Identification of New Mutations in Two Phosphoglycerate Kinase (PGK) Variants Expressing Different Clinical Syndromes: PGK Créteil and PGK Amiens

By Michel Cohen-Solal, Colette Valentin, François Plassa, Gilles Guillemin, François Danze, Françoise Jaisson, and Raymonde Rosa

Phosphoglycerate kinase (PGK) deficiency is generally associated with chronic hemolytic anemia, although it can be accompanied by either mental retardation or muscular disease. Genomic DNAs of two PGK-deficient patients previously described in France were sequenced directly after polymerase chain reaction amplification. The PGK Créteil variant arises from a G → A nucleotide interchange at position 1022 in cDNA (exon 9), resulting in amino acid substitution 314 Asp → Asn in the C-terminal domain, which contains the nucleotide binding site. It is associated with rhombomycosis crises but not with hemolysis or mental retardation. In the other case, which is associated with chronic hemolytic anemia and mental retardation (PGK Amiens), an A → T nucleotide interchange was found at position 571 in cDNA (exon 8), this leads to amino acid substitution 163 Asp → Val in the N-terminal domain, which contains the catalytic site for phosphoglycerate binding. These results corroborate the kinetic data observed. In the two cases, the mutations are distinct from others previously reported and no significant relationship could be observed between the location of the amino acid substitution and its clinical consequences.

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PHOSPHOGLYCERATE KINASE (adenosine triphosphate [ATP] 3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3: PGK) is a glycolytic enzyme catalyzing the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate that plays a key role in ATP generation during glycolysis. Erythrocyte PGK is a monomeric enzyme that is expressed in all tissues. It is encoded by a single structural gene located on chromosome X in humans, although a second gene (PGK2), which is expressed only in testicular tissue, is located on chromosome 19, whereas several pseudogenes are present on chromosomes X and 6.

The complete amino acid and genomic sequences of human PGK are known. The tertiary structure of PGK has been extensively studied in the past at the protein level, using horse-muscle enzyme as a model. Although the same study was not performed on the human enzyme, their amino acid sequences are closely related and therefore the data may be readily extrapolated. Crystallographic studies and x-ray analysis of horse and yeast PGKs have been reported.

Two globular domains contribute to the enzymatic mechanism; they are separated by two stretches of amino acids, one of which is an α helix and the other a random coil. The N-terminal domain contains the catalytic site for phosphoglycerate binding and the C-terminal domain that for nucleotide binding (ATP or adenosine diphosphate [ADP]).

Deficiency of PGK was first described in 1968, and until now several cases have been reported. Such a deficiency is generally associated with hemolysis and neurologic disorders, the latter being variable, often including mental retardation, seizure, emotional lability, and extrapyramidal tract disease. A few variants give rise to muscular symptoms that may be associated with chronic hemolysis. As the PGK gene is linked to the X-chromosome, deficiency is expressed at the clinical level only in males, whereas females, in general, have a normal phenotype, or minor disorders correlated with low PGK activity. The enzyme deficiency could be detected in red blood cells (RBCs), leukocytes, or in any other tissue by enzymatic assay, as a result of the ubiquitous expression of PGK.

At the present time, the structure of seven mutants have been described: PGK Shizuoka (157 Gly → Val), PGK München (267 Asp → Asn), PGK Uppsala (205 Arg → Pro), PGK II (351 Thr → Asn), PGK Michigan (315 Cys → Arg), PGK Matsue (88 Leu → Pro), and PGK Tokyo (265 Val → Met) (all residues reported are referred as the methionine initiator being residue −1). DiMauro’s group described two other PGK variants that are associated with muscular disorders, named PGK New Jersey and PGK Alberta on the basis of enzymatic study and phenotype, but they remain to be studied at the gene level.

This report deals with the reinvestigation at the gene level of two cases of PGK deficiency previously described by their phenotype in France, ie, PGK Amiens and PGK Créteil, and the assignment of the abnormality involved in enzyme deficiency. These studies have allowed us to draw conclusions concerning structure-function relationships in the enzyme and to relate them with the clinical disorders.

MATERIALS AND METHODS

Enzymatic Analysis

Erythrocyte PGK activity was determined by previously reported methods.

DNA Samples

Approximately 5 mL of peripheral blood was withdrawn on EDTA from members of families B and V. DNA was prepared using standard techniques.

In Vitro Amplification

Amplification was performed as previously described. The oligonucleotides used were designed according to the position of introns...
and exons to give polymerase chain reaction (PCR) products around 500 to 800 bp. Exons 3 and 4; 5 and 6; 7 and 8; 9 and 10 were amplified during the same reaction together with introns 3, 5, 7, and 9, respectively. Oligonucleotides used in the study are summarized in Table 1 and the position on the PGK gene is indicated in Fig 1. All oligonucleotides were synthesized by the phosphoramidite chemistry in the laboratory using an Applied Biosystem Instrument apparatus (model 390; Foster City, CA).

Sequence Determination

PCR products were reamplified using a ratio of 0.1:1 or 0.5:1 of the same oligonucleotides to give rise to single-stranded DNA, which was further sequenced using the Sequenase kit (US Biochemicals, Cleveland, OH). All sequences were made in duplicate on both DNA strands, ie, producing single-stranded DNA with two different asymmetric PCR amplifications on both sides. Alignments of human and animal amino acid sequences PGKs (from version 39 of NBRF-PIR data base) were performed by computer analysis.

RESULTS

Case Reports

Family B. As previously reported, the proband was a 31-year-old man who was generally in good health with normal intellectual development. He never showed any sign of chronic hemolysis, but since his childhood presented several symptoms during physical exercises, notably rhabdomyolysis crises. Biologic studies showed a decreased PGK activity in erythrocytes, leukocytes, platelets, and on muscular biopsy. The family study showed a low PGK activity in the RBCs of the mother and two daughters of the proband, whereas the PGK activity in his father was normal. These data confirmed the X-chromosome-linked genetic inheritance of the disease. In addition, physical and kinetics studies of the proband’s erythrocyte PGK showed a slow-moving electrophoretic band, thermal instability, and increased Km values for ATP and ADP; by contrast Km values for phosphoglycerates were similar to control values.

Family V. This case was first reported in 1974 when the proband was 3 years old. At this time, the patient manifested chronic hemolytic anemia that was occasionally exacerbated by acute hemolysis crises and associated with retardation in language development. Biologic analyses showed a decreased PGK activity in erythrocytes and leukocytes. The electrophoretic mobility of PGK, its Km for 3-phosphoglycerate and ATP, and its thermostability were normal. A slight

Table 1. Oligonucleotides Used in the Study of the PGK Gene (F for sense DNA strand and R for antisense DNA strand)

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Regions of Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGK 22 F</td>
<td>5’CGGAAGGCCGACGTCCTC3’</td>
<td>Promoter, E1, junction E1-E11</td>
</tr>
<tr>
<td>PGK 11 R</td>
<td>5’AGAAACAGTCGCCGGCC3’</td>
<td>E2, junctions I1-E2 and E2-I2</td>
</tr>
<tr>
<td>PGK 12 F</td>
<td>5’GCATCTCTAGTCTTTTAG3’</td>
<td>E3, E4, I3, junctions I2-E3 and E4-I4</td>
</tr>
<tr>
<td>PGK 13 R</td>
<td>5’CATCTCTAGACATACACAC3’</td>
<td>E5, E6, I5, junctions I4-E5 and E6-I6</td>
</tr>
<tr>
<td>PGK 14 F</td>
<td>5’GTCCCACTAGCTTAGGAG3’</td>
<td>E7, E8, I7, junctions I6-E7 and E8-I8</td>
</tr>
<tr>
<td>PGK 15 R</td>
<td>5’CATCTCTAGACATACACAC3’</td>
<td>E9, E10, I9, junctions I8-E9 and E10-I10</td>
</tr>
<tr>
<td>PGK 16 F</td>
<td>5’GGAGGTGGTGGTTAAATAGC3’</td>
<td>Junction I10-E11 and coding region of E11</td>
</tr>
<tr>
<td>PGK 17 R</td>
<td>5’TGCTGCTATGATGTGCG3’</td>
<td></td>
</tr>
<tr>
<td>PGK 18 F</td>
<td>5’AGTACCCACAGATACACAC3’</td>
<td></td>
</tr>
<tr>
<td>PGK 19 R</td>
<td>5’GGAGGTGGTGGTTAAATAGC3’</td>
<td></td>
</tr>
<tr>
<td>PGK 20 F</td>
<td>5’GGAGGTGGTGGTTAAATAGC3’</td>
<td></td>
</tr>
<tr>
<td>PGK 5 R</td>
<td>5’CTATGCGCAAGTGGATGC3’</td>
<td></td>
</tr>
</tbody>
</table>

Fig 1. Strategy followed in the study of PGK variants. Primers (sequences shown in Table 1) were designed after the PGK gene sequence. Sense primers are indicated as F and antisense primers as R. PCR amplification was obtained for one exon (or two adjacent exons plus intron) as well as the corresponding intron-exon boundaries. Nucleotide sequences of amplified segments were performed using the same oligonucleotides that were used for PCR experiments as well as internal oligonucleotides (data not shown).
Amplification Results

The sequence of this band was consistent with this hypothesis. Studies in normal regions covering the coding regions and flanking sequences. The primers indicated in Table 1. Preliminary studies in normal subjects indicated that a single product was obtained in accord with the mosaicism caused by X-linked genetic transmission.

The erythrocyte PGK activity in family V indicated a marked defect in enzyme activity in the proband (2.2 U/g Hb; normal 142 ± 33 U/g Hb). His father displayed a normal value (147 U/g Hb), as did his brother (123 U/g Hb) and sister (122 U/g Hb), whereas an intermediate value (96 U/g Hb) was found in his mother.

Amplification of the PGK gene was performed in seven parts according to the strategy described in Fig 1 and using the primers indicated in Table 1. Preliminary studies in normal subjects indicated that a single product was obtained in regions covering the coding regions and flanking sequences. This result indicated that none of the primers, which were designed in intron sequences, hybridize with the multiple PGK pseudogenes. In an experiment using primers located in exons 7 and 8, we observed two PCR products, one with the normal size (corresponding to exons 7 and 8 plus intron 7) and a second shorter product lacking intron 7 (data not shown), which probably originated from a pseudogene. The sequence of this band was consistent with this hypothesis.

The results indicate that patient B has a G → A interchange at position 1022 in cDNA (exon 9), which corresponds to the amino acid substitution 314 Asp → Asn (Fig 2a). In the case of family V (Fig 2b), the propositus was shown to have an A → T interchange at position 571 in cDNA (exon 5), which is responsible for the amino acid substitution 163 Asp → Val. His brother and his sister were normal, whereas the mother exhibited the same abnormality in the heterogeneous state (ie, both a normal and abnormal bases were found in the same position). No other difference was observed, with the exception of position 27 upstream to the cap site of mRNA in the promoter region, where a T nucleotide was found in the place of a C for patient B; by contrast, members of family V, as well as the nine normal control DNAs (six of which were females), did not display this substitution, which could be classified as a spurious mutation for patient B.

DISCUSSION

The technique followed in this report was derived from that used in our laboratory for another gene, bisphosphoglycerate mutase. Its use obviates subcloning of PCR products, which would have detected an error introduced by Taq polymerase, such an error being interpreted subsequently as a mutation during the sequencing step. In the case of PGK, which is an X-linked gene, a point mutation appears as an interchange for male patients, because there is only one allele present and it appears as the combination of the normal base with an abnormal base in the heterogeneous state for females.

In the case of PGK Crétteil (nucleotide 1022 G → A in cDNA, 314 Asp → Asn), and in addition to the enzymatic deficiency, the accompanying disorders mainly concerned rhabdomyolysis with acute renal failure, but no neurologic defect was observed. This case was extensively described previously by Rosa et al. A deficient enzyme that possesses a mutation at the adjacent residue in PGK Michigan (nucleotide 1025 T → C in cDNA, 315 Cys → Arg) has been described. This latter mutant is associated with hemolytic anemia and mental retardation, which is the more common pattern in PGK deficiency, but not with muscular disorders.

Both the 314 and 315 residues belong to the C-terminal domain in the nucleotide binding site. In these cases, the mechanism of PGK deficiency is easily understood if one considers the tertiary disruptions introduced by larger residues associated with the appearance of the new positive charge introduced by the arginine residue in PGK Michigan and by the replacement of aspartic acid, a negatively charged residue, by asparagine, a neutral residue, in PGK Crétteil.

In the case of PGK Amiens (nucleotide 571 A → T in cDNA, 163 Asp → Val), the mutation occurs in the N-domain in a position that is localized to the catalytic domain. This localization accounts for the defect in enzymatic activity, as the change of a neutral residue for a negatively charged residue in PGK Amiens disrupts the tertiary structure of the molecule. Another mutant was recently described in the vicinity of residue 163, ie, PGK Shizuoka (nucleotide 552 G → T in cDNA, 157 Gly → Val). In this case, it was supposed that the occurrence of a large residue instead of a very small one disrupted the tertiary structure, thus impairing the functional properties. PGK Shizuoka is associated with hemolytic anemia like in PGK Amiens but, in contrast to our case, it is not associated with mental retardation.

Both 167 and 315 aspartic acid residues were found to be invariant in all PGK amino acid sequences described up to now for humans, eukaryotes, and proeukaryotes. This indicates their functional importance in the enzymatic mechanism and that mutations observed in PGKs Crétteil and Amiens should impair it.

The nucleotide substitution observed in the promoter region in patient B could be explained either as a sporadic mutation found only in this given family or as a rare polymorphism, because it was not found in all the individuals studied, including members of the V family. For reasons given above (ie, Taq errors that are not detected in our strategy), and because it was the only base found at this position in a male patient, it is not an artefact but rather a real polymorphism. As described in other studies, polymorphic nucleotide interchanges leading to neutral mutations occur more frequently in regions of the gene where the selective pressure is less stringent, such as introns or flanking regions, or in
Two new mutations in PGK gene found in France

Fig 2. Results of PCR amplification followed by nucleotide sequencing of patients with PGK Créteil (A) and PGK Amiens (B). Nucleotide sequence of exon 9 in patient B indicated a G → A nucleotide replacement at position 1022 in cDNA sequence that leads to an Asp → Asn amino acid substitution at position 314 of the PGK protein. In family V (B) only T was found in position 571 in cDNA sequence (codon 163) in the hemizygote condition in the propositus and T and A were found in the same position for his mother (because of heterozygote conditions in the female). This leads to an Asp → Val amino acid substitution in the PGK protein. All other subjects of the family were normal, including his sister who is not a carrier of the disease.

As shown above, the mechanism of the enzymatic defect may be readily understood because the amino acid substitution involves modifications in the tertiary structure. On the other hand, the relationship of the molecular PGK defect to the clinical features remains conjectural. The PGK Créteil and PGK Michigan mutations are located on adjacent residues, but the former is associated with a muscular defect only, whereas the latter is associated with hemolytic anemia and mental retardation. A parallel observation can be made on comparison of PGK Amiens and PGK Shizuoka, whose respective mutations are located six residues apart but are associated with distinct clinical features. However, with the exception of triosephosphate isomerase deficiency and G6PD deficiency, no muscle manifestations have been reported in association with inherited defects of RBC metabolism, indicating that these two symptoms, ie, muscle mani-
festations and mental retardation, are not relevant of strictly identical causes.

Therefore, it is possible that other parameters are involved in the generation of the observed clinical symptoms. Hemolytic anemia is almost always found in PGK deficiency as a result of RBC deficiency. Muscular defects are found in a few PGK deficiencies, including PGK Shizuoka (157 Gly → Val) and PGK Créteil (314 Asp → Asn). The latter are the only two cases in which PGK enzyme deficiency is associated with muscular disorders. Other cases of PGK deficiency associated with myoglobinuria have been described by DiMauro’s group,22,23 but until now, the molecular defects of these mutants are not described. The explanation provided by Rosa et al23 for PGK Créteil was supported by the fact that the enzymatic defect in muscle was responsible for the decrease in muscle glycogen content, which is needed for exercise. In the case of violent or prolonged muscular effort, the energy stocked in glycogen is rapidly consumed and myoglobin occurs. However, it is surprising that PGK deficiency is not generally associated with muscular problems, because the enzyme is ubiquitous and the level of PGK activity is decreased in muscle as well as in other tissues.

On the other hand, the relationship between PGK deficiency and neurologic disorders remains to be established. Mental retardation is often associated with PGK deficiency. It is tempting to imagine that the chronic deficit of ATP induced by the decrease in glycolysis is responsible for the destruction of nervous cells to some degree, although such a mechanism seems to be more complex. Nevertheless, it is known that some enzymatic defects which do not impair glucose metabolism can be associated with mental retardation.22,23

Mechanistic explanations for the clinical defects observed in RBC glycolytic enzyme deficiencies are lacking. However, it is to be anticipated that the description of further cases of deficient patients in association with the determination of the molecular defect by application of molecular techniques will allow more precise correlation between the location of the defect in the enzyme molecule, the decrease in enzymatic activity and, possibly, the clinical pattern observed.

ACKNOWLEDGMENT

The authors thank Serge Amselem and Michel Videau for helpful technical discussions, Jean Rosa for encouraging us throughout this study, Anne-Marie Dulac for design of the figures, and John Chapman for revision of the manuscript.

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