The Wright (Wr+/Wr+) blood group polymorphism is defined by an allelic change (Lys658Glu) in the band 3 protein; nevertheless, the Wrb antigen apparently requires glycoporin A (GPA) for surface presentation. To gain insight into the structural basis for this protein-protein interaction and delineate its relationship with Wrb 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 Wrb from human red blood cells (RBCs) exhibiting the M*M*, En(a-), or MiV phenotype. However, the SAT homozygous cells carried a Glu658 form of band 3 and a hybrid glycophorin with the entire GPA extramembrane domain from residues 1 through 71, yet expressed no Wrb antigen. This finding suggests that formation of the Wrb 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 Wrb positive or negative status on nonhuman primates. In particular, the chimpanzee RBCs expressed Wrb and the Glu658 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-Glu658 charge pair are not crucial for Wrb antigen exhibition and (2) the role of GPA for interaction with band 3, including Glu658, probably involves a number of amino acid residues located in the α-helical region and transmembrane junction.

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: M*M*, En(a-), and SAT bands 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).
HUMAN RBC WRIGHT ANTIGENS

We addressed the antigen status on RBCs of animals with human antisera (MF) using standard hemagglutination techniques.

Isolation of DNA and RNA. Genomic DNAs were prepared from peripheral blood leukocytes as described.

Total RNA was isolated from hemolysates using a sequential cDNA synthesis reaction (RT-PCR), as previously described. To obtain band 3 cDNA products, two primers that define, respectively, exons 16 and 17 of the human gene were used: AE1, 5'-AAATCTCCTGCTGACTTGAGCCT-3' (nt 1891-1914, sense), and AE2, 5'-GACCCCTTGACCATCTTGCCGCTCA-3' (nt 2076-2099, antisense). After first-strand synthesis, the GPA cDNA product was amplified 30 cycles in the presence of two primers, AEI and AE2, 5'-GTATGGAAAAATAATTTC-3' (nt 2076-2099, antisense), and AE3, 5'-TCTCACGTGGATCTGAGACTCC-3'.

To obtain GPA cDNAs from higher primates, the following human primers were used: GPI, 5'-GTATGGAAAAATAATTTC-3' (nt 2076-2099, antisense), and AE3, 5'-TCTCACGTGGATCTGAGACTCC-3'.

DNA sequence determination. The PCR-amplified cDNA and genomic DNA products were purified by native agarose gel electrophoresis and stained with ethidium bromide.

Expression and inheritance of the Wp antigen in SAT (T.K.) family. SAT is a private RBC antigen associated with the expression of two glycoporphin isoforms in different families. 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. RT-PCR analysis of this three-generation family showed a specific interaction between the band 3 and SAT antigens.

RESULTS

Band 3 and GPA expression in Wr(a−b−) and Wr(a−b+) individuals with MNS-related variants. To further delineate the structure-phenotype relationship for the Wr+ antigen, we investigated the expression of two glycophorins and band 3 in 7 individuals with MNS-related variant pheno-

types. These phenotypes included M'M', En(a−), S-s-U-, St', MIV, Dantu, and SAT. Of the 7 individuals examined, all but the Dantu-positive proband were homozygous. RBCs from these individuals were either deficient in glyco-

porphins or associated with expression of hybrid glyco-

porphins resulting from unequal crossovers. As determined by sequencing of PCR-amplified cDNA or genomic DNA products, all individuals carried the Glu658 but not the Lys658 form of band 3 (data not shown), indicating that they were all homozygous for the Wp+ allele. In M'M', En(a−), and S-s-U- cells, the Wp+ status apparently paralleled the absence or presence of GPA and correlated with the genetic status of the GPA gene. For RBCs bearing glycoporphin hybrids except GPSat, the pattern of Wp+ antigen expression com-
pressed (see below). These data showed that, without GPA, the Glu658 form of band 3 alone does not display the Wp+ determinant.

Conformational dependence of the Wp+ antigen and role of GPA TM junction. It has been suggested that the region of GPA, from residues 62 through 70, involves the Wp+ antigen and forms part of an α-helix immediately adjacent to the TM segment. Definition of the sequence as part of the Wp+ epitope is supported by the absence of the Wp anti-
Thus, GPSat retains the entire extramembrane domain of that interfaces the extracellular and membrane-spanning domains of glycophorins is indicated. The interaction may not always lead to Wrh antigen expression. GPA and GPB, the parent molecules, are shown above GPSat, MiV, Dantu, and Sat, the hybrid molecules. The GPA sequence that may engage the leaflike epitope structure of the Wrh 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 Wrh are shown. Note that GPSat and GPB 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.

Fig 2. Comparison of the amino acid sequences between the parent and hybrid glycophorins in the region relevant to Wrh 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 leaflike epitope structure of the Wrh 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 Wrh are shown. Note that GPSat and GPB 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.

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Sequence polymorphisms of GPA and band 3 protein homologues in nonhuman primates. Among nonhuman primates, only chimpanzee RBCs express the Wrh antigen at a level comparable to humans, whereas RBCs from orangutans, gibbons, and rhesus monkeys essentially lack the serologic reaction. Because the definition of such positive or negative status could show the underlying structural diversity, we determined the Wrh 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 Wrh domain (Fig 3) showed that (1) the GPA homologues are more divergent...
than the band 3 homologues in different species, but the former have a number of conserved amino acids in positions 62 through 71, including Glu65, Leu66, His67, and Phe68. Ser69; and (2) both Arg67 of GPA and Glu68 of band 3, the two residues thought to be critical in forming the Wrβ antigen, are variable from rhesus to chimpanzees.

As shown for GPA sequences (Fig 3, top), Arg67 was present in the orangutan and rhesus but was replaced by Gly and Trp in the chimpanzee and gibbon. Scattered changes in positions 51 through 82 included unique insertion or random mutations, but positions 65 and 67 were occupied by reiterated substitutions in different species. Accordingly, Val65Ala and Arg67His distinguish chimpanzees from humans. In orangutan or gibbon, one more change, Val → Gly62, made the GPA sequence in positions 62 through 67 be identical with the human GPA sequence in positions 30 through 35 (Gly-Gln-Leu-Val-His-Arg; Figs 2 and 3). In the rhesus, its GPA differed from human GPA by three residues in positions 62 through 71 (Val65Ala, Gly67His, and Leu71Pro; Fig 3). In mice, a low sequence identity (34%) is mainly confined to the TM segment and there is no significant homology in the Wrβ 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 shared 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 Lys65 form of band 3 was detected, suggesting that the Wrβ antigen may also be rare in nonhuman primates. Nevertheless, Glu68 occurred in chimpanzees, but it had been replaced by a histidine residue in the TM domain.

Correlation of sequence variation with Wrβ antigen expression in nonhuman primates. It is evident that the proposed Arg67-Glu68 charge pair cannot be formed in the animals studied due to the substitution of one or both residues (Fig 3). This finding suggests that Arg67 of GPA is dispensable or replaceable with respect to the Wrβ reactivity, at least in the case of chimpanzees. Comparable Wrβ expression in human and chimpanzee RBCs (Fig 3) also implied that the two other changes in the helical region, Ala → Val5, and His → Arg5, may not affect the overall structure of the antigen. In the orangutan and gibbon, the absent Wrβ expression was apparently correlated with changes of Glu → His65 on band 3 and Val → Gly62 on GPA. On the other hand, although Arg67Glu change introduced a negative charge, and the substitution by Leu of conserved Pro67, a helix breaker, might perturb the local conformation and thus alter the interaction. It is not that, Ser69Glu67 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 Wrβ antigen that may be formed by band 3 alone, there is considerable evidence indicating the dependence of Wrβ 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 Wrβ 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 Lys65 polymorphism on the band 3 protein. This report describes two novel observations concerning the expression of the Wrβ 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 Wrβ status 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 Wrβ exhibition.

The definition of Wrβ 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 Glu65 of band 3 and VQL67-66, His67, and FSEP69-71 of GPA are important for the GPA-band 3 interaction and Wβ antigen expression, whereas Arg67, Ala68, and His70 are not so crucial. Although scattered amino acid variations proximal to position 58 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 Wrβ reactivity. Apparently, the role of the GPA moiety for epitope formation mainly involves amino acids that are located in the helical region and TM junction. In addition, the associated Wβ expression in the absence of Arg67, but presence of Arg67, in chimpanzee GPA suggests that the overall surface charge of the α-helix, rather than the specific location of Arg residues, would be more important, if the ionic interaction with Glu68 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 Wrh antigen by forming additional contacts with the residues nearby Glu635 of the band 3 protein.

In summary, the present studies have provided evidence for the conformational dependence of the Wrh antigen and led to the identification of certain amino acid residues that may be important for its exhibition. Nevertheless, although the Wrh 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 Wrh and whether they reshape the interaction to elicit new antigenicities, such as SAT and Str, 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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