Molecular Characterization of Erythrocyte Glycophorin C Variants

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Human erythrocyte glycophorin C plays a functionally important role in maintaining erythrocyte shape and regulating membrane mechanical stability. Immunochemical and serologic studies have identified a number of glycophorin C variants that include the Yus, Gerbich, and Webb phenotypes. We report here the molecular characterization of these variants. Amplification of glycophorin C mRNA from the Yus phenotype, using two oligonucleotide primers that span the coding domain, generated a 339-bp fragment compared with a 395-bp fragment generated by amplification of normal glycophorin C mRNA. Sequencing of the mutant 339-bp exon 2 deletion that corresponds to exon 2 of the glycophorin C gene. Similar analysis showed deletion of 84-bp exon 3 in the Gerbich phenotype. In contrast to the generation of shorter than normal DNA fragments from mRNA amplification in the Yus and Gerbich phenotypes, amplification of mRNA from the Webb phenotype generated a normal-sized fragment. Sequencing of this DNA fragment showed an A → G substitution at nucleotide 23 of the coding sequence, resulting in the substitution of asparagine by serine. This modification accounts for the altered glycosylation of glycophorin C seen in this phenotype. These results have enabled us to characterize glycophorin C variants in three different phenotypes that involve deletions of exons 2 and 3 of the glycophorin C gene, as well as a point mutation in exon 1 that results in altered glycosylation of this protein.

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ERYTHROCYTE GLYCOPHORIN C VARIANTS

30 seconds at 94°C; reannealing, 30 seconds at 60°C; extension, 1 minute 45 seconds at 72°C. DNA fragments were analyzed by 5% PAGE. The oligonucleotides used in this report are as follows. For most experiments, sequences extending from −8 to +387 in glycophorin C cDNA were amplified with the following primers: 5’-GCCAGGAATGTGGTCGACGAGA-3’ (sense strand), and 5’-TCAATAAAGTACTCTTCTT-3’ (antisense). Because these primers overlap the coding region, a new pair of oligonucleotides located entirely within 5’ and 3’ untranslated sequences was used to amplify and sequence the complete Webb glycophorin sequence: 5’-TCCCCGCGCTI~T(I~T(I~TGCCCGGGI~T(I~TC~ACG-~’ (sense) and 5’-GAGGAATTCAGGGAAGTGAAGTCTGTT-3’ (antisense). For direct sequencing of double-stranded PCR fragments, oligonucleotide primers were phosphorylated with γ[32P]-ATP using polynucleotide kinase, annealed to heat-denatured PCR product, and sequenced by the dideoxynucleotide termination method. Glycophorin PCR products from the Webb reticulocytes were also blunt-end ligated into the EcoRV site of Bluescript and sequenced in the double-stranded plasmid.

RESULTS

Immunochemical characterization of mutant glycophorins. The presence of abnormal glycophorin C in RBCs of Gerbich, Yus, and Webb phenotypes was confirmed by immunochemical analysis of RBC membranes obtained from these donors (Fig 1). A diffusely migrating band of glycophorin C with lower than normal molecular weight was seen in both homozygous Gerbich and homozygous Yus phenotypes. The observed decrease in molecular weight was more pronounced for the Gerbich phenotype. Normal as well as a variant glycophorin C species of a slightly lower molecular weight were detected in membranes of RBCs from a heterozygous Webb phenotype.

PCR analysis of mutant glycophorin C mRNA. To deduce the alterations in the glycophorin C gene responsible for the observed differences in the polypeptide in different variants, the entire coding region of glycophorin C mRNA was amplified using specific oligonucleotides. Amplification of reticulocyte RNA from an individual with normal glycophorin C generated a 395-bp DNA fragment that encompassed the entire coding domain (Fig 2). A band of identical size was also seen after amplification of RNA from a human erythroleukemia cell line (HEL) using glycophorin C-specific oligonucleotides. Amplification of reticulocyte RNA from Gerbich and Yus phenotypes generated shorter than normal DNA fragments for the coding domain, while a normal-sized DNA fragment was obtained following amplification of reticulocyte RNA from an individual heterozygous for the Webb phenotype (Fig 2). The appearance of unique, shortened DNA bands for the coding sequence in Gerbich and Yus phenotypes implies a major abnormality in the coding sequence. To precisely define these abnormalities, the amplified DNA fragments were sequenced.

Nucleotide sequence analysis of the shortened DNA band in the Yus phenotype showed the deletion of a 57-bp sequence in the coding region that corresponded precisely to exon 3 of the glycophorin C gene (Fig 3), resulting in the shortening of the polypeptide by 19 amino acids. Sequence analysis of the DNA fragment from the Gerbich phenotype showed the deletion of an 84-bp sequence that corresponds precisely to exon 3 of the glycophorin C gene (Fig 3), resulting in the shortening of the polypeptide by 28 amino acids.

The finding of only a normal-sized DNA fragment after reticulocyte RNA amplification in the heterozygous Webb phenotype suggested that, in contrast to the Gerbich and Yus phenotypes, the mutation in this phenotype may not involve a major deletion in the coding sequence. Earlier studies showed that the Webb glycophorin C polypeptide is abnormally glycosylated, suggesting a possible mutation around the consensus sequence for N-linked glycosylation at asparagine residue 8. Direct DNA sequence analysis of the uncloned PCR product showed heterogeneity in the mRNA sequence at codon 8: both AAC (asparagine) and AGC (serine) were detected in approximately equal amounts. The A → G mutation at nucleotide 23 results in loss of the single site of N-linked glycosylation in glyco-
phorin C (Fig 3). The decreased glycosylation, in turn, accounts for the lower molecular weight polypeptide seen in the immunochemical analysis. Subsequently, the entire coding region of the mutant Webb allele was subcloned into a plasmid vector and sequenced completely; no other amino acid changes were detected.

The predicted amino acid sequence of the extracellular and transmembrane domains of the three mutant glycophorin C polypeptides as derived from our sequencing of amplified cDNA is shown in Fig 3. The Yus phenotype has a deletion of 19 amino acids, encoded by exon 2, in the extracellular domain, while the Gerbich mutation has a larger deletion of 28 amino acids, encoded by exon 3, that extends into the predicted transmembrane domain. The Webb phenotype, on the other hand, is characterized by a substitution of serine for asparagine at residue no. 8, the single site of N-linked glycosylation in glycophorin C.

**DISCUSSION**

We have characterized three variants of erythroid glycophorin C by molecular cloning of mutant mRNA sequences using PCR techniques. Two of the mutant forms, the Gerbich and Yus phenotypes, involve deletion of one of the four exons in the glycophorin C mRNA. Our finding of deletion of exon 3 in the Gerbich phenotype confirms the earlier finding based on an analysis of genomic DNA. Moreover, the present study provides direct evidence for the earlier suggestion that the Yus phenotype is likely to involve deletion of exon 2. A genetic model for the generation of the Yus and Gerbich glycophorin C variants is illustrated in Fig 4. Unequal crossing over between the homologous 3.4-kb repeat sequences described earlier by Le Van Kim et al and Tanner et al can account for the two types of deletion within the glycophorin C gene. 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 phenotype). Alternatively, crossover 3' to misaligned exons 2 and 3 would yield a gene containing an exon 3 deletion (Gerbich phenotype). This model would also predict the existence of reciprocal recombination products, ie, glycophorin C genes containing duplications of exons 2 and 3.

![Fig 2](image-url)  
**Fig 2.** PCR analysis of glycophorin C mRNA from individuals with normal and abnormal glycophorin C proteins. (A) A model of glycophorin C mRNA with the coding region shaded and exons 1-4 indicated above. Arrows show location of 5' (sense strand) and 3' (antisense strand) oligonucleotides used to amplify a 395-bp band including the entire coding region and few flanking bases of untranslated sequence. (B) PCR products obtained following amplification of cDNA from individuals with the phenotypes indicated above each lane. Molecular weight standards are in the left lane (±x174 DNA cut with Hae III). Deduced structure of each glycophorin C mRNA is shown at the right.

![Fig 3](image-url)  
**Fig 3.** Predicted amino acid sequence of the extracellular and transmembrane domains of mutant glycophorin C polypeptides derived from nucleotide sequence analysis of the PCR-amplified cDNA. Mutants of the Yus phenotype have a deletion of 19 aa (encoded by exon 2) in the extracellular domain, while the Gerbich mutants have a larger deletion of 28 aa (encoded by exon 3) that extends into the predicted transmembrane domain. The Webb phenotype is characterized by a substitution of serine for asparagine at residue no. 8, the single site of N-linked glycosylation in glycophorin C.
Fig 4. Genetic model for generation of the Yus and Gerbich glycophorin C variants. Unequal crossover between the homologous 3.4-kb repeat sequences can produce two types of deletion within the glycophorin C gene. 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 phenotype). Alternatively, crossover 3' to misaligned exons 2 and 3 would yield a gene containing an exon 3 deletion (Gerbich phenotype). This model would also predict the existence of reciprocal recombination products, i.e., glycophorin C genes containing duplications of exons 2 and 3, respectively (not shown).

The mutation in the Webb glycophorin C variant is the result of a single nucleotide change that results in the substitution of serine for asparagine in the mutant polypeptide. The A → G mutation at nucleotide 23 was detected in the uncloned PCR product comprising both normal and Webb alleles, as well as in subcloned derivatives of the amplified DNA. The mutation thus represents the bona fide Webb sequence rather than a PCR artifact. Previous studies on the glycosylation state of mutant polypeptide in the Webb phenotype, moreover, have suggested that this is the likely site of the mutation.15 Our data provide direct support for this thesis.

It is interesting to note that in none of these three mutations is there an abnormality involving the cytoplasmic domain of glycophorin C that is involved in its interaction with protein 4.1. As such, these findings imply that the membrane properties of RBCs from these variants should be normal. This indeed appears to be the case, in that cell shape as well as membrane mechanical stability is normal for RBCs containing these variant forms of glycophorin C.26

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