO Cells*</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Anti-Vw</td>
<td>-</td>
</tr>
<tr>
<td>Anti-Hut</td>
<td>-</td>
</tr>
<tr>
<td>Anti-M</td>
<td>-</td>
</tr>
<tr>
<td>Anti-N (or -D)</td>
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</table>

Abbreviation: WT, wild-type recombinant glycophorin A.

The adsorbed antisera were tested with antigen-typed human red blood cells of the appropriate types as described in Materials and Methods. The ability of the acetone powder to adsorb (+) or not adsorb (-) the antisera was determined. In each case the agglutinating activity of the antisera either was completely removed or was unaffected, as measured by degree and titer of agglutination. None of the antisera bound to acetone powder derived from untransfected CHO cells (data not shown).

* Acetone powders were prepared from clonal CHO cell lines expressing recombinant wild-type, Mi.I, Mi.II, M3, and M4 glycophorin A molecules as described in Materials and Methods.
oligosaccharides, N-linked and O-linked, can independently facilitate cell surface expression of this protein.

There are several possible mechanisms for the poor cell surface expression of abnormally glycosylated or unglycosylated glycophorin A. The abnormally glycosylated or unglycosylated glycoprotein may not be stabilized in its appropriate conformation, leading to aggregation in the endoplasmic reticulum, or rapid proteolysis or internalization after expression on the plasma membrane. The mechanism responsible for poor surface expression of unglycosylated glycophorin A is currently under investigation.

The Mi.I and Mi.II mutations result in both loss of the N-linked oligosaccharide at Asn29 and the creation of new antigenic determinants recognized by the human anti-Vw and anti-Hut antisera. The results presented in Table 2 show that when the N-linked oligosaccharide is deleted alternatively by the conservative amino acid substitutions Thr29 → Ala (M3) or Asn29 → Gln (M4), the mutant proteins are not recognized by either anti-Vw or anti-Hut. This finding suggests that the Mi.I and Mi.II antigenic determinants are defined by the specific amino acid polymorphisms at position 28 and not by cryptic determinants uncovered by the loss of the N-linked oligosaccharide.

The use of genetic engineering to construct insoluble immunoadsorbents expressing recombinant human blood group antigens may be of benefit in transfusion medicine. The acetone powders prepared from CHO cells are dry, stable, insoluble, and can be stored at room temperature. These recombinant reagents may be useful in characterizing antibodies to red blood cell antigens when red blood cells expressing their naturally occurring counterparts are rare or difficult to obtain. Availability of recombinant reagents may make it unnecessary to maintain stocks of some rare blood cells in liquid nitrogen, and may facilitate the dissemination to routine hospital-based laboratories of reagents previously available only in regional reference laboratories. In addition, generation and analysis of recombinant forms of red blood cell antigens will lead to a more detailed understanding of the biochemical nature of clinically important human blood group antigens.

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