

The Characterization of Gene Mutations for Human Glucose Phosphate Isomerase Deficiency Associated with Chronic Hemolytic Anemia

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Abstract

DNA was isolated from four unrelated glucose phosphate isomerase-deficient patients. Seven new mutations in the coding region were found: 247 C→T, 671 C→T, 818 G→A, 833 C→T, 1039 C→T, 1459 C→T, and 1483 G→A. Three patients were compound heterozygotes, and one patient was a homozygote of 247 C→T/247 C→T. Six mutations were found to involve highly conserved amino acids of glucose phosphate isomerase, suggesting that these residues are crucial for the maintenance of biological activity. Two polymorphic sites were also identified, 489 A→G and 1356 G→C, which do not produce a change in the amino acid sequence. (*J. Clin. Invest.* 1994, 94:2326–2329.) **Key words:** restriction digestion • base substitution • polymorphism • single strand conformation polymorphism • DNA sequencing

Introduction

Glucose phosphate isomerase (GPI, glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)¹ is an enzyme that catalyzes the interconversion of fructose-6-phosphate and glucose-6-phosphate (G6P). GPI plays an essential role in carbohydrate metabolism in all tissues. The substrates of this enzyme, fructose-6-phosphate and G6P, are intermediates in glycolysis and gluconeogenesis, as well as intermediates in the pentose phosphate cycle. In humans, GPI deficiency comprises the third most common hereditary enzyme defect of red cells after G6P dehydrogenase deficiency and pyruvate kinase deficiency and has the typical manifestation of nonspherocytic hemolytic anemia (1–9). It has been reported that severe GPI deficiency can be a cause of hydrops fetalis in humans (10). In addition, neurological impairment has been associated with this disease. GPI deficiency was first described by Baughman et al. (11) in 1967, and by 1990 over 40 unrelated affected families have been characterized by assay of enzyme activity (12). Approximately half of the affected individuals are thought to be homozygotes, while the other half appear to be compound heterozygotes (13).

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1. *Abbreviations used in this paper:* G6P, glucose-6-phosphate; GPI, glucose phosphate isomerase; SSCP, single strand conformation polymorphism.

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GPI may also have a function outside of carbohydrate metabolism. GPI is identical to neuroleukin (14–16), a protein produced by activated T cells that induces immunoglobulin secretion. GPI/neuroleukin, found in large amounts in muscle, brain, heart, and kidneys, has also been shown to support the survival of embryonic spinal neurons, skeletal motor neurons, and sensory neurons (17).

The gene encoding human GPI is located on the long arm of chromosome 19 (18). The 1.9-kb GPI cDNA predicts a protein of 558 residues. The sequence of the 5' noncoding region, exon 1, intron 1, and exon 2 of the GPI gene has been reported and data suggest that the gene is at least 50 kb in length (19). Recently, two patients with chronic hemolytic anemia caused by GPI deficiency were shown by reverse transcription PCR to exhibit point mutations (20). One patient (GPI-LM) was a compound heterozygote of two mutations (475 G→A/1040 G→A). The other patient (GPI-KS) was homozygous for a T→C transition at nucleotide (nt) 1574.

We have recently determined the gene structure and the sequences of the portions of introns flanking its exons (our unpublished data) and now describe seven new mutations causing hemolytic anemia and two polymorphisms in the coding region of the GPI gene.

Methods

Patients. Four cases of GPI deficiency were diagnosed in patients with chronic hemolytic anemia by assay of erythrocyte enzymes. The clinical data and enzyme activities are summarized in Table I. A complete panel of other red cell enzymes showed normal or increased activities. DNA from peripheral blood leukocytes was isolated by standard methods.

PCR and single strand conformation polymorphism (SSCP) analysis. Scanning for mutations by SSCP analysis was performed on DNA samples from GPI-deficient patients. Intron primer pairs sufficiently distant from the intron/exon borders to avoid the consensus sequence were designed and are listed in Table II. DNA was initially denatured at 98°C for 7 min. Each exon was then amplified by PCR using intron primers (25 ng), denatured DNA (200 ng), [α -³²P]dATP (1 μ Ci), 0.2 mM dNTPs, 1.65 mM MgCl₂, and 33.5 mM Tris/HCl, pH 8.8, in a 10- μ l reaction mix. 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s were performed in a Thermalcycler (Perkin-Elmer Corp., Norwalk, CT). The PCR product was mixed with 8 μ l of 95% formamide, 5 mmol/liter NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol. The samples were heated to 95°C for 8 min, cooled on ice, and 4 μ l applied on a 5% polyacrylamide gel containing 5% glycerol and 0.5 \times TBE. Electrophoresis was performed at 25 V/cm for 6 h at room temperature.

DNA sequencing and mutation analysis. The samples that demonstrated the presence of a possible mutation by SSCP were subjected to sequence analysis. The double stranded PCR products were purified on quick spin columns (QIAGEN Inc., Chatsworth, CA), and 10 ng of the purified DNA was sequenced by using a cycle sequencing kit (Stratagene, La Jolla, CA) and the original primer pairs. Cycle sequencing was performed using 30 cycles of 95°C for 30 s, 58°C for 30 s, and

Table I. Clinical Data and Characterization of Gene Mutations for Patients with Hemolytic Anemia Due to GPI Deficiency

Patient	Ethnic group	Age	Sex	Hb	Reticulocyte count	GPI activity	Mutation	Amino acid change
		yr		g	%			
1	White American	3/12	M	8.7	11.2	9.82	833 C → T/1459 C → T	278 Ser → Leu/487 Leu → Phe
2	African American	12	M	10.6	17.0	15.9	818 G → A/1039 C → T	273 Arg → His/347 Arg → Cys
3	African American	8	M	10.3	15.0	12.25	671 C → T/1483 G → A	224 Thr → Met/495 Glu → Lys
4	Native American	4	M	9.8	11.0	16.44	247 C → T/247 C → T	83 Arg → Trp/83 Arg → Trp
Normal values				14-17	1.6-2.7	38.8-82.8		

72°C for 1 min. Each mutation was verified by sequencing in both directions.

Mutations were also confirmed by endonuclease digestion when the mutation resulted in a change in a restriction site. After PCR, 10 µl of the purified DNA was digested with the appropriate enzyme (Table III) for 2 h and analyzed on a 10% polyacrylamide gel. To confirm the 1459 C→T mutation which neither creates nor destroys a restriction site, a mismatched sense primer, 5'CCAAGCTCACACCATTCAG3', was used in PCR reaction with the normal antisense primer, 5'ATGATGCCCTGAACGAAGATCT3'. PCR with this mismatched primer generates a new HindIII site with wild-type GPI as a template, but does not do so with the 1459 mutant.

The 818 G→A mutation was also confirmed by PCR with a sense primer containing the mutant residue at the 3' end, 5'CTAGTGGGTGGGAGGACA3', and the normal antisense primer, 5'CTGCAACAC-TGTCTCCAC3'. PCR with this primer pair would yield a product of the appropriate size only if the 818A mutation was present.

Results

Table I summarizes the seven new mutations found in four patients with GPI deficiency. All of these mutations, nt 247

Table II. Primer Sequences for Detecting Mutation in the Human GPI Gene

Mutation	Primer pairs	Primer sequences
247 C → T	E3L	5'GACACTTGGGGTGGCCAG3'
	E3R	5'TGCCGGCTAACAGTCAG3'
671 C → T	E7L	5'GTCACTGTCACCTGACCTGC3'
	E7R	5'GCTCTTCCCAGCCAGAG3'
818 G → A 833 C → T	E10L	5'GCCCCTGTGCAAGACCAG3'
	E10R	5'CTGCAACACTGTCTCCAC3'
1039 C → T	E12L	5'CAGAGGCGCGTGTGTTGGT3'
	E12R	5'AAAGAGCTCCTGGAGGAGCT3'
1459 C → T	E16L	5'CAGGACTCTCTGGAGACAT3'
	E16R	5'CAAGCCCATTCCAACCTAC3'
1483 G → A	E17L	5'GTAGGTTGGAATGGGCTTG3'
	E17R	5'CGTTGCTACTGAAATCCCTG3'

C→T, nt 671 C→T, nt 818 G→A, nt 833 C→T, nt 1039 C→T, nt 1459 C→T, and nt 1483 G→A, were found to be in the coding region. Three of the patients were compound heterozygotes. One of the patients who is of Native American descent is homozygous for C→T transition at nt 247. The amino acids corresponding to these seven mutant positions in five species are presented in Table IV. Six mutations occur in highly conserved amino acids.

Changes of amino acids may simply be due to polymorphisms present in the normal population. To eliminate this possibility, 28 normal individuals of European American origin

Table III. Verification of Mutations of the Human GPI Gene by Restriction Analysis

Mutation	Enzyme	Size (bp)	
		Normal	Mutant
247 C → T	AciI	10, 79, 107	79, 117
671 C → T	BsmAI	18, 96, 143	114, 143
818 G → A	HgaI	90, 111	201
833 C → T	MscI	201	79, 122
1039 C → T	AciI	29, 89, 117, 119	29, 89, 236
1459 C → T*	HindIII	18, 143	161
1483 G → A	Bsp1286 I	26, 32, 131	26, 163

* 1459 C → T mutation was confirmed by making a mismatched oligonucleotide to create a HindIII site in the wild-type GPI gene (see Methods).

Table IV. Comparison of Amino Acid Residues of GPI among Five Species

Organism	Position of human amino acid residue						
	83	224	273	278	347	487	495
<i>T. brucei</i>	Ser	Ser	Arg	Ser	Arg	Leu	Glu
Yeast	Ala	Thr	Arg	Ser	Arg	Leu	Glu
Mouse	Asn	Thr	Arg	Ser	Arg	Leu	Glu
Pig	Arg	Thr	Arg	Ser	Arg	Leu	Glu
Human	Arg	Thr	Arg	Ser	Arg	Leu	Glu
Mutation	Trp	Met	His	Leu	Cys	Phe	Lys

Table V. Polymorphism at nt 489 in Human GPI Gene and Gene Frequencies

Ethnic group	n	Genotype			Frequencies	
		489A/489A	489A/489G	489G/489G	A	G
American White	40	39	1	0	0.987	0.013
African American	39	18	21	0	0.731	0.269

Table VI. Polymorphism at nt 1356 in Human GPI Gene and Gene Frequencies

Ethnic group	n	Genotype			Frequencies	
		1356G/1356G	1356G/1356C	1356C/1356C	G	C
American White	37	37	0	0	1.000	0.000
African American	29	27	2	0	0.966	0.034

were examined for the presence of these 7 base substitutions. The base substitutions found in the GPI-deficient patients were not found in any of these DNA samples.

SSCP analysis and DNA sequencing identified two additional silent base substitutions in a patient with 818 G→A/1039 C→T. The two additional base substitutions, an A→G substitution at nt 489 and G→C substitution at nt 1356, resulted in no change in the deduced amino acid sequence. Both silent mutations were found to be polymorphic sites by performing population studies. The results demonstrated the frequency of the 489G allele is 0.269 in 78 alleles in the African American population and 0.013 in 80 alleles in the White American population (see Table V). The 1356C polymorphic site was not found in 74 alleles in the White American population and was found with a frequency of 0.034 in 58 alleles in the African American population (see Table VI).

Discussion

Seven new mutations were identified in the GPI gene. Six of the seven mutations were found to involve highly conserved amino acids. The high degree of conservation of these residues is remarkable since the overall levels of conservation between human and the other species are 57% identity to *Trypanosoma brucei*, 58% identity to yeast, 89% identity to mouse, and 93% identity to pig (14, 17, 21, 22). The conservation of these residues and the loss of activity associated with these mutations suggest that they are most likely involved in maintaining the activity of GPI. Interestingly, the C→T substitution at nt 1039 is adjacent to the known 1040A mutation which affects the identical amino acid, and results in a Arg→Cys at residue 347 rather than Arg→His by 1040A. This arginine is in close proximity to Asp 342, an important residue localized to the catalytic site in the pig protein (20).

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