Molecular Abnormalities of a Phosphoglycerate Kinase Variant Generated by Spontaneous Mutation

By Masato Maeda, Erawati V. Bawle, Roshni Kulkarni, 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 transcription 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 isoschismic) 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.

PHOSPHOGLYCERATE 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 in various tissues, is encoded by a single structural gene on X-chromosome q13. The complete amino acid sequence, cDNA sequence, and genomic structure of the ubiquitous PGK have been elucidated.4-6 Thus far, 14 rare variants of the X-chromosome linked PGK with enzyme deficiency have been found in unrelated families. Most deficient subjects exhibit chronic nonspherocytic hemolytic anemia and mild to severe neurologic disease. Occasionally, the sole clinical manifestation has been metabolic muscle disease. We report here enzymatic properties and genomic abnormalities of a variant PGK that had been generated by spontaneous mutation.

MATERIALS AND METHODS

Clinical history. J.P. was born in 1976 as the first child of an 18-year-old white mother. Language development was delayed, the child was not enrolled in kindergarten until he was 6 years old, and by the time he was 7 he was placed in special education classes because of inability to keep up with academic work.

He had been placed on Ritalin (Ciba Pharm Co, Summit, NJ) because of a short attention span. He became aggressive toward schoolmates and destroyed objects around him when he was angry. At age 10 his IQ was in the 70 to 79 range. By the time he was 14 he was being counseled by a psychiatrist twice a month and was functioning at about the 5th to 6th grade level.

At 8 or 9 years of age he began to complain of dizziness and was discovered to be anemic with hemoglobin of 7.5 g/dL, and a mean corpuscular volume (MCV) of 105.4 fl, but a month later his hemoglobin had increased spontaneously to 13 g/dL and the MCV to 106.4 fl. The reticulocyte count was 1.6%. No splenomegaly could be detected on physical examination.

At age 14 the facial appearance was normal. Ophthalmologic and neurologic examinations were normal except for a rather clumsy gait. Sexual development was normal. A brain magnetic resonance imaging was normal and an electromyelogram showed paroxysmal bursts of generalized spike and wave activity. His hemoglobin was 12 g/dL, the reticulocyte count 5%. No splenomegaly could be detected on physical examination.

Cell lines. The fibroblast cell line was prepared from the foreskin obtained from the cytogenetics laboratory, City of Hope Medical Center (Duarte, CA). The fibroblast cells were cultured in Eagle’s minimum essential medium supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY), 24 mmol/L sodium bicarbonate, penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37°C in 5% CO₂/95% air.

Characterization of enzyme properties. Harvested cells were extracted with about 3 vol of 10 mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L 2-mercaptoethanol, 1 mmol/L EDTA, and 5 mmol/L NADP, by freezing/thawing five times and then centrifuging. PGK and glucose-6-phosphate dehydrogenase (G6PD) activities of the supernatants were assayed by the methods described,8,9 and enzymatic properties were determined as previously described.9 Protein concentration was assayed by the method of Lowry et al.10 Starch gel electrophoresis was performed using a Tris-citrate buffer system at pH 7.5, and stained for enzyme activity as previously described.11

Quantification of messenger RNA (mRNA). Cellular RNA was extracted from the cultured cells by extraction with guanidine thiocyanate, and density gradient centrifugation in a cesium chloride solution.12 Approximately 1 μg of total cellular RNA was subjected to a coupled reverse transcription and polymerase chain reaction (PCR)13 using two sets of primers, ie, one specific for PGK and the other specific for human G6PD that serves as an internal reference. The primers for G6PD mRNA are 5'-AGGTCGAGCGACTTCC-3' and 5'-TCTCAGGTGACCCGATGGC-3', which produce 165-bp fragments corresponding to the 5'-region of G6PD mRNA. The primers for PGK were

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.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.
mRNA are 5'-TCCTTAGAGCCAGTTGCTGT-3' and 5'-TTCTGTGTGGCCAGATTGACTCC-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. 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 M13 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'-CTTGCTGTTTCCGACCAC-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 I (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/g Hb 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.

![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.](image1)

**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.

![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).](image2)

**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). (—•—) Normal PGK; (—○—) variant PGK.

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

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

**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...
comparable with that of normal fibroblasts. A single T/A \(\rightarrow\) C/G base transition in PGK exon 9 was found in his gene. The mutation should induce an amino acid substitution Cys \(\rightarrow\) Arg at position 315 from the NH\(_2\) terminal of the PGK molecule. The substitution position is in \(\alpha\)-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 severely manifested in RBCs than in fibroblasts. 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

Molecular abnormalities of a phosphoglycerate kinase variant generated by spontaneous mutation

M Maeda, EV Bawle, R Kulkarni, E Beutler and A Yoshida