Red Blood Cell Glycophorins

By Joel Anne Chasis and Narla Mohandas

GLYCOPHORIN-A (GPA), GPB, GPC, and GPD constitute a group of red blood cell (RBC) transmembrane proteins that, although perhaps not widely appreciated in clinical hematology, have been important players in the fields of membrane biochemistry and cellular biology for several decades. GPA was the first membrane protein to be sequenced and has subsequently served as a model for topology of receptors and other transmembrane glycoproteins in both erythroid and nonerythroid cells. Initially, hematologic interest in the glycophorins was limited to blood bank serologists and the characterization of blood group antigens located on these sialoglycoproteins. However, with emerging data from functional studies, it is becoming apparent that certain glycophorins play important, but differing, roles in regulating RBC membrane mechanical properties and in maintaining RBC shape. Because several of these glycophorins are also expressed in various nonerythroid tissues, the functional importance of their interactions with the membrane skeleton may have a widespread biologic significance.

The presence of glycophorins in the RBC membrane was initially detected by Fairbanks et al. The four varieties of glycophorin comprise approximately 2% of the total RBC membrane protein, with GPA as the major component present at 5 to 9 x 10^6 copies per cell, while the less abundant GPB, GPC, and GPD are present at 0.8 to 3 x 10^6, 0.5 to 1 x 10^6, and 0.2 x 10^6 copies per cell, respectively. Because of their high sialic acid content, these molecules account for approximately 60% of the RBC’s negative surface charge. As such, they play a pivotal role in modulating RBC-RBC interactions, as well as RBC interactions with the vascular endothelium and other circulating blood cells. Over the past 2 decades, an unfortunate confusion was created in the glycophorin field by the appearance of four different nomenclatures (Table 1). Fortunately for us, a consensus has recently been reached among the investigators in the field, who have agreed to designate the various glycophorins as GPA, GPB, GPC, and GPD.

Protein, cDNA and genomic sequence analysis have provided a detailed characterization of the primary structure of GPA, GPB, and GPC. The primary structure of GPD is currently under study, but immunochromatographic and biochemical data imply that this is a protein closely related to GPC. Although these four sialoglycoproteins share the “glycophorin” name, suggesting a common genetic origin, this is partially a misnomer, because recent molecular biologic studies have firmly established that three of these glycophorins constitute different gene products. GPA, GPB, and GPC are encoded by three different genes on two different chromosomes. GPA and GPD do, however, appear to be closely related, arising from the same gene through use of alternative translation initiation sites. Recently, a novel GPE gene was isolated that might have evolved from GPA by homologous recombination at Alu repeats. Although the genomic structure of GPE is well-characterized, search for the protein encoded by this gene has yielded negative results. All the expressed glycophorins are O-glycosylated proteins with their amino terminal domains exterior to the lipid bilayer and a single membrane-spanning domain. GPA and GPB share extensive sequence homology with one another, while GPC and GPD are closely related proteins—with no structural homology to GPA and GPB.

In this review we will discuss the genomic organization and primary structure of cDNA and protein for each of the glycophorins, with particular emphasis on the relationship of these biochemical characteristics to the functional role of the proteins in the cell membrane. Recent molecular biologic analyses have also provided a detailed characterization of the primary structure of naturally occurring mutant forms of these molecules. This information, in association with biophysical studies of normal and mutant RBCs, has enabled us to begin to understand the contributions of glycophorins to the regulation of membrane material behavior. In the sections that follow we will describe these recent studies and the new insights that they have stimulated.

GPA AND GPD

Structural characterization of GPA. Because of extensive similarities in genomic organization, it appears that the genes for GPA and GPB arose from a common ancestral gene through homologous recombinant events involving Alu sequences. The GPA gene, located on chromosome 4q28-q31, contains 7 exons. The first exon, as well as part of the second, encode a cleavable leader peptide. The exoplasmic domain of GPA, composed of 70 amino acid residues (Fig 1), is encoded by the second through fourth exons with the codons for M- and N-blood group antigens contained in the NH2-terminal 26 residues encoded by exon 2. M- and N-phenotypes differ from one another at amino acid residues one and five, with the N-phenotype containing leucine at residue 1 and glutamic acid at residue 5, while the M-phenotype is characterized by serine at residue 1 and glycine at residue 5. A membrane-spanning domain of 22 amino acids and a cytoplasmic domain of 39 residues are encoded by exons 5 and 6, respectively. With its cleavable signal peptide and single membrane-spanning domain,
GPA is characterized as a class I transmembrane protein. Analysis of the secondary structural organization of GPA based on circular dichroism spectra and conformational prediction from primary structure suggests that the molecule is about 20% beta sheet, 3,31,32 One short stretch of beta sheet, composed of residues 90 through 93, is of particular interest, as it may play a role in the formation of GPA dimers, which are the predominant species in the native membrane. This segment of beta sheet lies closely adjacent to the helical region within the bilayer; formation of intermolecular parallel beta sheets between the two monomers could then control alignment and packing of the helical regions of the two molecules in the bilayer.32

Structural characterization of GPB. Five exons of the GPB gene27,28 localized to chromosome 4 encode a 70 amino acid class I transmembrane protein that possesses significant structural similarity to GPA (Figs 1 and 2). The nucleotide sequences of exons 1 through 5 of the GPB gene are very similar to exons 1 through 5 of the GPA gene; however, the 3′ proximal sequences differ. The NH2-terminal 26 amino acids of GPB, encoded by exon 2, are homologous to those of GPA molecules of the blood group antigen N-phenotype. Although GPB genomic DNA contains nucleotide sequences quite similar to GPA exon 3 and its flanking introns, these sequences are not expressed in GPB messenger RNA (mRNA). The cDNA of GPB thus lacks nucleotides that encode residues 27 through 55 of GPA, which includes the site at which GPA is N-glycosylated. The extracellular domain of GPB, encoded by exon 4, expresses the SS-blood group polymorphism at amino acid residue 29, with methionine and threonine imparting S- and s-phenotypes, respectively.3,12 The transmembrane domain of GPB encoded by exon 5, like that of GPA, contains about 20 hydrophobic amino acids. In both sialoglycoproteins, the cytoplasmic-transmembrane junction contains 3 to 4 basic amino acids, which function as stop transfer signal33 and, in addition, may interact with the phospholipids to anchor the proteins in the bilayer.34 Of potential functional significance is the fact that the cytoplasmic domain of GPB is shorter than that of GPA, containing only 3 residues in addition to the 3 membrane-anchoring amino acids.

Structural characterization of GPE gene. A new member of the GPA and GPB gene family has very recently been isolated and characterized.23,24,35 Initially called invariant (inv) but renamed GPE, this gene may have evolved from the GPA gene in a fashion analogous to that postulated for the GPB gene. As both the GPE and GPB genes contain similar 3′ sequences and 3′ Alu repeats, they may have arisen from GPA by homologous recombination at Alu repeats.24 Although this newly discovered gene is effectively transcribed, there is to date no evidence of protein expression. cDNA sequencing studies show that the gene would encode a 78 amino acid protein containing a 19 residue leader peptide.23,24 The 29 N-terminal amino acids are identical to those of blood group M-type GPA, but residues 27 through 59 differ significantly from GPA and GPB. Comparison of genomic and cDNA sequence shows that the gene consists of 4 exons, with the nucleotide sequence of exons 1 and 2 homologous to GPA and GPB. In contrast, exon 3 differs from the GPB gene by several point mutations, a 24-bp insertion, and a stop codon that shortens the reading frame. However, in the region 3′ of exon 3, the sequence of GPE is virtually identical to that of GPB.

Along with the genes for GPA and GPB, the gene for GPE has been localized to chromosome 4 (Fig 2). Based on genomic analysis of glycophorin variants, an interesting model has been proposed for the tandem organization of the three genes along chromosome 4 with the order GPA, GPB, and GPE.35 Deletion of structural genes within this region could position the promoter of one glycophorin gene upstream from the body of another glycophorin gene, thereby generating hybrid gene structures.35 In support of this model are the observations that, except for a few point mutations, the sequences of both the promoter region and exon 1 of the GPA, GPB, and GPE genes are highly homologous.35 Moreover, the rare point mutations do not affect the potential cis-acting elements (CACC, NF-E1, and NF-E2) that are present in the promoter region.

Glycophorin variants. RBC membranes containing hybrid glycophorin variants and glycophorin deficiencies were initially identified by serologic and immunochromatographic assays, but have recently been characterized at the molecular level.

Table 1. RBC Membrane Glycophorins

<table>
<thead>
<tr>
<th>Alternative nomenclature</th>
<th>GPA</th>
<th>GPB</th>
<th>GPC</th>
<th>GPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal location</td>
<td>PAS-1</td>
<td>PAS-3</td>
<td>PAS-2</td>
<td></td>
</tr>
<tr>
<td>Copies per cell (× 10^12)</td>
<td>500-900</td>
<td>80-300</td>
<td>50-100</td>
<td>20</td>
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<tr>
<td>Apparent molecular mass</td>
<td>Kd</td>
<td>36</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>O-linked Oligosaccharide side chains</td>
<td>131</td>
<td>72</td>
<td>128</td>
<td>~107</td>
</tr>
<tr>
<td>N-linked Oligosaccharide side chains</td>
<td>15</td>
<td>11</td>
<td>12</td>
<td>~6</td>
</tr>
<tr>
<td>Blood group antigens</td>
<td>MN</td>
<td>Ss</td>
<td>Ge-3</td>
<td>Ge-2,3</td>
</tr>
<tr>
<td>RBC specific</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Fig 1. Schematic representation of extracellular, intramembranous and cytoplasmic domains of GPA, GPB, GPC, and GPD. GPA and GPB amino acids 1-26, homologous (solid areas); GPA 71-101 and GPB 34-72, strikingly similar (very heavily shaded areas); GPA 58-71 and GPB 26-34, some homology (heavily shaded areas); GPA 26-58 and 101-131, no homology (open areas). GPC amino acids 1-128 and GPD 1-107, homologous (lightly shaded areas). Extracellular domains are on the left side of the bilayer and cytoplasmic domains on the right side of the bilayer.
Among these variant phenotypes are several that have been of particular interest because of the insight they provide into the functional role of glycophorin in the membrane. This group of mutants includes Miltenberger V, in which the RBC membrane contains biochemically altered GPA molecules,28,36,37 as well as the En(a-) and M\textsuperscript{m}\textsuperscript{m} phenotypes, in which the membrane is totally deficient in GPA.28-40 The Miltenberger V gene, as a consequence of unequal crossing over between GPA and GPB genes, is composed of exons 1 through 3 of the GPA gene and exons 3 through 5 of the GPB gene (Fig 2).28,35,37 As a result, the glycoprotein encoded by the Miltenberger V gene is a hybrid molecule composed of the exoplasmic domain of GPA (residues 1 through 58) fused to the transmembrane and cytoplasmic domains of GPB (residues 27 through 72).

The extremely rare En(a-) phenotype characterized by membranes totally lacking in GPA results from several different mutations. In what is categorized as the Finnish En(a-) phenotype, En(Fin), the individual is homozygous for a complete deletion of the GPA gene with normal genes encoding GPB and GPE (Fig 2).41 However, the English variant of En(a-), En(UK), is a more complex genetic story, in part, because the individual is assumed to be heterozygous for En(UK) and M\textsuperscript{m}. The presence of the M\textsuperscript{m} gene supresses the expression of GPA and GPB, due to deletion of both the GPA and GPB genes (Fig 2).42

Although molecular biologic studies are still incomplete, conclusions from protein and DNA analysis suggest that the En(UK) gene is a fusion product formed from GPA and GPB genes, which encodes a hybrid glycoprotein with the N-terminal domain of blood group M-type GPA and the C-terminal domain of GPB.41,43-45 Together, the Miltenberger V, En(Fin), and M\textsuperscript{m} mutations have been extremely useful in studies defining the biologic functions of GPA, as described below.

**Biologic function of GPA.** GPA expression is uniquely erythroid. Studies of multiple nonerythroid tissues and cell lines by both Northern analysis of RNA preparations and immunocytochemical analysis of cell surface expression have confirmed that GPA expression is restricted to the erythroid lineage.46-49 During normal erythropoiesis, GPA can be detected on the surface of the proerythroblast, but not on the surface of the earlier burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) progenitors.46,50-52 Because the biologic function of GPA during terminal erythroid differentiation is entirely unknown, this review will focus on functional studies in the mature, circulating RBC.

GPA, with its high sialic acid content, is the major contributor to the net negative surface charge of the mature RBC membrane. This underappreciated biologic role is critical for minimizing RBC-RBC interactions and preventing RBC aggregation (Fig 3). In circulation, the net aggregation energy between adjacent RBCs is determined by the balance between the aggregating energy from macromolecules bridging RBCs and the disaggregating energy produced by the mechanical shear stress and the electrostatic repulsive energy (as recently reviewed by Chien and Sung).53 The net negative electrical charges contributed by sialic acid residues on glycophorin produce the electrostatic repulsive energy. Biochemical studies of two mutant phenotypes, En(a-) Finnish type and M\textsuperscript{m}\textsuperscript{m}, suggest that a mechanism may exist in the RBC to maintain the surface content of sialic acid within a certain range. In both En(a-) and M\textsuperscript{m}\textsuperscript{m} cells, which completely lack GPA,58,39 an intriguing, associated surface change is an increased glycosylation
of band 3 resulting from the addition of sialic acid moieties.38,39,54 This posttranslational modification of band 3 partially compensates for the loss of sialic acid residues on GPA. As a consequence, these mutant RBCs maintain a net negative surface charge that is only 20% less than normal, rather than a 60% decrease, which would be expected in a GPA-deficient RBC. Individuals with En(a- and M^M^6 RBCs are clinically well. Thus, increased glycosylation of band 3 in En(a-) and M^M^6 cells functionally substitutes for the loss of GPA and accounts for the normal RBC behavior observed in these individuals. Mutations that result in GPA deficiency are extremely rare and we speculate that without concomitant increase in glycosylation of other membrane proteins these mutations may be incompatible with survival. Further support for the thesis that the erythrocyte harbors a mechanism for preserving surface glycosylation is provided by Dantu-positive erythrocytes. Although these cells lack 57% of their normal GPA, they contain a GPB-A hybrid molecule in a hybrid:GPA ratio of 2.4:1.55 Compensating for the increased glycosylation contributed by the glycophorin hybrid is a decrease in the glycosylated residues on band 3. From this body of data, we conclude that maintaining its negative surface charge is crucial to the RBC and that the critical biologic function of GPA is to minimize cell-cell interactions in circulation.

Characterization of the cellular consequences of En(a-) and M^M^6 mutations have also provided crucial leads for understanding the role of GPA in regulating membrane biophysical properties. Individuals with these two phenotypes have normally discocytic RBC morphology and no clinically significant anemia. When membrane material properties of En(a-) RBCs were characterized by ektacytometry,56 both membrane deformability and membrane mechanical stability were found to be normal (Fig 4). These studies unequivocally show that GPA plays no role in regulating cell shape, membrane deformability, or membrane mechanical stability.

Although the findings outlined above suggest that in its native state GPA does not regulate either cell shape or membrane material properties, a number of recent studies have shown that, following binding of a ligand specific for this protein, profound changes occur in membrane material behavior. Such an inducible change in membrane behavior initiated by ligand-receptor interaction is a form of signal transduction akin to ligand-induced changes in granulocyte and lymphocyte function. The study of this form of signal transduction in the RBC began with the initial observation that binding of the lectin, wheat germ agglutinin, to the RBC surface inhibited chemically induced echinocytic transformation57 and also markedly reduced RBC membrane deformability.58 Subsequent studies showed that monoclonal antibodies (MoAbs) specific for the exoplasmic domain of GPA, as well as their monovalent Fab fragments, also decreased membrane deformability, confirming that this process was mediated by GPA and suggesting that the process involved a transmembrane communication rather than the formation of an external lattice of GPA molecules cross-linked by lectin or divalent IgG.58 The importance of the cytoplasmic domain in this transmembrane process was underscored by the finding that ligand binding decreased the lateral mobility of normal GPA molecules within the membrane,53 but did not change the lateral mobility of the Miltenberger V hybrid glycoporphin A,60 which has a significantly truncated cytoplasmic domain of only six amino acids (Fig 5, left and middle panels).28,36,57 Based on these findings, the following model has been proposed. In the native state, the cytoplasmic domain of GPA has little or no interaction with the skeletal network. However, ligand binding induces a conformational change in the cytoplasmic domain that results in its increased association with the

Fig 4. Membrane deformability and mechanical stability of RBCs deficient in various glycoporphins. (Left) RBC membranes deficient in GPC and GPD (—) required significantly higher values of applied shear stress as compared with normal membranes (shaded area) to reach equivalent deformation. The lines are parallel; thus, GPC- and GPD-deficient membranes required 2.5-fold higher shear stress than did normal membranes to reach equivalent deformation at all points along the curve, indicating that the membranes had 0.4 times normal deformability. In contrast, GPA-deficient (--) and GPB-deficient (---) membranes required the same shear stress as normal membranes to reach equivalent deformation, implying normal membrane deformability. (Right) The deformability index of RBC membranes deficient in GPA and GPD (—) decreased more rapidly with time than normal RBC membranes (shaded area) when exposed to constant shear stress, implying decreased mechanical stability. In contrast, the rate of decline of deformability index of RBC membranes deficient in GPA (--) and GPB (---) was normal, implying that these membranes had normal membrane mechanical stability. (Reprinted with permission.)

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Fig 5. Schematic diagram of normal and variant Miltenberger V GPA and the effect of R-10 and 10F7 on the relative rigidity of normal and Miltenberger V RBCs. (Left) Normal GPA with cytoplasmic domain containing 39 amino acid residues. (Middle) Hybrid Miltenberger V molecule composed of the exoplasmic domain of GPA fused to the transmembrane and cytoplasmic domains of GPB and containing 6 amino acid residues in its cytoplasmic tail. (Right) The deformability of nonliganded normal cells (lane 1) and Miltenberger V cells (lane 4) were normal with a relative rigidity of 1. The relative rigidity of normal cells after R10 (lane 2) and 10F7 (lane 3) binding was 13.1 and 13.9, respectively. The rigidity of Miltenberger RBCs after R-10 (lane 5) and 10F7 (lane 6) binding was only minimally increased. These results imply that marked increases in antibody-induced rigidity require the presence of the cytoplasmic domain of GPA. (Reprinted with permission.62)

An important feature of this ligand-induced membrane rigidity is that the extent of rigidity can be modulated by varying the site on the exoplasmic domain to which the ligand binds (Fig 6).62 For example, binding of an MoAb to an epitope close to the N-terminus of GPA increased membrane rigidity 5.8-fold, binding to an epitope in the midregion of the exoplasmic domain resulted in a 10.8-fold increase in membrane rigidity, while binding to an epitope

Fig 6. Schematic diagram of antibody binding sites on GPA and the maximum relative rigidity induced by antibody binding. (Left) The peptide backbone with its 15 O-linked (C) and 1 N-linked (△) tetrasaccharide is shown traversing the lipid bilayer. The blood group antigens M and N are determined by variations within the first five amino acids in the amino terminal end of the molecule. The MoAb 9A3 has anti-M specificity and binds to the amino terminus. Antibodies R-10 and 10F7 bind in the midregion of the extracellular portion of GPA distal to the trypsin cleavage site. B14 detects an epitope closely adjacent to the lipid bilayer, between residues 56 and 67. (Right) Antibody binding to different regions of GPA induces different degrees of increase in membrane rigidity. Control MM RBCs with no bound antibody (lane 1) have normal deformability and relative rigidity of 1. Binding of 9A3 (lane 2) produces cells with a relative rigidity of 5.8 ± 1.5; 10F7 (lane 3) cells with a relative rigidity of 10.8 ± 1.4; R-10 (lane 4) cells with a relative rigidity of 10.8 ± 2.1; and B14 (lane 5) cells with a relative rigidity of 18.2 ± 2.7. (Reprinted with permission.62)
between residues 56 and 67 close to the bilayer increased rigidity 18-fold (Fig 6). While the mechanism for this modulatable process has not yet been characterized, we speculate that the site of ligand binding determines the extent of conformational change in the cytoplasmic domain. This modulatable process represents an intriguing mechanism whereby a single receptor could communicate across the membrane in multiple ways.

Although these studies clearly establish a ligand-induced increased association of the cytoplasmic domain of GPA with the membrane skeleton, the specific nature of this association has not yet been characterized. What remains to be elucidated is whether the changes in membrane material properties result from a specific protein-protein interaction or whether the ligand-induced conformational change in the GPA cytoplasmic domain causes this region to become physically entangled with the dense underlying skeletal lattice. Two membrane proteins that GPA might specifically interact with after ligand binding are protein 4.1 and band 3. Anderson and Marchesi have reported that an association occurs between protein 4.1 and GPA that is modulated by phosphoinositides. However, it is doubtful that this proposed interaction plays any role in the observed membrane changes because RBCs totally deficient in protein 4.1 become rigid after ligand binding. Parenthetically, it is difficult to assign a physiologically relevant role for a GPA and protein 4.1 interaction in light of the observed normal membrane behavior of En(a−) cells completely deficient in GPA. A second candidate protein for interaction with GPA is the other major RBC transmembrane protein, band 3. Accumulated data suggest that at least some subpopulations of band 3 and GPA molecules are in close proximity within the bilayer. For example, antibody-induced cross-linking of GPA molecules has been shown to affect the rotational mobility of band 3. Moreover, studies on Wrb blood antigen expression showed that MoAbs specific for Wrb immunoprecipitated both GPA and band 3 and reacted by radioimmunoassay only with cells in which both of these proteins were expressed. A novel concept that emerges from these observations is that interaction between these two major integral proteins may contribute significantly to the biologic function of the RBC membrane.

A number of bacterial antigens bind to carbohydrate residues on GPA. An intriguing, but as yet untested hypothesis, is that such binding induces membrane rigidity that then stimulates increased entrapment and phagocytosis. In this scenario, GPA binding would provide a mechanism for enhanced antigen clearance. While much remains to be learned regarding ligand-induced signal transduction and GPA-band 3 associations in the membrane, it can be stated with certainty that a major physiologic function of GPA is to bestow negative surface charge to the membrane, thus minimizing cell-cell interactions.

We have limited our discussion in this section to GPA because, to date, no biologic function has been ascribed to GBP other than that of carrying blood group antigens Ss and U.

**GPA AND GPB**

Structural characterization of GPA and GPB. Unlike GPA and GPB, which are encoded by two distinct genes, GPA and GPB are encoded by a single gene located on chromosome 2q14-q21. Although GPB contains a truncated amino terminal domain, the remaining polypeptide (residues approximately 21 to 128) is identical to that of GPA (Fig 1). While the mechanism of production of these two proteins from the same gene is currently under active investigation, evidence gathered to date suggests that these polypeptides arise from the use of two different translation initiation sites (AUGs) within the same reading frame through a leaky scanning mechanism. Translation initiated at the first AUG generates GPA, while initiation at the second AUG gives rise to GPB.

Structurally, the GPA gene is organized (over 13.5 kb) into four exons with exons 1 through 3 encoding the extracellular domain and exon 4 encoding both the membrane spanning and carboxy-terminal domains. Interestingly, exons 2 and 3 are within a 3.4-kb DNA fragment containing two repeated domains with less than 5% nucleotide sequence divergence. These two exons vary from one another only in that exon 3 contains 27 additional nucleotides, which encode residues 42 through 50 of the exoplastic domain. Therefore, it appears likely that these tandem repeated domains result from duplication of a region of an ancestral gene. In contrast to GPA, GPB does not express a cleavable signal sequence and thus belongs to the type II class of membrane proteins. GDNA analysis suggests a protein 128 amino acids in length. The extracellular amino terminal domain, composed of 57 hydrophilic residues, contains 12 O-glycosylation sites and one N-glycosylation site, as well as the Gerbich (Ge:3) blood group antigens (residues 41 through 50). The membrane spanning domain contains 24 nonpolar residues (58 through 81), while the cytoplasmic domain is composed of 47 residues (82 through 128). It may be functionally significant that the cytoplasmic domains of GPA and GPB are the longest of the four glycoporphins.

Several variant forms of GPA initially identified by immunochemical and serologic assays have recently been characterized on a molecular level. These variant phenotypes include Gerbich, Yus, and Webb, in which the RBC membrane contains normal amounts of GPA, but with altered biochemical composition, as well as the Leach phenotype, in which the membrane is totally deficient in GPA. Polymerase chain reaction (PCR) amplification of Yus mRNA and sequencing of the mutant fragment showed a 57-bp deletion corresponding to exon 2 that resulted in a deletion of N-terminal amino acids 17 to 35 (Fig 7). Similar analysis of the Gerbich phenotype showed a deletion of the 84-bp exon 3, which produced a deletion of N-terminal amino acids 36 to 63 (Fig 7). It is postulated that the Yus and Gerbich deletions within the GPA gene were produced by an unequal crossover between the homologous 3.4-kb repeat sequences. If a crossover occurs 5′ to misaligned exons 2 and 3 in the two chromosomes, an altered gene exhibiting an exon 2 deletion would be produced (Yus-type). Alternatively, crossover 3′ to mis-
alignexons2and3wouldyieldagenecontainingexon
3deletion(Gerbichphenotype).Incontrasttotheexon
deletionsseenintheYusandGerbichphenotypes,thef-webphenotypeistheresultofasubstitutionofserinefor
asparagineatposition8,75

todate,twodifferentmutationshavebeencharacterized
thatresultinataotaldeficiencyofGPCinthemembrane,
knownastheLeachphenotype.Southernblotanalysisof
 genomic DNAsuggeststhatthemostcommongeneticbasis
forthisdeficiencyinvolvesadeletionofexons3and4of
theGPCgene.22,76,77Thesecondmutationtobeidentifiedisas
single nucleotide deletion in codon 45 within exon 3,which
causesaframeshiftmutationinthemRNA,resultinginaprema-
ture stop codon.77Proteinstranslatedfromthis
mRNAwouldpresumablybetruncatedandnotinserted
into the lipid bilayer. Characterization of the Leach, Yus,
and Gerbich mutations has provided important insights
into the biologic function of GPC, as we will discuss in the
following section.

Biologic function of GPC. It is clearly apparent that
GPC, unlike GPA and GPB, has a pattern of expression
that is not limited to the erythroid lineage. Immunocy-
tochemical analysis of cell surface expression as well as
NorthernanalysisofRNApreparationsconfirmthepres-
ence of GPC in multiple nonerythroid tissues, including
breast, liver, and kidney.22,77 Interestingly, the level of
mRNAexpressionislowerinnonerythroidtissues.This
difference may be the consequence of the use of different
transcription start sites in different tissues; for example,in
a lymphoid cell line transcription begins 11 nucleotides up-
stream from the site used in erythroid cells, while in a
megakaryocytic cell linethe transcription start site is 57
nucleotides upstream.78 Similar differences in the level of
expression of other genes such as c-myc and band 3 have
been attributed to variable transcription sites.79,80

TwomurineMoAbs, one glycosylation dependent (MR4-
130) and the other sialic acid independent (AP03), have
been useful probes in examining the developmentally regu-
lated expression of GPC during terminal erythroid differen-
tiation.66,78,81 While CFU-E-derived erythroblasts react with
both antibodies, erythroblasts derived from BFU-E are
AP03-positive but MR4-130-negative, indicating that a
desialatedformofGPCisinsertedintothemembranesof
earlier progenitors. With this pattern of expression, GPC,
like carbonic anhydrase I and blood group antigen A, can
be used as a marker for identifying very early erythroid
precursors during normal and leukemic erythroid differen-
tiation.81

In the mature RBC, GPC, in contrast to GPA, appears to
play a critical role in regulating cell shape, membrane
deformability, and membrane mechanical stability. A number
of mutations involving the GPC gene (which have been
described above), result in RBCs that are either completely
deficient in GPC or else contain a variant form of the
glycoprotein.4,70,76 Characterization of the cellular conse-
quences of these mutations56,82 have provided crucial leads
for understanding the function of GPC. Although a com-
plete deficiency of GPC, as seen in the Leach phenotype,
doesnotcausclinicallysignificantanemia,itisdoesresultin
elliptocytosis,4 as well as a decrease in membrane deform-
ability and a marked reduction in membrane mechanical
stability (Fig 4).56 These abnormalities in cell shape and
membrane properties are in dramatic contrast to the
normal discocytic morphology and normal membrane prop-
erties observed in the En(a−) GPA-deficient RBCs. The
data obtained with Leach RBCs thus imply that GPC plays
a critical role in regulating both RBC shape and membrane
material properties.

Yus, Gerbich, and Webb mutations, as described above,
result in qualitative changes in the GPC rather than
quantitative changes of the Leach mutations.30,75,76 Interest-
ingly,these threemutationsare all limited to the region of
the gene encoding the exoplasmic domain, leaving the
primary structure of the cytoplasmic domain unperturbed.75
By characterizing the cellular properties of these mutant
phenotypes, we have been able to define the role of various
domains in regulating membrane function. In contrast to
RBCs of the Leach phenotype, Gerbich, Yus, and Webb
RBCs have normal discocytic morphology and exhibit
normal membrane deformability and mechanical stability
(Fig 8).82 The normal membrane properties of these pheno-
types, in the context of a structurally normal cytoplasmic
domain, implies that the cytoplasmic domain of GPC is the
critical region of the molecule for maintaining normal
shape and for regulating membrane properties.

An abundance of information is currently available
regarding the role of band 3 in anchoring the spectrin-based
membrane skeleton to the lipid bilayer through interaction
of its cytoplasmic domain with ankyrin. Less well-appreciated, but strongly substantiated by the observations outlined above, is a similarly important anchoring function for the cytoplasmic domain of GPC. Evidence to date implies that protein 4.1 is the membrane skeletal component with which GPC interacts. Mueller and Morrison originally suggested this specific interaction based on the observation that nonionic detergents do not extract GPC from normal membranes, but do extract this sialoglycoprotein from membranes deficient in protein 4.1. Subsequent studies substantiating this hypothesis include the observation that GPC and protein 4.1 copurify after extraction from the membrane and that GPC content of the membrane is related to protein 4.1 content. For example, flow cytometric analysis showed that the GPC content of membranes with 50% deficiency of protein 4.1 was 44% of normal, while it was only 9% of normal in membranes totally deficient in protein 4.1. In these individuals, molecular analysis of both GPC and protein 4.1 genes documented only an abnormality in the protein 4.1 gene, implying that GPC deficiency in these membranes is secondary to protein 4.1 deficiency rather than the result of a primary defect in the glycoprotein itself. Convincing evidence that protein 4.1 anchors GPC in the bilayer has recently been provided by a series of experiments in which detergent extractability of GPC was examined in protein 4.1-deficient RBCs in their native state and after reconstitution with exogenously purified protein 4.1. While GPC was readily extractable from protein 4.1-deficient membranes, the glycoprotein was retained with the skeletal proteins after reconstitution of the 4.1-deficient membranes with purified protein 4.1 (Fig 9). These data, considered with that obtained with the mutant RBCs, strongly suggest that a physiologically relevant interaction does indeed occur between the cytoplasmic domain of GPC and protein 4.1.

Although the molecular nature of the interaction between the two RBC membrane constituents, GPC and protein 4.1, remains to be elucidated, it seems clear that GPC plays a functionally important role in regulating RBC shape and membrane properties, and that protein 4.1 serves as a membrane anchor for this sialoglycoprotein.

**CONCLUSIONS**

GPA and GPB, having arisen from a common ancestral gene through homologous recombinant events involving Mhu sequences, share extensive sequence homology with one another. GPA and GPD, which have no structural homology to GPA and GPB, are encoded by a single gene, probably through the use of different translation initiation sites via a leaky scanning mechanism. Translation initiated at the first AUG generates GPC, while initiation at the second AUG gives rise to GPD, which contains 21 fewer amino acid residues in its amino terminal domain than does GPC. The structural data accumulated on the GPA gene family serves as a springboard for detailed analysis of multiple GPA variants. It has already been shown that certain of these variants are encoded by genes produced by a variety of recombinations and deletions within and between the glycophorin genes. With their extensive diversity, the glycophorins could, therefore, serve as an impor-
tant model for future study of polymorphisms in other human genes.

Certain important and previously unsuspected functions for various RBC sialoglycoproteins have recently been identified. GPA, with its abundant sialic acid content, is the major contributor to the net negative surface charge of the mature RBC membrane. The electrostatic repulsive energy produced by these negative charges plays a critical role in minimizing cell-cell interactions in the circulation. An intriguing hypothesis is that the erythrocyte harbors a mechanism for preserving negative surface charge. This thesis is suggested by the apparent compensatory changes in glycosylation of band 3 observed in mutant phenotypes [En(a−), M4M3, Dantu-positive] in which GPA molecules are either deficient or present in an overly glycosylated variant form.

While GPA, in its native state, does not appear to regulate either cell shape or membrane material properties, ligand binding induces both a profound increase in membrane rigidity and a decrease in the lateral mobility of GPA molecules. This ligand-induced change in membrane properties results from increased association of the cytoplasmic domain of GPA with the skeletal network and can be modulated by varying the site on the exoplasmic domain to which the ligand binds. This inducible change in membrane behavior, mediated by the cytoplasmic domain, can be considered a form of signal transduction akin to ligand-induced changes in cellular functions described in granulocytes and platelets. Moreover, recent studies show that different VLA integrin α subunit glycoprotein domains mediate distinct cellular functions.86 Molecular characterization of the mechanism involved in GPA signal transduction may, therefore, provide insights into novel cell communication pathways present also in nonerythroid cells.

GPC in the native state, in contrast to GPA, appears to play a pivotal role in regulating cell shape, membrane deformability, and membrane mechanical stability. The GPC-related regulation of these membrane functions appears to be through the interaction of the cytoplasmic domain of the sialoglycoprotein with protein 4.1. A deficiency in the anchoring protein 4.1 results in GPC-deficient RBC membranes. The coexistence of GPC and protein 4.1 in a wide variety of tissues raises the possibility that the interaction between these two proteins may have important but as yet undefined functions in these nonerythroid cells. Because the level of GPC mRNA transcription is higher in erythroid than in nonerythroid tissues, this sialoglycoprotein will be a relevant molecule for future study of differential tissue specificity and factors regulating gene expression.

It is now abundantly clear that the glycoporphins function as more than just blood group antigens. Indeed, the glycoporphins can continue to serve as models for exploring mechanisms of human gene polymorphisms, differential tissue expression, signal transduction, and surface component glycosylation, thereby contributing to our understanding of the biology of both erythroid and nonerythroid cells.

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Red blood cell glycophorins

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