Molecular Abnormalities of a Phosphoglycerate Kinase Variant Generated by Spontaneous Mutation

By Masato Maeda, Erawati V. Bawle, Roshni Kulikarni, Ernest Beutler, and Akira Yoshida

A new case of X chromosome-linked phosphoglycerate kinase (PGK) abnormality is described. The male proband was mentally retarded, had behavior disorders, and displayed episodes of hemolytic anemia. The enzyme activity of red blood cells from the patient was about 10% of normal, and that of the cultured fibroblasts was about 50% of normal cells. The variant PGK was characterized by a lower affinity for the substrates, reduced thermostability, and increased anodal electrophoretic mobility. The pH activity profile of the variant enzyme was different from that of normal. The amount of messenger RNA (mRNA) in the variant fibroblasts was comparable to that of normal fibroblasts. The mRNA coding for PGK was subjected to coupled reverse transcriptase followed by amplification by the polymerase chain reaction. Nucleotide sequence of the variant cDNA showed a point mutation, T/A → C/G transition, in exon 9 of the variant gene. No other mutation was found in all coding regions of the variant. The mutation should cause Cys → Arg substitution at the 315th position from the NH₂-terminal Ser of PGK, and it created an additional Ava II (or isoschimatic) cleavage site in the variant gene. Because the variant gene was not detected in the proband's mother and siblings, it must have been generated by spontaneous mutation during oogenesis.

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From the Department of Biochemical Genetics, Beckman Research Institute of the City of Hope, Duarte, CA; Division of Genetics, the Department of Pediatrics, Children’s Hospital of Michigan, Detroit, MI; the Department of Pediatrics and Human Development, Michigan State University, East Lansing, MI; and the Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA. Submitted October 2, 1991; accepted January 20, 1992. Supported by US Public Health Service Grants No. HL29515 and HL25552 and the Sam Stein and Rose Stein Charitable Trust Fund. Address reprint requests to Akira Yoshida, PhD, Department of Biochemical Genetics, Beckman Research Institute of the City of Hope, 1450 E Duarte Rd, Duarte, CA 91010.

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mRNA are 5'-TCCTTAGACGGAGGCTGTG-3' and 5'-TTCTTGTTGCACTGCAGATC-A-3', which produce 338-bp fragments corresponding to PGK mRNA (nt 229 to nt 551). One-fifth of the PCR products was electrophoresed on a 1.5% agarose gel and transferred onto nitrocellulose filters. The filters were hybridized with the full-length human PGK cDNA clone 825,14 deprobed, and subsequently rehybridized with the full-length human G6PD cDNA clone RXG-5 cloned in this laboratory.

**Determination of cDNA sequence.** Detailed procedures for determination of cDNA produced from the variant mRNA were previously described.7 Briefly, the entire coding sequences of mRNA were reverse transcribed and amplified in six overlapped segments, using six sets of sense and antisense primers as previously described. The amplified DNAs were digested with appropriate restriction enzymes and subcloned into MI3 vector. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al.15

To avert possible errors in the PCR, DNA fragments obtained from at least two separate reactions were sequenced in both orientations.

**Restriction fragment analysis of amplified genomic DNA.** Genomic DNAs were prepared from fibroblasts and blood using the standard method.12 The DNA samples were amplified using a pair of primers, 5'-GTCTTGGTGGAGGTGGTG-3' and 5'-CTTGCTGTTTACGACAC-3', by PCR. The primers used correspond to a 3' part of intron 8 (nt -70 to -51, from the intron-exon junction) and 5' part of exon 9 (nt 942 to 966, counting from the adenine of the initiator codon). Therefore, the amplified product of 100 bp should contain the mutation site. Half of the amplified products from the normal and variant DNAs were digested by Ava II (NEB, Beverly, MA). The digested and undigested samples were electrophoresed on 4% agarose gel in a Tris-acetate buffer system and stained by ethidium bromide.

**RESULTS**

**Enzymatic studies.** PGK activity of the patient was severely diminished; that is, about 10% of normal in red blood cells (RBC). However, PGK activity of variant fibroblasts was about 50% of that of normal cells referring to both extractable proteins and G6PD activity (Table 1). The anodal electrophoretic mobility of the variant’s PGK was slower than that of the normal enzyme (Fig 1). The variant enzyme was more labile than normal enzyme; ie, about 40% inactivation occurred within 30 minutes at 45°C and at pH 7.0, whereas the normal enzyme was not inactivated. The effect of pH on the variant enzyme activity was different from that on the normal enzyme, ie, an optimal pH of the variant enzyme shifted towards the acidic side (Fig 2). In comparison with the normal enzyme, the variant enzyme exhibited lower affinity to both ATP and 3-phosphoglycerate (Table 1).

In contrast to the partial enzyme deficiency commonly observed in heterozygous females, the PGK activity of the patient’s mother was normal, ie, about 290 U/gHb in both reticulocyte-rich (5.1%) younger RBC fractions and aged RBC fractions.

**Quantification of PGK mRNA.** Two major DNA fragments, a 165-bp fragment hybridizable with the G6PD cDNA probe, and a 338-bp fragment hybridizable with the PGK cDNA probe, were produced from the proband’s cellular RNA and the control cellular RNA by PCR (Fig 3). In comparison with the amount of G6PD fragment, which served as an internal reference, the amount of PGK fragment was roughly the same in the variant and the control products. The result implied that the PGK mRNA level was not diminished in the variant fibroblasts.

![Fig 1. Starch gel electrophoresis patterns. 1, variant PGK; 2, normal PGK. Electrophoresis was performed in 13% starch gel at pH 7.5. Arrow indicates the sample origin.](image)

![Fig 2. Effect of pH on enzyme activity. Buffer solutions used were acetate buffer (pH 4 to 6), imidazole-chloride (pH 6 to 7), Tris-chloride buffer (pH 7 to 9), and carbonate/bicarbonate buffer (pH 9 to 10.4).](image)

### Table 1. Characteristics of PGK of the Variant and Control Cells.

<table>
<thead>
<tr>
<th>Origin of PGK</th>
<th>U/mg Protein</th>
<th>Relative to G6PD</th>
<th>Michaelis Constant (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>PGK Michigan</td>
<td>1.9</td>
<td>5.8</td>
<td>230</td>
</tr>
<tr>
<td>9024 (normal)</td>
<td>4.1</td>
<td>11.3</td>
<td>170</td>
</tr>
</tbody>
</table>

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Fig 3. Southern blot hybridization of PCR amplification products with specific cDNA probes. The RNA preparations were amplified using the two sets of primers (see Materials and Methods). The products were separated by agarose gel electrophoresis and transferred onto a filter. The filter was hybridized, deprobed, and rehybridized successively with (A) PGK cDNA probe and (B) G6PD cDNA probe. Lane 1, product from normal cellular RNA; lane 2, product from variant cellular RNA.

DNA sequencing of amplified DNAs. Amplification of total cellular RNA by PCR using six sets of sense and antisense oligonucleotide primers produced six overlapping cDNA fragments that were hybridizable with the PGK cDNA probe and covered entire coding sequences. Nucleotide sequence analysis of the amplification products originating from the variant mRNA showed only one nucleotide difference in comparison with the reported sequence of normal PGK. A part of the nucleotide sequence ladders of the variant and normal PGK is shown in Fig 4. A thymine residue at position 946 (counting from the adenine residue of the initiation codon of mRNA) was found to be substituted by a cytosine residue in the variant.

Fig 4. Partial nucleotide sequence of the normal and variant PGK. The coding sequence is shown aligned with the sequence ladders. The encoded amino acid residues are shown. Numerals correspond to residue number, counting from the NH₂-terminal Ser of the enzyme protein. *Substitution site of the variant gene.

Restriction fragment analysis of amplified genomic DNA. Based on the known genomic structure of the PGK locus, the mutation site is located in exon 9. The nucleotide substitution T/A → C/G is expected to generate a new Ava II and Asu I (and isochromatic Cfr13I and Sau96I) cleavage site. The 100-bp mutation region originating from the proband’s genomic DNA was in fact cleaved by Ava II, but the corresponding region from the normal gene was not cleaved (Fig 5).

The PCR product of the targeted 100-bp region produced from his mother’s genomic DNA and his siblings’ genomic DNA were not cleaved by the restriction enzyme, indicating that they do not have the variant gene (Fig 5).

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 observed behavioral and neurologic abnormalities, in addition to hemolytic anemia, in certain affected members. By now, 14 rare PGK variants associated with enzyme deficiency have been found in unrelated families. The RBC PGK activity in affected males ranged from 3% to 20% of the normal level. The deficiency was observed also in nucleated tissues. Except for PGK München, which exhibits relatively mild deficiency, all deficient variants are associated with chronic nonspherocytic hemolytic anemia, mental disorders, or rhabdomyolysis. The molecular abnormalities of four deficient variants have been elucidated, and all of these variants were found to be associated with single amino acid substitutions caused by point mutations.

The present subject has RBC enzyme deficiency (about 10% of normal) and suffered from chronic nonspherocytic hemolytic anemia and mental disorders. The content of mRNA in the proband’s fibroblasts was found to be
substitution caused molecular instability in vitro and presumably also in vivo, and thus the enzyme deficiency is more severely manifested in RBCs than in fibroblasts. A single T/A → C/G base transition in PGK exon 9 was found in his gene. The mutation should induce an amino acid substitution Cys → Arg at position 315 from the NH₂-terminal of the PGK molecule. The substitution position is in α-helices X (residues 315 through 325) of the PGK molecule.¹⁸ The substitution caused molecular instability in vitro and presumably also in vivo, and thus the enzyme deficiency is more severe than in fibroblasts. The mother and siblings do not have the variant gene associated with phosphoglycerate kinase deficiency in erythrocytes. The RBC PGK activity of the proband’s mother was normal. Moreover, the mother and siblings do not have the variant gene (Fig 5). Because the mutation is not present in the genomic DNA of somatic cells from the mother, the variant gene must have been generated by spontaneous mutation during oogenesis. Because the variant was found in Michigan, it is designated PGK Michigan.

REFERENCES

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