Molecular Analysis of Glycophorin C Deficiency in Human Erythrocytes

By R. Winardi, M. Reid, J. Conboy, and N. Mohandas

Human erythrocyte glycophorin C plays a functionally important role in maintaining erythrocyte shape and regulating membrane mechanical stability. We report here the characterization of the glycophorins C and D deficiency in erythrocytes of the Leach phenotype. Glycophorin C gene is encoded by 4 exons. Amplification of reticulocyte cDNA from Leach phenotype and normal individuals generated a 140-bp fragment when using primers spanning exons 1 and 2. However, no polymerase chain reaction (PCR) products were detected in the Leach phenotype using primers flanking either exons 1 and 3 or exons 1 and 4, suggesting that the 3' end of the mRNA was missing or altered. Exon 4 also appeared to be missing from Leach genomic DNA, based on both Southern hybridization and PCR. These results indicate that an absence of glycophorin C and glycophorin D in erythrocytes from these Leach phenotype individuals is a consequence of a deletion or marked alteration of exon 3 and exon 4 of their glycophorin C gene. Surprisingly, the mutant gene encodes an mRNA stable enough to be detected in circulating reticulocytes. Although this mRNA could encode an N-terminal fragment of glycophorin C, these protein isoforms would not be expressed in the membrane because they lack the transmembrane and cytoplasmic domains.

Immunohistochemical analyses. The methods for preparation of erythrocyte membranes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and periodic acid Schiff's base (PAS) staining have been described previously. Immunoblotting was performed as described before, except 5% wt/vol low-fat milk in phosphate-buffered saline (PBS) at pH 7.4 was used as the blocking agent. Monoclonal antibodies (MoAbs) used in this report included NBTs/BRIC10 (BRIC10) and BRIC4, which recognize epitopes on the extracellular domain of the glycophorin C; BRIC100, which recognizes an epitope on the cytoplasmic domain of glycophorins C and D; and R1.3, which recognizes an epitope on glycophorins A and B. These antibodies were provided to us by Dr D.J. Anstee (South Western Regional Blood Transfusion Centre, Bristol, UK.).

Reverse transcription/polymerase chain reaction (RT/PCR) analysis. One to 2 μg of human reticulocyte total RNA was reverse transcribed with 200 U murine Moloney's leukemia virus reverse transcriptase (RTase) into cDNA at 37°C for 2 hours in a 50 μl reaction containing 40 mmol/L KCl, 50 mmol/L Tris-CI (pH 8.3), 0.8 mmol/L MgCl₂, 0.5 mmol/L dNTPs, 100 ng random hexamer (Boehringer Mannheim, Indianapolis, IN), and 1 U RNase inhibitor (Promega, Madison, WI). Ten microliters of this first-strand synthesis was used as a template for PCR amplification using 50 pmoles each of glycophorin C-specific primers (see below for nucleotide sequences) in 100 μl reaction containing 50 mmol/L KCl, 10 mmol/L Tris-CI (pH 8.3), 1.5 mmol/L MgCl₂, 0.1% gelatin, 0.2 mmol/L dNTPs, and 5 U Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). In similar experiments, 100 ng of genomic DNA was used as a template. Thirty cycles of amplification were performed using an automated Perkin Elmer-Cetus thermal cycler under the following conditions:

5 min at 94°C for denaturation,

30 sec at 94°C for denaturation,

1 min at 58°C for annealing,

1 min at 72°C for extension,

7 min at 72°C for final extension.

Experimental Procedures

Blood samples from two individuals of Leach phenotype (TL and JC) known to lack glycophorin C were collected and prepared as previously described. Informed consent was obtained from both subjects.

From the Lawrence Berkeley Laboratory, University of California, Berkeley, CA, and the New York Blood Center, New York, NY.

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Address reprint requests to N. Mohandas, DSc, Division of Cell and Molecular Biology, Lawrence Berkeley Laboratory, University of California, 1 Cyclotron Rd, MS 74-157, Berkeley, CA 94720.

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conditions: denaturation at 94°C for 30 seconds, reannealing at 60°C for 30 seconds, and extension at 72°C for 1 minute and 45 seconds. PCR products were analyzed by 5% PAGE and ethidium bromide staining. The glycophorin C-specific primers used and their position on the DNA sequence are as follows: p1, 5’CCCCCGGGTCTCCTCCGGGGCAGCG 3’, nt-34 to -8; p2, 5’TGCAATGGGTGTAGTATGCATTGT 3’, nt 106 to 82; p3, 5’CTGCAATGGACAAATGTCCATT 3’, nt 190 to 167; p4, 5’GAGAATTCAGGAATGTCCG 3’, nt 419 to 393; p5, 5’TTGCTGAGAGTGACATG 3’, nt 301 to 319; p6, 5’GCTGGGTACATTTCAATATA 3’, nt 916 to 895. The identity of PCR products was confirmed by hybridization as follows. After electrophoresis, amplified DNA was transferred to a nylon filter and subsequently subjected to Southern hybridization protocol.

Nucleotide sequence analysis. PCR-amplified cDNA fragments were subeloned using the TA cloning kit (Invitrogen, La Jolla, CA). Spun-column (Pharmacia, Piscataway, NJ) purified plasmid DNA from several independent clones was alkali-denatured and sequenced using Sequenase (US Biochemicals, Cleveland, OH).

Southern blot analysis. DNA samples were digested with restriction enzymes (Boehringer Mannheim). After phenol extraction and ethanol precipitation, 10 to 15 µg cleaved DNA samples were separated by electrophoresis in 1% agarose gel, vacuum-blotted onto Duralon-UV filter (Stratagene, La Jolla, CA), hybridized with radiolabeled cDNA probe, and autoradiographed.

The probes used for these analyses were as follows: probe “12,” p1-p2 PCR-amplified product recognizing exons 1 and 2 of the glycophorin C gene; probe “14,” p1-p4 fragment encoding the whole coding region; probe “4,” p5-p6 fragment containing exon 4 (including the 3’ untranslated region). A plasmid containing the translated region of normal glycophorin C cDNA was used as a template for amplification.

RESULTS

Immunoochemical characterization of Leach phenotype. The absence of normal glycophorins C and D in erythrocytes from TL and JC was confirmed by hemagglutination and immunoblotting. Hemagglutination was noted using BRIC4 and BRIC10 (anti-glycophorin C) with normal erythrocytes but not with erythrocytes from either TL or JC.

In addition, PAS staining indicated that membranes prepared from TL were deficient in glycophorin C (Fig 1). Western blotting of erythrocyte membranes using BRIC10 showed a band of molecular weight (Mr) 40,000 in normal membranes and no bands in TL or JC membranes (Fig 1). Western blotting with BRG1000 (anti-glycophorins C and D) showed bands of Mr 40,000 and 30,000 in normal membranes only (data not shown). The banding pattern obtained by Western blotting of membranes from TL and JC using R1.3 (anti-glycophorins A and B) was indistinguishable from that of the normal control (data not shown). These results indicate that erythrocyte membranes from TL and JC do not possess glycophorin C or glycophorin D, but do possess normal glycophorin A and glycophorin B.

RT/PCR analysis of glycophorin C mRNA and genomic DNA. To determine whether glycophorin C mRNA was expressed in Leach reticulocytes, cDNA was amplified with oligonucleotide primers specific for each exon (Fig 2B). Using primers that amplify the mRNA sequence derived from the first two exons, both normal and Leach (TL and JC) samples yielded a band of 140 bp (Fig 2C, lane a). However, although primers spanning exons 1 to 3 and 1 to 4 produced DNAs of expected sizes in normal control, no amplified products were detected in cDNAs from TL or JC (Fig 2C, lanes b and c).

These results suggest that Leach reticulocytes in these individuals express a stable, mutant glycophorin C mRNA that retains exons 1 and 2, but has lost exons 3 and 4. To confirm and explore this hypothesis further, additional control experiments were performed. First, the identity of the Leach PCR product spanning exons 1 and 2 was verified by hybridization to probe “12” derived from normal glycophorin C cDNA. Both normal and Leach 140-bp DNA fragments hybridized with equal intensity to this probe (Fig 2D, lane a). Normal PCR products spanning exons 1 to 3 or 1 to 4 also hybridized to this probe, but no hybridization signal was observed in the Leach samples (Fig 2D, lanes b and c). Fur-
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**A**

Glycophorin D
Glycophorin C

**B**

mRNA

probe

"12"

"14"

"4"

**C**

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**Fig 2.** PCR analysis of glycophorin C mRNA prepared from normal and Leach phenotype reticulocytes. (A) Distinct domains of glycophorin C and glycophorin D polypeptides (ec, extracellular; tm, transmembrane; cp, cytoplasmic). (B) A model of glycophorin C mRNA with its coding regions (exons 1 to 4) shaded. A downstream AUG that may be used in translation initiation of glycophorin D is indicated in parentheses. Arrows show location of sense (p1 and p5) and antisense (p2, p3, p4, and p6) primers used to amplify exons 1 to 2 (a), exons 1 to 3 (b), exons 1 to 4 (c), and the coding and non-coding regions of exon 4 (d). PCR fragments that were amplified from a subcloned glycophorin C cDNA for use as probes in hybridization experiments are also indicated correspondingly. (C) PCR products of normal (N) and Leach (L) cDNAs showing amplification of the 140-bp fragments (lane a) in both normal and Leach cDNAs. PCR products of 224 bp (lane b) and 453 bp (lane c) were only detected in normal cDNAs. Letters a through d correspond to the designation used in the PCR scheme above. (D) To confirm their identity, the amplified DNA was transferred to a nylon filter and hybridized with probe "12." (E) Amplification of normal (N) and Leach (L) cDNAs (lane d) and genomic DNAs (lane e) using primers within exon 4 showed a 600-bp fragment present only in normal cDNA and genomic DNA. Control PCR using band 4.1-specific primers showed that the genomic DNA from Leach phenotype is viable (lane e). The molecular weight marker is *Hae* III-digested φX174.
thermore, the Leach cDNA fragment was cloned and analyzed; its nucleotide sequence was identical to that of normal glycophorin C cDNA (data not shown).

In another experiment, amplification of exon 4-specific sequences in the 3' untranslated region yielded the expected 600-bp fragment from normal reticulocyte cDNA, but not from Leach cDNA (Fig 2E, lane d). To test whether this exon was present in the Leach glycophorin C gene, the same primers were used to amplify genomic DNA. Again, normal DNA yielded the expected 600-bp fragment, but Leach genomic DNA did not (Fig 2E, lane d'). As a positive control to check the quality of Leach genomic DNA, amplification with erythroid protein 4.1-specific primers gave identical results with both normal and Leach samples (Fig 2E, lane e).

Southern blot analysis. To validate the PCR results obtained using both cDNA and genomic DNA, Southern blot analysis was performed on genomic DNA (Fig 3). Genomic DNA digested with BamHI, HindIII, and Pst I endonucleases was probed with a cDNA fragment encompassing the whole coding region of glycophorin C gene (probe “14”). Normal DNA hybridized to bands representing the 5' end (3.8-kb and 6-kb BamHI fragments, 9-kb HindIII fragment, 1.6-kb and 1.2-kb Pst I fragments) as well as the 3' end (10-kb BamHI fragment, 2.7-kb HindIII fragment, 3.4-kb and 0.9-kb Pst I fragments) of the gene.3 DNA from JC exhibited the 5' bands, but not the 3' bands, indicating the presence of the 5', but not the 3', region of glycophorin C gene. A 6.7-kb HindIII fragment not present in normal samples was found in the genome of JC, which was previously shown to contain sequences from exon 2.12 Furthermore, reprobing with cDNA containing the coding as well as the noncoding regions of exon 4 (probe “4”) showed that normal Pst I-digested DNA, but not DNA from JC, hybridized to bands of 3.4 and 0.9 kb, suggesting that the whole exon 4 is missing from the Leach genome (data not shown).

DISCUSSION

We have shown that the glycophorin C gene in individuals with Leach phenotype erythrocytes possesses exon 1 and exon 2, but not normal exon 3 and exon 4. Similar conclusions have been reported previously after Southern blot analysis of genomic DNA from three Leach phenotype individuals.12,13 A unique feature of our studies is the finding that the mutant glycophorin C gene, apparently lacking its 3' terminal exon containing the normal polyadenylation signal, can still express a stable mRNA. This transcript must be able to exit the nucleus because it is detectable in an enucleated reticulocyte population from peripheral blood. The mutation breakpoint must have occurred on the 3' end of exon 2 in the glycophorin C genomic DNA. Therefore, we hypothesize that the Leach gene rearrangement has serendipitously juxtaposed a polyadenylation signal close to exon 2 (or the downstream intron), allowing normal mRNA 3' end processing to occur.

Our results indicate that an absence of glycophorins C and D in erythrocytes from these Leach phenotype individuals is a consequence of a deletion or marked alteration in the segment of glycophorin C gene encoding exons 3 and 4. Several other glycophorin C gene variants have been described showing that glycophorin C deficiency has a heterogeneous genetic basis. A nondeletional Leach phenotype involving a frameshift mutation at codons 44-45 that introduces a premature stop codon has been reported recently.13 Another variant known as the Webb phenotype involves a point mutation in exon 1 that results in altered glycosylation of glycophorin C.20 In addition, two other glycophorin C variants characterized by partial internal deletions have been analyzed: one, the Yus phenotype, lacks exon 2, whereas the other, the Gereich phenotype, lacks exon 3.14 Both exon-specific deletions are thought to arise from unequal crossing-over events between the homologous 3.4-kb repeat regions of the glycophorin C gene.4,21 The existence of these variant glycophorin C genes leads us to suggest that the glycophorin C deficiency in the Leach phenotype individuals tested is most likely due to deletion of the region surrounding exons 3 and 4 rather than to the rearrangement of these exons.

Glycophorin C, possibly through its interaction with protein 4.1, plays a functionally important role in regulating

**Fig 3.** Southern blot analysis of normal (N) and Leach (L) DNA digested with BamHI, HindIII, and Pst I followed by hybridization with probe “14.” The glycophorin C gene of Leach phenotype lacks restriction fragments corresponding to the 3' region of the normal gene.
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erythroid shape and membrane mechanical properties because erythrocytes lacking glycophorins C and D are elliptocytic and have markedly decreased membrane mechanical stability and deformability. Although quantitative and qualitative defects in protein 4.1, as well as defective spectrin oligomerization, are the two most common causes of elliptocytosis, these defects have not generally been reported in these glycophorin C- and D-deficient cells (with one exception in which a slight reduction in protein 4.1 content was observed).8,9,13 Further studies will be needed to distinguish whether the elliptocytosis observed in Leach phenotype is a direct manifestation of glycophorin C deficiency or a secondary effect due to abnormal assembly or retention of protein 4.1 in the erythrocyte membrane.

Glycophorin C, like all integral membrane proteins, has a hydrophobic peptide segment that spans the lipid bilayer. Because glycophorin C lacks a cleavable N-terminal signal sequence,21,22 the hydrophobic domain is thought to contain the information required for its insertion into the membrane and to stop its transfer through the membrane. If the truncated glycophorin C mRNA species in Leach phenotype individuals were translated, the protein(s) would lack both the transmembrane and the cytoplasmic domains of glycophorins C and D. It was originally suggested that such a gene product would not be inserted into the membrane or secreted from the cell.9 However, in view of the receptor-mediated translocation, the synthesized protein could potentially be transferred across, but not integrated into, the membrane because the membrane-associated domain is lacking. Translocation may be envisioned to occur through an aqueous, proteinaceous channel directed by internal uncleaved signal sequences.23 Alternatively, the defective gene product could remain in the cytoplasm and probably be degraded. Either scenario could account for the absence of glycophorin C and glycophorin D proteins from the erythrocyte membrane in the Leach phenotype individuals.

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REFERENCES

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