Life-threatening nonspherocytic hemolytic anemia in a patient with a null mutation in the \textit{PKLR} gene and no compensatory \textit{PKM} gene expression

Amalia Diez, Florinda Gilsanz, Joaquin Martinez, Susana Pérez-Benavente, Néstor W. Meza, and José M. Bautista

Human erythrocyte R-type pyruvate kinase (RPK) deficiency is an autosomal recessive disorder produced by mutations in the \textit{PKLR} gene, causing chronic nonspherocytic hemolytic anemia. Survival of patients with severe RPK deficiency has been associated with compensatory expression in red blood cells (RBCs) of M2PK, an isoenzyme showing wide tissue distribution. We describe a novel homozygous null mutation of the \textit{PKLR} gene found in a girl with a prenat al diagnosis of PK deficiency. The mutant PK gene revealed an 11–nucleotide (nt) duplication at exon 8, causing frameshift of the \textit{PKLR} transcript, predicting a truncated protein inferred to have no catalytic activity. Western blot analysis and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) detected no M2PK expression in the peripheral blood red cell fraction. The expression of mutant RPK mRNA in the RBCs was almost 6 times higher than that detected in a control patient with hereditary spherocytosis. This molecular phenotypic analysis of the null mutation in the \textit{PKLR} gene provides evidence for a lack of M2PK in the mature RBCs of this patient and suggests that normal red cell functions and survival are achieved through a population of young erythroid cells released into the circulation in response to anemia. (Blood. 2005;106:1851-1856)

Introduction

Pyruvate kinase (PK; EC 2.7.1.40) is a key enzyme of the glycolytic pathway responsible for irreversibly catalyzing the conversion of phosphoenolpyruvate to pyruvate. In humans, pyruvate kinase activity is provided by 4 isoenzymes encoded by 2 structural genes. The \textit{PKLR} gene on chromosome 1 codes for the R-type PK (RPK) (exclusively in mature red blood cells [RBCs]) and the L-type PK (in liver) isoforms under the control of tissue-specific promoters. RPK is a 200-kDa tetramer with 4 identical subunits, each consisting of 4 domains. The active site of RPK occurs at a domain interface, and the allosteric site is found in domain C. The \textit{PKM} gene on chromosome 15 codes for 2 different M-type PK (MPK) forms, M1 (brain and skeletal muscle) and M2 (fetal and most adult tissues), produced by alternative splicing. Undifferentiated erythroid precursor cells express the M2PK isoenzyme earlier than mature RBCs express the RPK isoenzyme. Because of the tissue-specific expression patterns of the genes, only mutations in the \textit{PKLR} gene lead to erythrocyte PK deficiency.

PK deficiency severely affects RBC metabolism, causing adenosine triphosphate (ATP) depletion, which ultimately leads to hemolysis. Clinical symptoms vary considerably from mild to severe anemia. For the patient, anemia may mean transfusion dependence and may even be life threatening. Pathologic signs of PK deficiency are usually observed when enzyme activity is less than 25% normal activity and patients are generally homozygotes or compound heterozygotes with 2 different mutant alleles. Most cases of PK deficiency are caused by the production of mutant enzymes with abnormal biochemical properties. Thus far, molecular analyses have identified at least 133 different mutations in the \textit{PKLR} structural gene. Most are missense mutations, but there are also reports of point mutations, deletions, or insertions that led to more drastic changes, such as alterations of the splicing site, frameshift, early termination mutations, and disruption of erythroid-specific promoters.

Most mutations constitute single-base substitutions, predicting amino acid changes of conserved residues in structurally and functionally important domains of the RPK tetramer. Mutations that affect \textit{PKLR} transcription, or processing of its pre-mRNA, are less common. To date, only a few homozygous PK null mutations have been identified. The first reported was the relatively common “PK gypsy,” consisting of a large deletion at exon 11 with premature termination of its translation lacking 10% of the original sequence at the C-terminus (exon 11 is deleted, and 35 aberrant residues are added); afterward, 2 one-base deletions and one transition at a split site were reported. More recently, the first homozygous nonsense mutation was reported, which causes premature termination of translation resulting in a truncated protein lacking a 33-residue C-terminal fragment.

Here, we report a novel homozygous \textit{PKLR} gene null mutation in a patient with severe PK deficiency. The mutation consists of an 11-nt insertion at exon 8 causing frameshift of the \textit{PKLR} transcripts and protein truncation. Our molecular results indicate the patient’s life-threatening anemia arises from the lack of the RPK functional
isozyme. Phenotype studies performed at the protein and RNA levels indicate no surrogate regulation by the PKM gene.

**Patient, materials, and methods**

**Patient and controls**

The patient is a Spanish girl in whom PK deficiency was diagnosed in utero by umbilical cord blood sampling at 30 weeks of gestation. She is the fourth child of 2 first cousins, who lost their 3 previous children before or immediately after birth to severe anemia, as has been previously described in the literature.25 Our patient was born by cesarean delivery. Because she had severe hydrops fetalis and her bilirubin level was 101 mg/L at 54 hours of life, she was given 2 exchange transfusions within the first 24 hours of life and phototherapy for 8 days. She remained transfusion dependent until the age of 3 years, during which time she received monthly RBC transfusions using 70 g/L hemoglobin as a transfusion trigger. At 3.5 years, she underwent splenectomy and since then her hemoglobin levels have remained at approximately 85 g/L (91 g/L is the highest and 54 g/L the lowest hemoglobin level recorded in this period), requiring at most one transfusion per year, mostly during periods of intercurrent infection. Since November 2002, she has required no blood transfusions.

At the time of this writing, the patient is 12 years old and well. Her current bone age is 10 years, her height is at the 75th percentile, and she is mildly obese (97th percentile). Her intellectual development is normal. Serum ferritin is 1150 ng/mL and serum enzyme levels are normal, although desferrioxamine was given immediately after transfusions. Computed tomography performed at 9 years of age showed no evidence of liver involvement, and this was confirmed during recent ultrasonography. Her current blood tests indicate chronic nonspherocytic hemolytic anemia (CNSHA) (Table 1).

As controls for our tests, we used healthy subjects and patients with hereditary spherocytosis. All tests were undertaken during long transfusion-free periods. Approval was obtained from the University Hospital 12 de Octubre Ethical Committee for these studies. Informed consent was provided according to the Declaration of Helsinki.

**Mutation analysis**

Genomic DNA was extracted from peripheral blood mononuclear cells of the patient and her parents using standard procedures. The coding region of the PKLR gene was then analyzed according to the cDNA sequence reported.26 Automated DNA sequence analysis was performed using the ABI Prism dRhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Sequencing reactions were all carried out in forward and reverse directions in duplicate in an ABI 3730 DNA Analyzer (Applied Biosystems). Alternatively, total RNA from fresh peripheral blood mononuclear cells (PBMCs) and reticulocytes was extracted using an RNeasy kit (Qiagen, Valencia, CA) and was reverse transcribed (by real-time polymerase chain reaction [PCR]) to detect the mutation using the GeneAmp RNA PCR Core Kit from Applied Biosystems according to the manufacturer’s instructions. Briefly, 1.0 µg extracted RNA was reverse transcribed using random hexamers as primers and M-MLV reverse transcriptase. The entire complementary DNA (cDNA) obtained was amplified by PCR using primers designed according to the reported RPK cDNA sequence.26 PCR products were purified and cloned into a pGMEM-T vector (Promega, Madison, WI). The insert was sequenced in an ABI PRISM 3730 genetic analyzer (Applied Biosystems) by the Big Dye termination method.

To corroborate the mutation in the patient and her parents, multiplex PCR was performed on PBMC cDNA with the following primers: F1PK8 5'-GTGATCTGGCCAGGTCTCCC-3' and R1PK8 5'-GGCAGACTGGGTAGTGAAAGG-3'. PCR was carried out with 200 ng cDNA in a final volume of 50 µL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each deoxynucleotide triphosphate (dNTP), 0.3 µM each primer, and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). After initial incubation for 10 minutes at 95°C, samples were subjected to 35 cycles of amplification with denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 45 seconds, followed by a prolonged extension time of 10 minutes after the last cycle.

**RPK and M2PK mRNA expression analysis**

Mononuclear cells were separated from whole blood using 6% hydroxyethyl starch (HES; Grifols, Barcelona, Spain). Saline suspensions of red cells were freed from leukocytes by passage over Sigmacell-cellulose columns (Sigma Chemical, St Louis, MO) and saline washed.27 No further method of reticulocyte enrichment was used. For transcription expression, real-time quantitative reverse transcription–PCR (qRT-PCR) was run on total RNA extracted from PBMCs and peripheral blood red cell (PBRC) fractions using the GeneAmp RNA PCR Core Kit from Applied Biosystems according to the manufacturer’s instructions. In brief, 1.0 µg patient and control cell RNA was reverse transcribed using random hexamers as primers and M-MLV reverse transcriptase. Real-time PCR was performed in a LightCycler instrument (Roche, Mannheim, Germany) using SYBR green as the fluorogenic dye. Three samples of each cDNA were subjected to 35 PCR cycles for 10 seconds at 95°C, 25 seconds at 60°C, and 2 seconds at 80°C in a 15-µL mixture containing 1 × LightCycler-DNA Master SYBR Green I (Roche), 4.5 mM MgCl2, and 0.4 µM each of forward and reverse gene-specific primers. SYBR green fluorescent emission was acquired at 80°C to minimize the formation of low-temperature–melting nonspecific DNA products. Oligonucleotide pairs used for qRT-PCR were: human RPK, forward primer 5'-GTAGTCGCGAGGCTCCC-3' and reverse primer 5'-CTCTCAAGAGCTGGTGGG-3'; human M2PK, forward primer 5'-GTGATGTTGCGAATGACTC-3' and reverse primer 5'-GTGAAGCCGGACAG-3'; designed according to the cDNA sequence reported.26,28 Controls with no RNA and controls in which the reverse transcription step was omitted were included. Expression of the β-actin gene was used as the housekeeping control. qRT-PCR expression was analyzed using the 2-ΔΔCT method, as previously described.29

**SDS-PAGE and Western blot analysis**

Peripheral leukocyte and erythrocyte fractions were separated from whole blood samples of a healthy control and of the patient through centrifugation at 500g for 15 minutes at room temperature. Antibodies against recombinant RPK20 were raised in immunized mice and rabbits. Mouse antibodies

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**Table 1. Relevant hematologic findings in the PKLR-deficient patient**

<table>
<thead>
<tr>
<th>Hematologic finding</th>
<th>Patient</th>
<th>Patient’s parents</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At diagnosis</td>
<td>After splenectomy</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>64</td>
<td>86</td>
<td>138</td>
</tr>
<tr>
<td>Reticulocytes, %</td>
<td>&gt; 95</td>
<td>&gt; 95</td>
<td>1.6</td>
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<tr>
<td>Erythroblasts, per 100 WBCs</td>
<td>15</td>
<td>26</td>
<td>UD</td>
</tr>
<tr>
<td>PK, IU/gL</td>
<td>&lt; 1</td>
<td>1.3</td>
<td>UD</td>
</tr>
<tr>
<td>ATP, mmol/g Hb</td>
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<td>12.8</td>
<td>15 ± 1.99</td>
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<td>4404</td>
<td>4540</td>
<td>4230 ± 290</td>
</tr>
<tr>
<td>AMP, mmol/g Hb</td>
<td>758</td>
<td>787</td>
<td>635 ± 105</td>
</tr>
</tbody>
</table>
| Hb indicates hemoglobin; ND, not determined; UD, undetected.
were specific for recombinant RPK, whereas rabbit antibodies cross-reacted with human MPK isoenzymes. For Western blot analyses, 100 µg/well soluble proteins from control and patient PBMCs and erythroid cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide gels) and were transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine, and 5% methanol, pH 8.3. Membranes were blocked with 5% skimmed milk in phosphate-buffered saline (PBS). Antibody binding (in 2.5% skimmed milk in PBS) was detected by incubation with secondary HRP-conjugated rabbit anti-mouse or sheep anti-rabbit immunoglobulin antibodies, followed by enhanced chemiluminescence (ECL) detection (Pierce Biotechnology, Rockford, IL).

Results

By sequencing the entire genomic DNA fragment encoding the 12 exons of the PKLR gene on chromosome 1, a homozygous 11-base pair (bp) insertion was detected. This insertion is presumably the result of an 11–nucleotide (nt) upstream duplication (GAGAGCAT-GAT) at nt 1133 in the patient; it occurs in heterozygosity in her parents (Figure 1A). This finding was confirmed in mRNA from PBMCs by RT-PCR amplification using a specific primer for the insertion and flanking sequences (Figure 1B) and by subsequent sequencing of the cDNA clones from the patient and her parents (data not shown). The inserted nucleotides create a frameshift at residue 377, producing a truncated protein as a consequence of an earlier stop codon at position 381. The truncated product has 380 amino acids (40 kDa) instead of the expected 574 amino acids of the normal RPK isoenzyme (63 kDa). This represents a 35% loss in the original RPK monomer, including the entire C domain, which contains the binding site for allosteric effector fructose-1,6-diphosphate and decisive regions for subunit assembly into the catalytically active tetrameric structure. Comparison analysis of the primary structures, along with identification of essential residues and inspection of the available 3-dimensional MPK and RPK structures, predicted a lack of PK activity for this truncated PKLR gene product resulting from the 11-bp insertion.

Western blot immunoanalysis of a hemolysate from the patient and a healthy control using highly specific mouse antibodies against human recombinant RPK clearly detected the wild-type erythroid enzyme bands expected in the control sample only: the 63-kDa band of the RPK monomers and a 57- to 58-kDa band corresponding to the proteolyzed form. In contrast, no wild-type RPK bands were detected in the erythroid fraction of the patient, and a faint, unidentified band of 22.5-kDa appeared that was absent from the control lysate (Figure 2A). This band was interpreted as an unspecific reaction of this rabbit antibody. If the 32.2-kDa band detected in the PBMC was a degradation product of M2PK, the same band appearing in the erythroid fraction from the patient but absent in the healthy control could be attributable to the presence of M2PK relics from the abundant erythroblasts in the patient’s erythroid fraction. It should be noted that reticulocytes/erythroblasts, mainly in the patient, could have been present in the mononuclear cells and mature red cell fractions when separation was performed by centrifugation. Molecular weights for the RPK monomers were calculated according to published data.

![Figure 1](image1.png)

Figure 1. Genetic analysis of the mutational insertion at exon 8 of the PKLR gene. (A) PKLR DNA sequence analysis of healthy exon 8 compared with patient exon 8. The homozygous 11-bp insertion site at the propositus exon 8 and the oligonucleotide primer used in multiplex RT-PCR are indicated. (B) Multiplex RT-PCR amplification using the specific insertion primer. Three different fragments were obtained: a single 284-bp fragment from the normal sequence (lane 1) and 2 mutant fragments (217 bp and 295 bp) from the inserional allele. Diagram below shows the multiplex assay. The patient (lane 2) is homozygous for the mutant allele. Her mother (lane 3) and father (lane 4) are heterozygous for the wild-type and mutant alleles.

![Figure 2](image2.png)

Figure 2. Immunodetection of RPK in the circulating erythroid fraction and peripheral mononuclear cells. (A) Western blot analysis using mouse antibodies against recombinant human RPK on PBMCs and red cells from a healthy control and the patient. The 11-bp insertion resulted in the lack of the wild-type subunit bands (61.7 and 57.2 kDa) from the red cell fraction of the patient. (B) Western blot analysis performed on the same set of samples using antibodies against recombinant human RPK raised in rabbits detecting the RPK (61.7 and 57.2 kDa) and the M2PK (58.1 kDa) wild-type subunit bands. M2PK was detected in PBMCs from the control and the patient, but not in the corresponding RBC fractions. The top extra band in all the tracks has a molecular weight not corresponding to any PK species and was interpreted as an unspecific reaction of this rabbit antibody. If the 32.2-kDa band detected in the PBMC was a degradation product of M2PK, the same band appearing in the erythroid fraction from the patient but absent in the healthy control could be attributable to the presence of M2PK relics from the abundant erythroblasts in the patient’s erythroid fraction. It should be noted that reticulocytes/erythroblasts, mainly in the patient, could have been present in the mononuclear cells and mature red cell fractions when separation was performed by centrifugation. Molecular weights for the RPK monomers were calculated according to published data.
Given this critical structural alteration, a complete lack of RPK residues contributing to the active site of the wild-type protein. This hypothesis of compensatory PK activity provided by an isozyme was first formulated to explain the results of zymogram analysis by thin-layer electrophoresis of red cell hemolysates from a patient who had PK deficiency with extreme reticulocytosis. In this study, the PK activity band with RPK mobility was not observed; rather, an activity band with the mobility of M2PK was detected. Under normal conditions, during erythropoiesis, PK expression switches from the M2 to the R isoenzyme in response to developmental signals. Thus, the hypothesis of persistent M2PK expression in PK-deficient patients would require overriding of isozyme switching by an unknown mechanism, which seems to differ among patients because the presence of M2PK in erythrocytes is unrelated to disease severity or to a specific gene mutation. Compensatory M2PK isozyme production is attributed to its overproduction in erythroblasts, analogous to the compensatory production of fetal hemoglobin in thalassemia major. In this pioneer report, the compensatory persistent M2PK expression in some severely PK-deficient subjects was assumed for the first time. However, at this early stage, experimental evidence for M2PK mRNA expression was unavailable. Although this idea of M2PK compensation to explain the survival of patients with severe PKLR deficiency is generally accepted, the detection of M2PK isozyme expression in mature RBCs using zymograms or immunodetection techniques has only really been possible in a few patients with severe PK deficiency and has been always associated with reticulocyte counts greater than 70%. Moreover, no M2PK was detected in hemolysates from PK-deficient dogs with 15% to 24% reticulocytosis using anti–M1PK antibodies that recognize dog leukocyte M2PK.

Although the RBCs of our patient lacked the RPK isoenzyme, we were unable to detect the expression of either of the 2 MPK isoenzymes. It may, therefore, be stated that the lack of enzyme activity in the RBCs of our patient with severe PK deficiency and its effects on energy metabolism were overcome by a mechanism other than the surrogate expression of the PKM gene.

In addition to any residual activity of the mutant or chimeric RPK enzymes in PK-deficient patients, the possible overexpression of deficient PK isoenzymes to partially compensate for the deficiency cannot be ruled out because abnormal levels of intermediate regulatory metabolites could act as signals to modulate expression at the PKLR promoter in erythroblasts. In effect, the qRT-PCR assay for RPK transcripts indicated their overexpression in our patient. Nevertheless, given that in our patient the mutation is highly deleterious, it is unlikely that this mechanism could account for the compensation and clinical improvement after splenectomy. Moreover, in PK-deficient persons, some mutational genotypes render abnormal mRNA expression profiles as a consequence of mutations in regulatory sequences reducing transcription or decreasing mRNA stability.

The lack of mature RBCs may be compensated by a physiopathologic adaptation mechanism, as indicated by the increased lifespan.

### Discussion

Herein, we describe the molecular basis of a PK deficiency in a young patient with severe hemolytic anemia. In both alleles, we detected an 11-nnt insertion at exon 8 of the PKLR gene. The amino acids comprising the active site are found in 7 clusters encoded by exons 5, 7, 8, and 9 of the human RPK gene. The insertion mutation identified gives rise to a mutant protein devoid of all residues contributing to the active site of the wild-type protein. Given this critical structural alteration, a complete lack of RPK activity in the RBCs would be expected.

The clinical severity of PK deficiency has been linked to the disruptive effect of the mutation found and to the compensatory expression of the widely distributed M2PK isozyme in the erythrocytes of some patients. This hypothesis of compensatory PK activity provided by an isozyme was first formulated to explain the results of zymogram analysis by thin-layer electrophoresis of red cell hemolysates from a patient who had PK deficiency with extreme reticulocytosis.
of immature erythroid cells in circulation in anemic animals.\textsuperscript{28} Thus, the slower removal from the circulation of younger erythroid cells to keep the mitochondrial machinery producing energy\textsuperscript{49} and to provide high levels of hemoglobin regulatory metabolites probably compensates for the lack of the mature red cell functions. Extreme reticulocytosis and the presence of erythroblasts in peripheral blood are common clinical signs in patients with severe PK deficiency, especially after splenectomy.\textsuperscript{11,24} Accordingly, hemoglobin levels higher than 85 g/L were maintained in our patient after splenectomy. Moreover, although basal reticulocyte counts in the patient were significantly elevated at birth, the splenectomy performed when she was 3.5 years old led to increased peripheral blood immature reticulocytes and erythroblasts, in parallel with clinical improvement. Thus, the physiopathologic adaptation of this patient with null PKLR seems to be attributable to circulating immature erythroid cells.\textsuperscript{50} This immaturity of the circulating cells could reflect the observed residual PK activity attributable to M2PK protein left after the PK isoenzyme gene switch, covering the patient’s minimal metabolic demands. Contrary to the expected reduced erythrocyte glycolytic rate in our RPK-deficient patient, we detected increased concentrations of the glycolytic pathway intermediates ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and 2,3-diphosphoglycerate (2,3-DPG). Besides impaired glycolysis, these high levels of intermediates could be explained by the fact that the high proportions of RPK-deficient reticulocytes in the circulating blood will almost entirely rely on mitochondrial oxidative phosphorylation. Glycolytic intermediates proximal to the pyruvate kinase step, such a 2,3-DPG building up in the RPK-deficient reticulocytes, would shift the oxyhemoglobin dissociation curve to the right,\textsuperscript{9} providing our patient with a life-compatible degree of anemia. This mechanism would explain her transfusion-independent survival with a tolerable hematologic profile after splenectomy.

Acknowledgments

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