Molecular Basis for Elliptocytosis Associated With Glycophorin C and D Deficiency in the Leach Phenotype

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Glycophorin C (GPC) and glycophorin D (GPD) are highly glycosylated integral membrane proteins of human erythrocytes encoded by the same gene and associated with expression of Gerbich blood group system antigens. GPC/D deficiency (the Leach phenotype) is a rare condition usually associated with elliptocytosis. Characterization of the molecular basis of this phenotype in three previously uninvestigated families has shown that the most common genetic basis of GPC/D deficiency is deletion of exons 3 and 4 of the GPC gene. However, in one family, the Leach phenotype appeared due to a deletion of one nucleotide in exon 3, causing a frameshift mutation in the messenger RNA and premature generation of a stop codon. The GPC and GPD protein sequences are therefore interrupted in the extracellular domain and probably intracellularly degraded.

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Materials and Methods

Materials. Restriction enzymes, bacterial alkaline phosphatase, and pUC vectors were from Appligene (Strasbourg, France), T4 DNA ligase and polydeoxynucleotide kinase were from Biolabs (Northbrook, IL) and radionucleotide nucleotides were from Amersham (Bucks, UK). Thermus aquaticus polymerase (Taq polymerase) was from Perkin-Elmer (Norwalk, CT). Avian myeloblastosis virus (AMV) reverse transcriptase and the random priming labeling kit were from Boehringer (Mannheim, Germany). T7 sequencing kit was from Pharmacia (Uppsala, Sweden).

RNA and genomic DNA preparations. Blood samples from donors with the normal (Ge-positive) or the Leach phenotype (Cud, MWB, and LN) were drawn into anticoagulant. Peripheral blood mononuclear cells were isolated by Hypeaque-Ficoll density centrifugation and used to prepare total RNA and genomic DNA by the guanidine isothiocyanate lysis method, followed by cesium chloride gradient centrifugation. In the case of the MWB donor, an Epstein-Barr virus-transformed B-lymphocyte cell line was established and used for nucleic acid preparation.

Southern blot analysis. Human genomic DNA was digested with restriction enzymes (10 U/µg DNA), resolved by electrophoresis in 0.8% agarose gels, and transferred as described by Southern to nylon membrane using 0.4 mol/L NaOH as blotting buffer. The complete GPC cDNA (pGCF23), as well as a 600-bp fragment (GPC600) extending from the unique Psi I site in the exon 4 coding sequence down to the noncoding region of the GPC cDNA, were radiolabeled by random priming and used as probes. Hybridizations were performed according to manufacturer's recommendations and final washes were performed at 65°C in 0.1 x SSC, 0.1% sodium dodecyl sulfate (SDS) for 15 minutes.

Polymerase chain reaction (PCR) amplification of RNA and genomic sequences. Primers used for PCR amplification of the GPC messenger RNA (mRNA) coding sequence were specific for nucleotides 484 to 465 (YC59) and -120 to -101 (JCP36), respectively (+1 taken as the first nucleotide of the translation initiation codon). Oligonucleotides YC566 and YC567 (5'-AGGGCTCTGTGATGATGAGA-3' and 5'-TTGTCCTCTGTTCACAG-3', respectively) complementary to intronic sequences of the GPC gene were used to amplify the exon 3 region from genomic DNA. First cDNA strand synthesis and PCR conditions were as previously described. Amplification products were electrophoresed from agarose gel and subcloned into pUC18 vector. Plasmid DNA from several independent clones was sequenced on both strands by the dideoxynucleotide chain termination method using universal or specific internal primers and the T7 sequencing kit.

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RESULTS

Comparative Southern analysis of the GPC gene from donors with the normal and Leach phenotypes. DNA from the three unrelated donors (Cud, MWB, and LN) with the Leach phenotype was digested with the restriction enzymes Pst I, BamHI, and HindIII and compared with DNA from a normal (Ge-positive) individual by Southern blot analysis using the complete GPC cDNA as probe. As shown in Fig 1A, donors Cud and MWB carry an extensively rearranged GPC gene. From the known organization of the normal GPC gene, which encodes both GPC and GPD, the lack of the 10-kb BamHI fragment, of the 3- and 0.85-kb Pst I fragments, and of the 2.7- and 10-kb HindIII fragments (which are replaced by an abnormal 5-kb fragment) indicates the deletion of a large genomic region encompassing exons 3 and 4 of this gene, while the fragments carrying exons 1 and 2 (Pst I, 1.6 and 1 kb; BamHI, 5.2 and 3 kb; HindIII, 9 kb) are normally present. This finding was confirmed by hybridization with probe GPC600 containing only the 3' end of the GPC cDNA (Fig 1B), because the hybridization bands characteristic of exon 4 were present in the normal donor and LN but absent from Cud and MWB. Therefore, the deletion occurring in the GPC gene of donors Cud and MWB is very similar to that found in another unrelated Leach individual (PL) and previously described by Tanner et al. On the other hand, the Southern analysis shown in Fig 1A and B shows no difference between the restriction pattern of the GPC gene carried by the normal donor and the Leach variant LN. This finding prompted us to investigate further why the GPC and GPD polypeptides were not expressed on the red cell membranes of donor LN.

Identification of mutations in the GPC gene of LN. Because it has been previously shown that an identical form of GPC is expressed but differently regulated in erythroid and nonerythroid cells, total RNA from LN peripheral blood mononuclear cells (lymphocytes, monocytes, and reticulocytes) was prepared to amplify, by PCR, the coding sequence of the GPC mRNA between primers YCS9 and JPC36. As expected, PCR amplification performed with mRNA from the normal donor yielded two PCR products, which have been previously identified as the GPC mRNA (larger and more abundant band) and a splicing isoform (smaller and minor band) lacking the 57 nucleotide residues of exon 2. PCR amplification of the mRNA from the Leach donor LN also yielded two amplification products. The larger and predominant PCR products from each amplification were cloned into pUC18 and several clones of each were sequenced. Two changes were observed in the 720-bp PCR product from LN (Fig 2A). The first nucleotide change is a G→A transition 509 nucleotides from the start codon (Fig 2B). This change results in the creation of a new Pst I site. The second nucleotide change is a G→T transition 512 nucleotides from the start codon, which represents a silent mutation in exon 2. Since this mutation is located proximal to the putative transcription start site, it is likely to affect GPC gene expression. This finding is consistent with the absence of the GPC polypeptide on the red cell membranes of LN.

Fig 1. Southern blot analysis of DNA from individuals with a normal Gerbich phenotype and with the Leach phenotype. DNA was digested with restriction enzymes as indicated and hybridized with GPC cDNA probes. (A) Hybridization performed with the full-length probe pGCF23. (B) Hybridization with a 600-bp fragment (probe GPC600) specific for the 3' region of GPC cDNA. DNA from one individual with the Leach phenotype (LN) gave a normal pattern of restriction fragments, while DNA from the other two (Cud and MWB) did not contain restriction fragments corresponding to exons 3 and 4 of the GPC gene.
LEACH PHENOTYPE

independent comparison were sequenced on both strands. Sequence comparison showed four mutations within the GPC cDNA coding sequence of LN. A T → C change in codon 118 and a C rather than an A or G in codon 111 constitute silent polymorphisms, while a G → T substitution changes amino acid 44 from Trp to Leu. More important is the deletion of a C residue within codon 45 (Fig 2) that alters the reading frame of the LN GPC cDNA (+1 frameshift), resulting in a premature TAA stop codon (nucleotides 166 through 168) (Fig 3). To verify that this mutation was not due to a PCR artifact, the genomic region of LN encoding exon 3 of the GPC gene was also separately amplified, between primers YC566 and YC567, and sequenced. This experiment confirmed the deletion of a C residue in the LN GPC gene coding sequence.

DISCUSSION

The Leach or GPC/D-deficient phenotype has been reported to be associated with elliptocytosis, although all individuals with this phenotype have been identified due to the presence of anti-Gerbich antibodies in their sera rather than due to hematologic abnormalities. Some persons with this phenotype have, nevertheless, been reported to have mild anemia, presumably due to membrane instability, leading to mild hemolysis. Research to date has disagreed as to whether protein 4.1 is expressed normally in these GPC/D-deficient erythrocytes. Initial reports found normal expression of 4.1 protein in Leach erythrocytes. However, more recently, at least one family with the Leach phenotype (MWB) has been shown to have decreased protein 4.1 expression. In addition, such persons may be difficult or impossible to transfuse, as they can produce antibodies that react with red cells of all other phenotypes. Previous study of one individual (PL) with the Leach phenotype indicated that the phenotype was due to an apparent deletion of DNA containing exons 3 and 4 of the GPC gene, but whether this gene is transcribed was not determined. We have now studied three additional samples of the rare Leach phenotype. Although two of these (Cud and MWB) appear due to deletions in the same region as that previously described, a third case (LN) was associated with a grossly intact GPC gene. Furthermore, this gene appears to be functional, in that GPC mRNA is also present in these cells. Sequencing of the cDNA and corresponding genomic region from the Leach individual showed a consistent mutation represented by a single nucleotide deletion in codon 45, ultimately causing a stop codon before the domain encoding the transmembrane region of GPC. Thus, if this truncated form of GPC/D is translated, it would probably not be expressed at the cell surface, but should rather be secreted or degraded. Accordingly, the cells from LN are deficient for GPC, GDP, and Gerbich antigens, and they are elliptocytic presumably because of their decreased mechanical stability and deformability as a result of a defective interaction with the membrane skeleton protein 4.1.

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These data therefore indicate that, although Southern blotting analysis of DNA restriction fragments will likely prove helpful in identifying and confirming variant red cell antigen phenotypes, some phenotypes considered “null” from the viewpoint of antigen and protein may nevertheless not be “null” on the genetic level. It is known for instance that the Cromer-null phenotype (also called Inab phenotype for the protein decay accelerating factor). This study now shows that a grossly intact GPC gene may also be present in cells with the GPC/D “null” Leach phenotype. Thus, when evaluating potential causes of elliptocytosis, Southern blotting is not sufficient to determine whether GPC/D deficiency is responsible for the membrane defect. Interestingly, the deletion of a C residue within the LN GPC coding sequence abolishes the uniqueMspI restriction site normally present in exon 3. This observation, together with the possibility of amplifying genomic sequences from minute quantities of whole blood, could be useful for rapid preliminary screening of undiagnosed elliptocytosis.

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