Human Red Blood Cell Wright Antigens: A Genetic and Evolutionary Perspective on Glycophorin A-Band 3 Interaction

By Cheng-Han Huang, Marion E. Reid, Shen-Si Xie, and Olga O. Blumenfeld

The Wright (Wr/Wr) blood group polymorphism is defined by an allelic change (Lys658Glu) in the band 3 protein; nevertheless, the Wr antigen apparently requires glycophorin A (GPA) for surface presentation. To gain insight into the structural basis for this protein-protein interaction and delineate its relationship with Wr antigen expression, we investigated GPA and band 3 sequence polymorphisms occurring in rare humans and nonhuman primates. The lack of GPA or amino acid residues 59 through 71 of GPA results in the absence of Wr antigen expression in human red blood cells (RBCs) exhibiting the M0M0, En(a−), or MiV phenotype. However, the SAT homozygous cells carried a Glu668 form of band 3 and a hybrid glycophorin with the entire GPA extramembrane domain from residues 1 through 71, yet expressed no Wr antigen. This finding suggests that formation of the Wr antigenic structure is dependent on protein folding and that the transmembrane junction of GPA is important in maintaining the required conformation. Comparative analyses of GPA and band 3 homologues led to the identification in the interacting regions of conserved and dispensable amino acid residues that correlated with the Wr positive or negative status on nonhuman primates. In particular, the chimpanzee RBCs expressed Wr and the Glu668 form of band 3, which is identical to humans, but their GPA contained the Gly rather than Arg residue at position 61. Taken together, the results suggest that (1) Arg61 of GPA and the proposed Arg61-Glu668 change pair are not crucial for Wr antigen exhibition and (2) the role of GPA for interaction with band 3, including Glu668, probably involves a number of amino acid residues located in the α-helical region and transmembrane junction.

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Materials and Methods

Blood samples and serologic testing. Blood samples used as controls were obtained from normal human blood donors. Blood samples exhibiting variant phenotypes of the MNS blood group system were gifts from the following sources: M0M0, En(a−), and SAT bloods were from the Osaka Red Cross Blood Center (Osaka, Japan); MiV blood from the American Red Cross (Los Angeles, CA); Dantu and S-s-U- bloods were from the Community Blood Center (Dayton, OH); and Sr blood was from the Miyagi Red Cross Blood Center (Sendai, Japan). Blood samples of nonhuman primates were provided by the Wildlife Conservation Society (Bronx, NY), the Yerkes Primate Center (Atlanta, GA), or the Laboratory for Experimental Medicine or Surgery in Primates (Tuxedo, NY). The
HUMAN RBC WRIGHT ANTIGENS

Wr<sup>b</sup> antigen status on RBCs of animals was tested with human antisemipur (MF) using standard hemagglutination techniques. Isolation of DNA and RNA. Genomic DNAs were prepared from peripheral blood leukocytes as described.<sup>22</sup> Total RNA was isolated from hemolysates using a sequential cell lysis method<sup>2</sup> and then extracted with Trizol reagent (BRL, Gaithersburg, MD).

Synthesis and amplification of cDNA. Synthesis and amplification of cDNAs from erythroid total RNA was performed by reverse transcription-coupled polymerase chain reaction (RT-PCR), as previously described.<sup>23</sup> To obtain band 3 cDNA products, two primers that define, respectively, exons 16 and 17 of the human gene were used: AE1, 5'-AAACCTGCTGGTCATGCGCTTC-3' (nt 1891-1914, sense), and AE2, 5'-AGGCCCTTGACCATCTGGCCTCA-3' (nt 2076-2099, antisense).<sup>24,25</sup>

To obtain GPA cDNAs from higher primates, the following human primers were used<sup>26,27</sup>: GPI, 5'-GTATGGAAAAATAATCTTGGATTT-3'; GP3, 5'-ATCACTTGTCTCTGGATTTTCTATTTC-3' (nt 421-447, exon 6-7, antisense); and GP4, 5'-TCCACATTGGTTTGGTGTGAACAGATTC-3' (nt 454-480, exon 7, antisense). GP3 and GP4 could only prime the synthesis of GPA cDNAs, because their sequences are located in the last two exons encoding the cytoplasmic domain and are not present in the GPB gene.<sup>28,29</sup> After first-strand synthesis, the GPA cDNA product was amplified 30 cycles in 50 μL volume on a thermocycler (Ericomp, San Diego, CA). The first 29 cycles were each run at 94°C for 1 minute, at 55°C for 45 seconds, and at 72°C for 1 minute. For the last cycle, annealing at 55°C and chain extension at 72°C was for 2 and 7 minutes, respectively.

Amplification of genomic DNA sequences. Exon 16 of the band 3 gene spanning the A1972G (Lys<sup>658</sup>Glu) polymorphism was amplified from total genomic DNA in the presence of two primers, AE1 (see above) and AE3 (5'-TCTCAGTGGTGATCGACTCC-3').<sup>28</sup>

DNA sequence determination. The PCR-amplified cDNA and genomic DNA products were purified by native 5% polyacrylamide gel electrophoresis and their nucleotide sequences were directly determined on an automated DNA sequencer (Applied Biosystem, Foster City, CA).

RESULTS

Band 3 and GPA expression in Wr<sup>a</sup>-<sup>b</sup>- and Wr<sup>a</sup>-<sup>b</sup>+ individuals with MNS<sub>5</sub>-related variants. To further delineate the structure-phenotype relationship for the Wr<sup>b</sup> antigen, the band 3 polymorphism and glycophorin expression were revisited in 7 individuals with MNS<sub>5</sub>-related variant phenotypes. These phenotypes included M<sup>M</sup>M<sup>+</sup>, En<sup>a</sup>-<sup>+</sup>, S<sup>s</sup>-<sup>+</sup>-<sup>+</sup>, St<sup>c</sup>, MIV, Dantu, and SAT. Of the 7 individuals examined, all but the Dantu-positive proband were homozygotes. RBCs from these individuals were either deficient in glycophorins<sup>23,30-32</sup> or associated with expression of hybrid glycophorins resulting from unequal crossovers.<sup>33-37</sup> As determined by sequencing of PCR-amplified cDNA or genomic DNA products, all individuals carried the Glu<sup>658</sup> but not the Lys<sup>658</sup> form of band 3 (data not shown), indicating that they were all homozygotes for the Wr<sup>b</sup> allele. In M<sup>M</sup>M<sup>+</sup>, En<sup>a</sup>-<sup>+</sup>, and S<sup>s</sup>-<sup>+</sup>-<sup>+</sup>-<sup>+</sup> cells, the Wr<sup>b</sup> status apparently paralleled the absence or presence of GPA and correlated with the genetic status of the GPA gene. For RBCs bearing glycophorin hybrids except GPsat, the pattern of Wr<sup>b</sup> antigen expression complied with the absence or presence of the GPA portion expressed (see below). These data showed that, without GPA, the Glu<sup>658</sup> form of band 3 alone does not display the Wr<sup>b</sup> determinant.

Expression and inheritance of the Wr<sup>b</sup> antigen in SAT (T.K.) family. SAT is a private RBC antigen associated with the expression of two glycophorin isofoms in different families.<sup>38</sup> In the T.K. family, expression of the SAT antigen was accompanied by the inheritance of a GPA-B hybrid gene, GPsat, that arose via a similar mechanism as GPMiV but differed from the latter in the site of crossover point.<sup>37</sup> RT-PCR analysis of this three-generation family showed a genetic association but a phenotypic dissociation of band 3 with the Wr<sup>b</sup> antigen. The erythroid cells from the five SAT-positive members all contained the band 3 and GPsat transcripts; nevertheless, in contrast to the heterozygotes, the homozygote (donor II-1) lacked both GPA and GPB transcripts (Fig 1). Although sequencing of the band 3 cDNAs showed that all individuals should be the Glu<sup>658</sup> homozygotes, the Wr<sup>b</sup> antigen was segregated from GPsat in donor II-1 but was cotransmitted with GPA in members of the first and third generations (Fig 1). This inheritance pattern reinforced the hypothesis that the Wr<sup>b</sup> antigen requires a specific interaction between the band 3 and GPA proteins.<sup>3,4,17,20</sup>

Conformational dependence of the Wr<sup>b</sup> antigen and role of GPA TM junction. It has been suggested that the region of GPA, from residues 62 through 70, involves the Wr<sup>b</sup> antigen and forms part of an α-helix immediately adjacent to the TM segment.<sup>11,12</sup> Definition of the sequence as part of the Wr<sup>b</sup> epitope is supported by the absence of the Wr<sup>b</sup> anti-
Thus, GPSat retains the entire extramembrane domain of that interfaces the extracellular and membrane-spanning domains of glycophorins is indicated. residues 59 through the interaction may not always lead to Wrh antigen expres-
tion of the s, s and U antigens and the importance of
tion. As shown, the SAT homozygous RBCs expressed the GluHss form of band 3 and the hybrid glycophorin, GPSat (Fig 1). Comparison of the amino acid sequences (Fig 2) shows that the hybrid structure of GPSat is reciprocal to that of GPDantu 
and distal to that of GPMiV or GPSt. Thus, GPSat retains the entire extramembrane domain of GPA from residues 1 through 71, including the moiety for the Wrb epitope, and differs from GPA in the extramembraneous junction and TM segment (Fig 2). The lost Wrb expression in the SAT homozygote strongly suggests that formation of the epitope depends on proper contact of the two proteins and that the TM junction of GPA plays an important role in maintaining the required conformation. This notion is consistent with our recent studies showing the conformational dependence of the S, s and U antigens and the importance of the GBP TM junction for their presentation.41 In the case of GPSat, it is likely that the insertion of three GBP residues (Ala-Pro-Val) at the TM junction could readjust the orienta-
tion of the preceding α-helix and thus alter the native antigen structure necessary for anti-Wrb binding. This is different from GPSt or GPMiV, in which the retention or loss of the Wrb epitope sequence (Fig 2) has caused a coprecipitation and a null reaction with the anti-Wrb antibody, respectively.11,43

Sequence polymorphisms of GPA and band 3 protein homologues in nonhuman primates. Among nonhuman primates, only chimpanzee RBCs express the Wrb antigen at a level comparable to humans, whereas RBCs from orangutans, gibbons, and rhesus monkeys essentially lack the serologic reaction.44,45 Because the definition of such positive or negative status could show the underlying structural diversity, we determined the Wrb antigen status on animal RBCs and sequenced their cDNAs encoding the homologues of GPA and band 3 proteins. Comparison of the deduced primary sequences encompassing the Wrb domain (Fig 3) showed that (1) the GPA homologues are more divergent

<table>
<thead>
<tr>
<th>GP</th>
<th>Wrb Status</th>
<th>Hybrid Arrangement</th>
<th>GPA moiety of the Wrb epitope</th>
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<tbody>
<tr>
<td>GPA</td>
<td>+</td>
<td></td>
<td>51 VYPPEETGERVQLAHFSEP EITLI</td>
</tr>
<tr>
<td>GPB</td>
<td>–</td>
<td></td>
<td>19 SYISSQNGETGQLVHRFTVPAPVV</td>
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<tr>
<td>GPSt</td>
<td>+</td>
<td>B1-26/A59-131</td>
<td>19 SYISSQNGETGQLVHRFTVPAPVV</td>
</tr>
<tr>
<td>GPMiV</td>
<td>–</td>
<td>A1-58/B27-72</td>
<td>51 VYPPEETGERVQLAHFSEP EITLI</td>
</tr>
<tr>
<td>GPDantu</td>
<td>–</td>
<td>B1-39/A71-131</td>
<td>19 SYISSQNGETGQLVHRFTVPAPVV</td>
</tr>
<tr>
<td>GPSat</td>
<td>–</td>
<td>A1-70/B40-72</td>
<td>51 VYPPEETGERVQLAHFSEPAPVV</td>
</tr>
</tbody>
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Fig 2. Comparison of the amino acid sequences between the parent and hybrid glycophorins in the region relevant to the Wrb antigen exhibition. GPA and GPB, the parent molecules, are shown above GPSat, Miv, Dantu, and Sat, the hybrid molecules. The GPA sequence that may engage the labile structure of the Wrb antigen is overlined. GPB and the GPB-derived sequences are shaded. The arrangement of hybrids with respect to the GPA and GPB polypeptides and their status associated with Wrb are shown. Note that GPSat versus GPMiV and GPDantu versus GPSat are two pairs of reciprocal protein products whose crossing-over points in the genes reside in introns 3 and 4, respectively. The TM junction that interfaces the extracellular and membrane-spanning domains of glycophorins is indicated.
than the band 3 homologues in different species, but the
former have a number of conserved amino acids in positions
62 through 71, including Glu, Leu, His, and Phe, Ser, and
Glu, Gln; and (2) both Arg, GPa and Glu, band 3, the
two residues thought to be critical in forming the Wr interaction,
are variable from rhesus to chimpanzee.

As shown for GPA sequences (Fig 3, top), Arg, was
present in the orangutan and rhesus but was replaced by Gly
and Trp in the chimpanzee and gibbon. Scattered changes in
positions 61 through 82 included unique insertion or random
mutations, but positions 65 and 67 were occupied by reiterated
substitutions in different species. Accordingly, Val,
Ala and Arg, His distinguish chimpanzee from human.

In orangutan or gibbon, one more change, Val — Gly, made
the GPA sequence in positions 62 through 67 be identical with
the human GPB sequence in positions 10 through 15
(Gly, Gin, Leu, Val, His, Arg; Figs 2 and 3). In the rhesus,
its GPA differed from human GPA by three residues in positions
63 and 71 (Val, Ala, Glu, His, and Leu, 17, Pro; Fig 3).
In mice, a low sequence identity (34%) is mainly
confined to the TM segment and there is no significant homology in the Wr domain.

Regarding the band 3 sequence (Fig 3, bottom), the region
encompassing the extracellular loop and the adjacent
membrane-spanning segment (TM 8th pass) is well conserved and,
even in mice, the sequence identity is as high as 84%.

In that region, the chimpanzee had an identical sequence
with humans. The orangutan/gibbon and rhesus/baboon pairs
each share the same sequence, with two or three substitutions
located between the N-glycosylation site and TM 8
(data for baboon not shown). Of all animals examined, no Ly, form of band 3 was detected, suggesting that the Wr antigen
may also be rare in nonhuman primates. Nevertheless,
Glutex occurred in chimpanzees, but it had been replaced
by a histidine residue in band 3.

Correlation of sequence variation with Wr antigen expression in nonhuman primates. It is evident that the proposed Arg, Glutex charge pair cannot be formed in the animals studied due to the substitution of either one or both residues (Fig 3). This finding suggests that Arg, GPA is dispensable or replaceable with respect to the Wr reactivity, at least in the case of chimpanzees. Comparable Wr expression in human and chimpanzee RBCs (Fig 3) also implied that the two other changes in the helical region, Ala and His, may not affect the overall structure of the antigen. In the orangutan and gibbon, the absent Wr expression was apparently correlated with changes of Glu — His, on band 3 and Val — Gly, on GPA, because the former removed a negative charge and the latter rendered the sequence more like GPB, which is known not to display the Wr reactivity (Fig 2). Similarly, the lack of Wr expression on rhesus RBCs could also be attributed to changes on both band 3 (Glu — His,5) and GPA. Regarding the rhesus GPA moiety, the Arg67Glu change introduced a negative charge, and the substitution by Leu of conserved Pro, a helix breaker, might perturb the local conformation and thus alter the interaction. It is notable that, Ser, Glutex in the TM
junction of GPA may also be important for the GPA-band 3 interaction, because they differ from human GPB (Fig 2) but are conserved from the rhesus to humans (Fig 3, top).

DISCUSSION

In contrast to the Wr antigen that may be formed by
band 3 alone, there is considerable evidence indicating the
dependence of Wr antigenicity on a specific GPA-band 3 interaction. Nevertheless, the nature of this protein-protein interaction as well as the role of individual amino acid residues in Wr antigen formation remains largely speculative. The difficulty in defining this interaction stems partly from the fact that single amino acid changes in the regions encompassing GPA residues 62 through 70 and band 3 residues 651 through 660 are seldom encountered in the human populations, despite the recent finding of a rare Ly, polymorphism on the band 3 protein. This report describes two novel observations concerning the expression of the Wr antigen. The first observation is that the alteration of TM junction in GPSat silences the human antigen. The second observation points to a correlation of the Wr domain with sequence variations on GPA and band 3 homologues in nonhuman primates. These data provide new insights, in the context of primary structures, into the molecular basis for the GPA-band 3 interaction and its relationship with Wr exhibition.

Of the Wr null phenotypes found in human RBCs, the one associated with SAT homozygous cells is particularly intriguing in that GPSat retains an apparently intact sequence essential for the antigen presentation. The antigen disruption in GPSat by a small insertion distal to the putative Wr domain raises the possibility that the extramembranous junction or adjacent TM residues participate in or influence the GPA-band 3 interaction. Although both contain the Wr moiety, GPSat makes a contrast with GPSat, because the latter expressed the antigen weakly and carried a GPB sequence proximal to the residues 62 through 70. This comparison indicates that the Wr antigen is sensitive to local structural changes and that the closer the alteration is to the TM segment, the more profound the conformational perturbation.

The definition of Wr positivity or negativity in nonhuman primates by inspection of sequence divergence and conservation broadens our view on the human counterpart from an evolutionary perspective. Our data suggest that Glu on band 3 and VQL, His, and FSEP on GPA are important for the GPA-band 3 interaction and Wr antigen exhibition, whereas Arg, Ala, and His are not so crucial. Although scattered amino acid variations proximal to position 68 of GPA occur in animals (Fig 3), they are unlikely to be crucial either, because GPSat carries a completely different sequence proximal to that position (Fig 2), yet still displays the Wr reactivity. Apparently, the role of the GPA moiety for epitope formation mainly involves amino acids that are located in the TM and helical region. In addition, the associated Wr expression in the absence of Arg, but presence of Arg, in chimpanzee GPA suggests that the overall surface charge of the a-helix, rather than the specific location of Arg residues, would be more important, if the ionic interaction with Glutex of band 3 occurs at all.
Because parallel packing of the TM α-helices of the two proteins would bring the preceding extramembranous portions in close proximity, the helical region of GPA may stabilize the interaction and therefore the Wrb antigen by forming additional contacts with the residues nearby Glu685 of the band 3 protein.

In summary, the present studies have provided evidence for the conformational dependence of the Wrb antigen and led to the identification of certain amino acid residues that may be important for its exhibition. Nevertheless, although the Wrb antigen may be considered a phenotypic indicator of the GPA-band 3 interaction, its lost expression does not necessarily mean the abolition of the interaction. How those local changes cause the phenotypic silencing of Wrb and whether they reshape the interaction to elicit new antigenticities, such as SAT and Sr, are some of the issues that remain to be investigated. Studies combining site-directed mutagenesis with cotransfection of GPA and band 3 cDNAs in an ex vivo system should allow one to dissect in depth the structural elements involved in such protein–protein interactions.

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