Molecular Defect of a Phosphoglycerate Kinase Variant (PGK-Matsue) Associated With Hemolytic Anemia: Leu → Pro Substitution Caused by T/A → C/G Transition in Exon 3

By Masato Maeda and Akira Yoshida

We have identified the mutation in a phosphoglycerate kinase variant (PGK-Matsue) associated with severe enzyme deficiency, congenital nonspherocytic hemolytic anemia, and mental disorders. The mRNA coding for PGK was reverse transcribed and amplified by the polymerase chain reaction. Nucleotide sequencing of the variant cDNA showed a point mutation, a T/A → C/G transition in exon 3 of the variant gene. No other mutation was found in all coding regions of PGK-Matsue. The nucleotide change created an additional Ncol cleavage site in the variant gene; thus, the Ncol fragment

PHOSPHOGYLCERATE kinase (ATP: 3-phosphoglycerate 1-phosphotransferase, EC 2.7.3.3 [PGK]) plays a key role for ATP generation in the glycolytic pathway. The PGK, which exists universally in various tissues of various organisms, is encoded by a single structural gene on the X-chromosome q13 in humans. The complete amino acid sequence, cDNA sequence, and genomic organization have been elucidated. An inherited deficiency of this enzyme is associated with chronic nonspherocytic hemolytic anemia and mental disorders. Occasionally, the sole clinical manifestation has been muscle disease. The patient with PGK-Matsue also had severe chronic hemolytic anemia and mental disorders. The activities of the enzyme in red blood cells (RBCs), muscles, and fibroblasts of the patient were about 5% of those of normal tissues. However, the content of mRNA in the patient’s fibroblasts was comparable with that of normal fibroblasts. We determined the nucleotide sequence of the variant cDNA to elucidate the molecular abnormality of PGK-Matsue.

MATERIALS AND METHODS

Cell lines. The fibroblast cell line from the PGK-Matsue male subject (identification GM00743) was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). A normal fibroblast cell line of human newborn foreskin (9024) was obtained from the cytogenetics laboratory, City of Hope National Medical Center (Duarte, CA). The fibroblast cells were cultured in MEM medium supplemented with 20% (vol/vol) fetal calf serum (GIBCO, Grand Island, NY), 24 mmol/L sodium bicarbonate, penicillin (50 U/mL), and streptomycin (50 mg/mL) at 37°C in 5% CO2, 95% air.

Amplification of mRNA. About 5 × 10^6 fibroblast cells of passages 17 and 26 were harvested from GM00743 and 9024, respectively. Total cellular RNA samples were prepared from the cells by extraction with guanidine thiocyanate followed by density gradient centrifugation in cesium chloride. The entire coding sequences of mRNA were reverse transcribed and amplified in six overlapped segments, using six sets of sense (A-1, B-1, ... F-1) and antisense (A-2, B-2, ... F-2) oligonucleotide primers (Table 1). To create adequate restriction sites, linker sequences were attached to the 5’ end of these primers (Table 1).

The first-strand cDNA copies of mRNA were produced by incubating total RNA (8 to 10 μg) with one of the antisense primers (A-2, B-2, ... F-2; 10 pmol) and 20 U of reverse transcriptase from avian myeloblastosis virus (Life Science, St. Petersburg, FL) in the reaction mixture (30 μL) as described previously. The reaction products were amplified by the polymerase chain reaction (PCR) using a corresponding set of sense and antisense primers (50 pmol each) in the reaction mixture (100 μL) containing 20 mmol/L Tris-HCl (pH 8.3), 3.1 mmol/L MgCl2, 80 μmol/L dTTP, 58 mmol/L KCl, 0.01% gelatin, dNTP (200 μmol/L each), and 5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). The reaction was performed with a programmable thermal controller (MJ Research, Inc, Watertown, MA). The cycle, which consisted of 1 minute of denaturation at 94°C, 2 minutes of annealing at 50°C, and 3 minutes of elongation at 72°C, was repeated for 40 times and followed by another 12 minutes of elongation at 72°C.

To verify the amplification products, 1/20 of each reaction mixture was separated by agarose gel electrophoresis, transferred onto nitrocellulose filters, and hybridized with a 32P-labeled full-length human PGK cDNA (1.8 Kb BamHI/Xho I insert of PGK 82s clone) in 50% formamide (vol/vol), 5X Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100 μg/mL salmon sperm DNA at 42°C.

Subcloning and sequencing of amplified DNA. The amplified DNAs were digested with appropriate restriction enzymes, whose recognition sites were created by PCR, and purified by electrophoresis in polyacrylamide gels followed by electro-elution. The purified DNAs were subcloned into the M13 vector as described. DNA sequencing was performed by the dideoxy-nucleotide chain termination method of Sanger et al. To avert possible errors in the Taq polymerase reaction, DNA fragments isolated from at least two separate amplification reactions were sequenced in both orientations.

Restriction-fragment analysis. Genomic DNAs were prepared from GM00743 and 9024 fibroblast cells as described. The DNA samples (approximately 1 μg each) were amplified using a pair of primers, 5’-GCGAACAGTCGTGATGCCTAGGG-3’ and 5’-CGCCA- CAAAGTCTACCAAGC-3’, by PCR. The primers used corre-
Table 1. Sequences of Synthetic Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (*)</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>(5'GCGagTCGTGACCGAATCACCGA-3' (-27)</td>
<td>SstI</td>
</tr>
<tr>
<td>A-2</td>
<td>(5'AAcagCACAAACCTCTTGCCCGAGC-3' (262)</td>
<td>PstI</td>
</tr>
<tr>
<td>B-1</td>
<td>(5'AGggaTTCTTAGAGCGATGCTGT-3' (248)</td>
<td>BamHI</td>
</tr>
<tr>
<td>B-2</td>
<td>(5'CCAGaaTCTCTGTCGAGATGTACTCC-3' (532)</td>
<td>EcoRI</td>
</tr>
<tr>
<td>C-1</td>
<td>(5'GGAAGCCTTTCCAGCCAAATCGG-3' (347)</td>
<td>HindIII</td>
</tr>
<tr>
<td>C-2</td>
<td>(5'CCATGAGAATCGGCTATAAGCC-3' (752)</td>
<td>PstI</td>
</tr>
<tr>
<td>D-1</td>
<td>(5'AGCTGGAGACAGAGATCCAGATCC-3' (671)</td>
<td>PstI</td>
</tr>
<tr>
<td>D-2</td>
<td>(5'ATAGCTGCTGTGCTGTGCT-3' (976)</td>
<td>HindIII</td>
</tr>
<tr>
<td>E-1</td>
<td>(5'AGCTGAGAGAAATGCTGCTG-3' (1188)</td>
<td>SstI</td>
</tr>
<tr>
<td>E-2</td>
<td>(5'AGTGGTTACAGCTGGCACTGTTG-3' (1350)</td>
<td>XbaI</td>
</tr>
<tr>
<td>F-1</td>
<td>(5'GCAaagCTTTGCCCCACTAGCTGAATC-3' (1394)</td>
<td>HindIII</td>
</tr>
<tr>
<td>F-2</td>
<td>(5'GCCAGCAAGAATGCTGCTG-3' (1394)</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

Oligonucleotides A-1, B-1, . . . F-1 correspond to the sense strand of DNA. Oligonucleotides A-2, B-2, . . . F-2 correspond to the antisense strand of DNA. Lowercase letters were altered to create corresponding restriction sites (underlined).

*Counting from the adenine residue of the initiation codon.

spond to a 5' part of exon 3 (nucleotides 163 → 183, counting from the adenine residue of the initiation codon of cDNA) and a 5' part of intron 3 (nucleotides 18 → 38, counting from the intron-exon junction). Therefore, the amplified product of 148 bp should contain the mutation site.

Half of the amplified products produced from the control and variant DNAs were digested by NciI (10 U, Stratagene, La Jolla, CA). The digested and undigested samples were electrophoresed on 4% agarose gel in the Tris-Acetate-EDTA buffer system, blotted onto nylon membranes, and hybridized with a PGK cDNA probe (5P-labeled cDNA fragment produced by oligonucleotide primers B-1 and B-2).

RESULTS

Amplification of total cellular RNA by PCR using six sets of sense and antisense oligonucleotide primers (Table 1) produced six overlapping cDNA fragments, which were hybridizable with the PGK cDNA probe and covered entire coding sequences.

Fig 1. Partial nucleotide sequence of normal and variant PGK-Matsue. The coding sequence is shown aligned with the sequence ladders. Asterisks indicate the substitution site of the PGK-Matsue gene. The encoded amino acid residues are shown. Numerals correspond to the residue number, counted from the NH2-terminal of the enzyme protein.

Nucleotide sequence analysis of these amplification products originating from PGK-Matsue RNA showed only one nucleotide difference between the variant PGK and the reported sequence of normal PGK.4 A part of the nucleotide sequence ladders of PGK-Matsue and the normal PGK is shown in Fig 1. A thymidine residue at position 266 (counting from the adenine residue of the initiation codon of mRNA) in the normal PGK was found to be substituted by a cytidine residue in PGK-Matsue. Based on the information of the genomic structure of the PGK locus,5 the mutation site is located in exon 3. The nucleotide substitution is expected to generate a new NciI (and isoschizomeric AhaI, BclI, HpaII, Msp I) cleavage site, CCGGG, in the PGK-Matsue gene. The 148-bp mutation region originating from the variant gene was in fact cleaved by NciI, but the corresponding region originating from the normal gene was not cleaved (Fig 2).

The transition T/A → C/G in the gene should result in
Fig 2. Southern blot hybridization of PCR products of the normal and PGK-Matsue DNAs. The 148-bp genomic region, which includes the mutation site, was amplified by the use of a pair of 5' and 3' primers. Half of the amplified product was digested by NcoI. Undigested and digested PCR products were subjected to agarose gel electrophoresis, and Southern blot hybridization using cDNA as a hybridization probe. (A) Undigested PCR product. Lane 1, product from normal DNA; lane 2, product from PGK-Matsue DNA. (B) PCR product digested by NcoI. Lane 1, normal DNA; lane 2, PGK-Matsue DNA. The left-hand column indicates the position of size markers. Note that a small fragment (44 bp), generated from PGK-Matsue DNA by NcoI digestion, cannot be hybridized with the cDNA probe because the fragment consists mostly of an intron sequence and contains a coding sequence of only 6 bp.

Discussion

Chronic hemolytic anemia associated with RBC PGK deficiency was first observed by Kraus et al. in a female patient. Subsequently, in their study of an unrelated large family, Valentine et al. found that the enzyme deficiency and clinical symptoms were more severe in male than female members, and suggested the X-chromosome linked inheritance of PGK deficiency. In addition to hemolytic anemia, behavioral and neurologic abnormalities were observed in certain affected members of this family. At this time, 14 rare PGK variants associated with enzyme deficiency have been found in unrelated families (Table 2). The RBC enzyme activity in affected males ranged from 3% to 20% of the normal level. The deficiency was observed also in nucleated tissues, such as the leukocytes and muscles of the subjects. Except for PGK-München, which exhibits relatively mild deficiency (about 20% of the normal level), all deficient variants are associated with chronic nonspherocytic hemolytic anemia, mental disorders, or rhabdomyolysis.

In addition to these rare deficient variants, several electrophoretic variants with normal enzyme activity have been found in certain populations.

The molecular abnormalities of three deficient variants, i.e., PGK-München, PGK-Uppsala, and PGK-Tokyo, and an electrophoretic variant, PGK-II, which is common among Southern Pacific populations, were previously elucidated by the peptide mapping analysis (Table 2). The impact of these structural abnormalities on PGK function has been predicted based on the three-dimensional structure of horse PGK, which is highly analogous (about 97% sequence identity) to the human enzyme.

Table 2. PGK Variants Associated With Enzyme Deficiency

<table>
<thead>
<tr>
<th>Investigator/Reference</th>
<th>Name of Variant</th>
<th>Sex</th>
<th>Activity in RBC ( % of normal)</th>
<th>Electrophoretic Mobility</th>
<th>Heat Stability</th>
<th>Hemolytic Anemia</th>
<th>Neurologic Abnormalities</th>
<th>Muscle Disease</th>
<th>Amino Acid Substitution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kraus et al.¹⁵</td>
<td>F</td>
<td>75</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Valentine et al.⁵</td>
<td>F, M</td>
<td>5 in M</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hjelm et al.¹⁸,²⁷,²⁸</td>
<td>Uppsala</td>
<td>M</td>
<td>10</td>
<td>Faster</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Arg → Pro at 205</td>
</tr>
<tr>
<td>Arse et al.³⁹</td>
<td>F</td>
<td>42</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cartier et al.⁴⁰</td>
<td>M</td>
<td>20</td>
<td>ND</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Miwa et al.³⁹,⁴⁰</td>
<td>Matsue</td>
<td>M</td>
<td>5</td>
<td>Slower</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Leu → Pro at 88</td>
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<tr>
<td>Konrad et al.³¹</td>
<td>M</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Akatsuka et al.²⁶,³²</td>
<td>Tokyo</td>
<td>M</td>
<td>15</td>
<td>Slower</td>
<td>Low</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Val → Met at 265</td>
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<tr>
<td>Boivin et al.³³</td>
<td>M</td>
<td>10</td>
<td>Normal</td>
<td>ND</td>
<td>+</td>
<td>±</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Krietsch et al.²⁷,³¹</td>
<td>München</td>
<td>M</td>
<td>21</td>
<td>Slower</td>
<td>Low</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Asp → Asn at 267</td>
</tr>
<tr>
<td>Rosa et al.³⁷</td>
<td>M</td>
<td>3</td>
<td>Slower</td>
<td>Low</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DiMauro et al.³⁵</td>
<td>M</td>
<td>17</td>
<td>Slower</td>
<td>Low</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sugie et al.³⁶</td>
<td>M</td>
<td>8.2</td>
<td>ND</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In addition to the 14 deficient variants listed above, several electrophoretic variants with normal activity were reported. Abbreviations: F, female; M, male; ND, not described.

*The position of amino acid substitution is counted from the NH₂-terminal Ser.
PGK-Matsue is associated with a very severe enzyme deficiency (about 5% of the normal level) in RBCs as well as in nucleated cells. The patient suffered from chronic hemolytic anemia and mental disorders, and died at age 9 from complications of pneumonia. The content of mRNA in the patient’s fibroblasts, estimated by the conventional Northern blot hybridization and by the more accurate quantitative liquid hybridization, was comparable with that of normal fibroblasts. The specific enzyme activity of PGK-Matsue was estimated to be about 35% to 40% of that of normal PGK from the quantitative immunoneutralization test. Because the PGK activity in the patient’s RBCs is only 5% of that of normal cells, the molecular concentration of PGK-Matsue in the variant cells should be about 10% to 15% of that of normal cells. PGK-Matsue exhibited higher Km values for all substrates, particularly for ATP and 1,3-diphosphoglycerates.

In the present study, a single T/A → C/G base transition and a consequent amino acid substitution Leu → Pro at position 88 from the NH2-terminal were found in PGK-Matsue. The substitution position is in a random coil between α-helices II (residues 77 through 87) and a β-strand C (residues 91 through 94) of PGK molecule. Because the pyrrolidine ring of the Pro residue causes a substantial change in backbone torsional angle in a peptide chain, structural instability and functional retardation are expected to be induced in PGK-Matsue by the mutation. Indeed, all human hemoglobin and other protein variants with the Leu → Pro substitution are unstable. The major cause of a diminished concentration (10% to 15% of the normal level) of PGK-Matsue in RBCs and other tissues is most likely a more rapid denaturation and degradation of the variant enzyme originating from the Leu → Pro substitution.

REFERENCES

27. Hjelm M, Wadam B: Nonspherocytic hemolytic anemia with phosphoglycerate kinase deficiency. XIIIth International Congress of Hematology, Munich, Germany, 1970, p 121
Molecular defect of a phosphoglycerate kinase variant (PGK-Matsue) associated with hemolytic anemia: Leu----Pro substitution caused by T/A- ---C/G transition in exon 3

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