Structural Polymorphism of Glycophorins Demonstrated by Immunoblotting Techniques

Yi-Qing Lu, Margaret E. Nichols, William L. Bigbee, Ronald L. Nagel, and Olga O. Blumenfeld

We have explored the polymorphism of the glycophorin system in the human erythrocyte membrane using the immunoblotting techniques and examining 52 individuals selected without prior bias as to their serologic state and ten documented serologic variants of M, N, S blood group system. Polyclonal antisera to α glycophorin and to α glycophorin CNBr carboxyl terminal fragment C (residues 82-131) and M and N specific monoclonal antibodies (MoAbs) were used. The first two reagents detect specific regions of the α glycophorin molecule and all electrophoretically resolved species of glycophorins immunologically related to α and δ glycophorins (δ glycophorin, [α–δ] hybrids and other glycophorins with an alteration in the carboxyl terminal segment); the M and N MoAbs identified the glycophorin species containing or lacking the M or N determinant in the amino terminal octapeptide structures. We find that (1) immunoblotting confirmed in all cases the serologically determined phenotype; we also find that polymorphic forms of the glycophorin system are relatively infrequent; (2) immunoblotting, independent from serologic testing, was capable of detecting five mutants, two most likely S-s-U-phenotypes; (3) a new glycophorin species was detected in normal red cells with both antiglycophorin and antipeptide C sera, which is not evident with MoAbs; (4) immunoblots of known glycophorin variants (En(a—), U—, M9, Mi I, II, III, V, and St*) confirmed but also extended our knowledge of the abnormal glycophorins involved; and (5) the He and Wrb(–) cells showed normal patterns.

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GLYCOPHORINS are a family of erythrocyte membrane glycoproteins, and in each individual several related forms are present.1,2 In the major M and N α glycophorins,3,4 the antigens that determine the M, N blood group resides; these glycoproteins differ in two amino acids at residues 1 and 5 of the polypeptide chain and are products of two codominantly expressed allelic genes.1-6 Moreover, β glycophorins, in which the S and s blood group antigens reside, are specified by other allelic genes5,6; these relatively less abundant glycophorins are related to the N form of α glycophorin but show amino acid sequence differences and lack the asparagine-linked oligosaccharide and the carboxy terminal segment of α glycophorin.1,3,7 The β and γ glycophorins are minor components whose serologic and structural characteristics are less clear.1,2

As shown by recent gel electrophoretic and structural studies, erythrocytes of serologic variants of the MNSs locus may exhibit additional variant forms of glycophorins. Blood group specific Mt*, Mt*, Mi I, and II erythrocytes contain products of variant alleles of the M or N genes,8-13 and those of St*, Miltenberger V, or Ph individuals contain fusion glycophorins originating from recombination events between α and δ glycophorin genes.14-18 Some individuals and their families are known whose erythrocytes do not bear either α (En(a–)) or δ glycophorins (S-s-U–)1,2; and others have cells that contain α glycophorin glycosylation variants, for example the Cad phenotype.19,20

In this report we examined two aspects of glycophorin polymorphism using immunoblotting approaches. First, the extent of occurrence of glycophorin variants in a population of 52 individuals, selected without prior bias as to their serologic state; and second, the ability to be detected and the nature of profile differences in erythrocytes of individuals with serologically defined variant M, N, S, and s phenotypes.

MATERIALS AND METHODS

Preparation of erythrocytes. Random control erythrocytes were obtained from laboratory personnel and from 41 medical students from Central Africa (Cameroon, Zaire, and Central African Republic).2 Venous blood (collected with heparin or citrate-phosphate-dextrose) was centrifuged at 3,000 rpm for 30 minutes and plasma and buffy coat removed by aspiration. The erythrocytes were washed three times with isotonic phosphate buffer pH 7.6, followed by two washes with the same buffer containing inhibitors of proteolysis: 1 mmol/L phenylmethylsulfonylfluoride (PMSF), 2 mmol/L N-ethylmaleimide, and 0.02% sodium azide. Washed cells were centrifuged at 2,000 rpm for 15 minutes and used for blood typing and gel electrophoretic studies.

Serologically variant erythrocytes were obtained from individuals referenced in the rare donor file at New York Blood Center; their phenotypes are noted in Table 1. These cells had been frozen in
Glycophorins of 20 slots of the slab gel. The remaining solution, in 0.1-mL 3% 10% to 15% gradient gels in 0.1% sodium dodecyl anti-N sera (Ortho Diagnostic Systems, Raritan, NJ). immersed for one minute followed by 0.25 mL of 3x sample buffer.23 The solution was ing for the freshly collected cells.

Hemagglutination was performed accord-

Blood group typing. 'See Figs 1 to 3;

Preparation and properties of antisera and antibodies. Antisera were prepared in rabbits against α glycophorin prepared from erythrocyte membranes of an MN individual and against carbohydrate-free carboxyl terminal peptide C (residue 82-131) obtained by CNBr cleavage of α glycophorin.16,26 Both antisera react with M, N α glycophorin and its carboxyl terminal peptide C but do not react with the internal carbohydrate-rich portion of α glycophorin (CNBr peptide B, residues 9-81).16 Preimmune rabbit sera showed no bands when tested with purified glycophorins; with preparations of intact M, N, or MN erythrocytes a very faint band was seen at the position of band 5 similar in intensity to that seen in Ena(−) cells (Fig 1; Ena(−) lanes A and B). More generally the specificity of the antisera is demonstrated by the pattern of reaction with the Ena(−) cells (lane B).

An M-specific MoAb (1-2-D9) was produced as previously described for MoAbs G8 and E3.27 The N-specific MoAb (NN 5) has been previously described.28 The M MoAb reacted exclusively with the M α glycophorin and the N MoAb reacted with N α and δ glycophorins when tested on Western immunoblots with M and N α glycophorins isolated from MM or NN erythrocytes.13 The specificities of antisera and antibodies are summarized in Table 2. Preim-

<table>
<thead>
<tr>
<th>Serologic Phenotype</th>
<th>Variant Bands*</th>
<th>MN Glycophorin</th>
<th>Peptide C</th>
<th>MoAbs M N</th>
<th>Possible Structural Characteristics†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIL, M, N, S, s</td>
<td>4'</td>
<td>+</td>
<td>+</td>
<td>− −</td>
<td>Glycophorin variant altered in the amino terminal octapeptide</td>
</tr>
<tr>
<td>MIL, M, S, s</td>
<td>4'</td>
<td>+</td>
<td>+</td>
<td>+ −</td>
<td>Glycophorin variant (M-like)</td>
</tr>
<tr>
<td>MIL, N, S</td>
<td>2'</td>
<td>+</td>
<td>+</td>
<td>− +</td>
<td>δ Glycophorin variant</td>
</tr>
<tr>
<td>2'</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+ +</td>
<td>δ Glycophorin variant</td>
</tr>
<tr>
<td>4'</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+ +</td>
<td>δ Glycophorin variant</td>
</tr>
<tr>
<td>MIL, N, S</td>
<td>2'</td>
<td>+</td>
<td>+</td>
<td>− +</td>
<td>α Glycophorin variant</td>
</tr>
<tr>
<td>2'</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+ +</td>
<td>α Glycophorin variant</td>
</tr>
<tr>
<td>2'</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+ +</td>
<td>α Glycophorin variant</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Ena(−)</th>
<th>S, s</th>
<th>Bands 1 and 4</th>
<th>−</th>
<th>−</th>
<th>−</th>
<th>−</th>
<th>Lack α glycophorin; “normal” δ glycophorin</th>
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<tbody>
<tr>
<td>MIL, s</td>
<td>s</td>
<td>Bands 1 and 4</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>α glycophorin altered within the amino terminal octapeptide; “normal” δ glycophorin</td>
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<tr>
<td>U−, N</td>
<td></td>
<td>Band 6</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>trace lack δ glycophorin; “normal” α glycophorin</td>
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<tr>
<td>He−, M, S, s</td>
<td>No obvious differences from normal.</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*See Figs 1 to 3; †Structural characteristics apparent from reaction with the antisera and the antibodies.

Table 1. Reaction of Variant Glycophorins in Erythrocytes with Rare Serologic Variants of M, N, S, s With Specific Polyclonal Antisera and MoAbs
RESULTS

Glycophorins in erythrocytes of MN individuals.
Erythrocytes of random individuals displayed seven bands on SDS-polyacrylamide gels when probed with the polyclonal antiserum to α glycophorin. This is shown in Fig 1 (lanes A), for erythrocytes of three representative MM, NN, and MN individuals of 52 individuals examined. Bands 1 and 4 correspond to the α glycophorin dimer and monomer, respectively; bands 3b and 6 migrate in the area of δ glycophorin dimer and monomer, and band 5 corresponds to τ glycophorin (Table 3). In addition to these well-established bands, an

Table 2. Specificity of Polyclonal Antisera and Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antiserum to</th>
<th>Reaction With</th>
<th>No Reaction With</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN α glycophorin</td>
<td>α, δ glycophorins and other minor glycophorins</td>
<td>CNBr peptide B*</td>
</tr>
<tr>
<td>Antiserum to carboxyl terminal peptide C of α glycophorin (residue 82–131)</td>
<td>α glycophorins and all other glycophorins related to α glycophorins in the carboxyl terminal</td>
<td>δ glycophorins and all glycophorins altered or lacking the carboxyl terminal region of α glycophorins</td>
</tr>
<tr>
<td>M-specific MoAb</td>
<td>M α glycophorin† (ser ser thr thr gly val ala met)</td>
<td>N α glycophorin δ glycophorin</td>
</tr>
<tr>
<td>N-specific MoAb</td>
<td>N α glycophorin † δ glycophorin (leu ser thr thr glu val ala met)</td>
<td>M α glycophorin</td>
</tr>
</tbody>
</table>

†Epitope is located within the amino terminal octapeptide as shown. Blumenfeld and Adamany

*Blumenfeld et al

See Fig 1.

Table 3. Reaction of Glycophorins in Erythrocytes of Randomly Chosen Individuals with Specific Polyclonal Antisera and MoAbs

<table>
<thead>
<tr>
<th>Antisera to</th>
<th>Reaction with</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN α glycophorin peptide C</td>
<td>MoAbs</td>
</tr>
<tr>
<td>Bands 1 and 4*</td>
<td>α glycophorin dimer and monomer</td>
</tr>
<tr>
<td>Bands 3b and 6</td>
<td>δ glycophorin dimer and monomer</td>
</tr>
<tr>
<td>Band 2</td>
<td>δ-δ dimer†</td>
</tr>
<tr>
<td>Band 5</td>
<td>γ glycophorin and possibly other glycophorins</td>
</tr>
<tr>
<td>Band 3a</td>
<td>glycophorin X</td>
</tr>
</tbody>
</table>

*See Fig 1.
additional band designated 3a is seen. β-Glycophorin bands, noted by others, were not visualized and probably co-migrate with the monomer of α glycophorin (band 4).

As shown in Fig 1 (lane B) and Table 3, the antiserum to the carboxyl-terminal peptide C does not react with δ glycophorin in bands 3b and 6 but reacts with the other glycophorins. This confirms that δ glycophorin differs from α glycophorin either by an alteration in the carboxyl-terminal region or by the absence of that region. This also suggests that the minor glycophorins in bands 2, 3a, and 5 are structurally related with respect to that polypeptide region to α rather than to δ glycophorin.

The monoclonal M- and N-specific antibodies react with α glycophorins according to the M or N serologic phenotype of the cells; in addition, the N-specific MoAb reacts with δ glycophorin, even in the M positive cells (Fig 1, lanes C and D, Table 3). It was observed that in all the erythrocyte samples tested, band 3a was not demonstrated by either MoAb whereas band 5 was evident with both. The reactivity of pre-immune mouse serum at band 5 but not at bands 1 and 4, for example, suggests a specific antigen/antibody reaction rather than a nonspecific binding effect. It would appear therefore that some of the glycophorins recognized by pre-immune mouse serum also carry M- and N-specific epitopes recognized by the monoclonal reagents.

The glycophorin band profiles observed were all essentially similar for the 52 randomly chosen individuals. The only deviations observed were the absence of δ glycophorin in two individuals and the absence of band 5 using antipeptide C serum in three individuals. The latter observation suggests that a structural alteration exists in the carboxyl terminal region of these glycophorins. The erythrocytes of the two former individuals could possibly exhibit the S-, S-, S., U− blood group phenotype. Unfortunately, fresh red cells were not available for confirmatory blood typing tests to be performed.

Glycophorins in erythrocytes with variant serologic phenotypes. Erythrocytes from ten different individuals exhibiting variant or null glycophorin phenotypes were examined (Table 1). The altered glycophorin profiles became apparent when the cells were probed with the polyclonal antibody to α glycophorin (Figs 1 to 3). Thus in En(a−) cells the usual α glycophorin bands were absent, and in U− cells the δ glycophorins were absent; and in Mi I, II, III, V, and St* cells, variant glycophorins (denoted as prime,') could readily be detected by their electrophoretic mobilities, which were distinct from the usual glycophorin bands. In the case of M* homozygous erythrocytes, the variant nature of α glycophorins was apparent because they did not react with the M- or N-specific MoAbs. These observations, summarized in Table 1, are essentially in agreement with reported findings.  

Additional aspects of the structure of the altered glycophorins were apparent from the known specificities of the antisera and antibodies used. For example, the variant glycophorin in band 4' in MiI erythrocytes did not react with either M or N MoAb; in contrast, this band was distinctly evident with MiII cells and the M MoAb (Fig 2). Clearly the amino terminal region of this glycophorin in Mi I and Mi II cells is different. Bands 3b and 6 in En(a−) erythrocytes appear to contain the dimer and monomer of δ glycophorin from their reaction with antiglycophorin serum, the N MoAb, and lack of reaction with antipeptide C serum. In addition to having no α glycophorin, these cells also lack the glycophorins in bands 2 and 3a seen in "normal" cells. However, at the position of band 5 these erythrocytes contain a glycophorin that reacts with both M and N MoAbs (a double band is seen with the N MoAb; Fig 1). Glycophorins in bands 2' and 4' in Mi III cells resemble δ glycophorin in that they fail to react with antipeptide C serum. They are probably variant forms of δ glycophorins whose usual 3b and 6 bands are less evident and appear to react with antipeptide C serum.

Glycophorin bands 2', 2", and 4' in Mi V cells appear to be variant forms of δ glycophorin, as they do not react with antipeptide C serum; indeed it has been proposed that they are (α-δ) glycophorin hybrids. In contrast, St* glycophorin variant bands 2', 2", and 2" react with antipeptide C serum.
and with the N MoAb; this supports their proposed (δ-α) hybrid structure.14,15,18

The glycophorin profiles of He+ and Wrb(−) cells are similar, under all conditions, to those of "normal" erythrocytes (Fig 3).

**DISCUSSION**

Using gel electrophoresis followed by immunoblotting, we demonstrate the extent of structural polymorphism of glycophorins in randomly chosen individuals, as compared to known serologic variants of the M, N blood group system.

The polyclonal antiserum to α glycophorin of an MN individual is an excellent reagent for the visualization of most molecules of the glycophorin family, and the antiserum to the carboxyl-terminal peptide of α glycophorin as well as the M and N MoAbs provide insight into the integrity of specific regions of the glycophorin molecule. In particular, this approach allowed us to identify the δ glycophorin and its variants and to detect glycophorins with altered amino terminal regions.

The presence of multiple bands in the gel patterns reflects partly the monomer-dimer relationship of α and δ glycophorins. Some bands observed in "normal" cells may represent other members of this family of proteins, for example, glycophorins in bands 3a and band 5. Glycophorin in band 3a may be a variant, and its lack of reaction with M and N MoAbs may be due to a sequence alteration or a lack of glycosylation within the amino terminal region. Reaction of the glycophorin in band 5 with both MoAbs in erythrocytes of all individuals of either M or N blood type is puzzling. Our findings could be explained by co-migration with γ glycophorin of minor glycophorins carrying M and N epitopes; no such glycophorins have been described. These glycophorins are thought to be related to α glycophorins since they all react with the antiserum to peptide C.

The mostly invariant glycophorin profiles as determined here among the 52 individuals suggest that electrophoretically definable variants are relatively infrequent. In addition, as part of another study,31 54 individuals (with different pathologies) were also found to have identical glycophorin patterns, confirming that immunoblotting-definable glycophorin variants are rare. These observations complement the serologic findings of low incidence of variants of M, N, S, s blood groups.6 Nevertheless, in our series of 41 Africans, two individuals whose profiles are different lack δ glycophorin, and their phenotype is most likely S-s-U−. The frequency of approximately 5% observed here confirms the reported serologically defined frequency for the ethnic group we examined (Bantu-speaking Africans).32 This frequency is high enough to qualify for a selection-balanced polymorphism and because glycophorins have been implicated in *plasmodium falciparum* invasion, this relationship is worth pursuing. The structural difference in glycophorin of band 5 that was observed with the other three individuals has not been reported previously.

Our results with the serologically variant erythrocytes confirm and amplify the observations of other laboratories. This proved particularly significant in the case of hybrid glycophorins as found in Mi V or Stα cells13–16,18 where reaction with antipeptide C serum or with the MoAbs could differentiate between the α-δ or δ-α hybrids and provided evidence that En(α−) cells used contained δ glycophorin. The use of the M and N MoAbs also confirmed the structural alteration in Mδ glycophorin10,11 and in addition allowed us to conclude that Mi I glycophorin in band 4′ was altered in the amino-terminal region and differed therefore from the analogous protein in MII cells.

The lack of an abnormal pattern in He+ and Wrb(−) erythrocytes could be due to structural alterations of glycophorins that cannot be detected by the antisera used or to
ERYTHROCYTE GLYCOPHORINS

mutations that do not alter the SDS-electrophoretic banding pattern.

Our results indicate that the erythrocytes of eight of ten MNSs blood group variants, documented serologically, show differences from the "normal" cells in electrophoretically resolved glycophorin profiles and clearly demonstrate that serologic deviation is a manifestation of structural variation in one or more members of the glycophorin family of molecules.

In a related approach, interactions with specific lectins have been used to probe variant glycophorin molecules resolved by gel electrophoresis of erythrocyte membrane proteins.33 Merry et al used a series of MoAbs to a glycophorin.

REFERENCES

31. Unpublished results


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YQ Lu, ME Nichols, WL Bigbee, RL Nagel and OO Blumenfeld