Molecular Abnormality of Erythrocyte Pyruvate Kinase Deficiency in the Amish

By Hitoshi Kanno, Samir K. Ballas, Shiro Miwa, Hisaichi Fujii, and Herbert S. Bowman

We describe the cellular and molecular biologic studies of the erythrocyte pyruvate kinase (PK) deficiency of the Amish deme in Pennsylvania. Nucleotide sequencing of the patient's PK gene showed a point mutation, CGC to CAC, corresponding to no. 1436 from the translational initiation site of the R-type PK (R-PK) mRNA, and it caused a single amino acid substitution from Arg to His at the 479th amino acid residue of the R-PK. The substituted Arg residue is located in the C domain of PK subunit, which is essential for both the intersubunit contact and the allosteric regulation. Because this enzyme shows the catalytic activity only as a dimer or tetramer, it is rational that the structural alteration would result in severe PK deficiency. To elucidate the effect of the PK deficiency on red blood cell (RBC) membrane, we performed the cellular studies of the patients' RBCs. Ouabain-insensitive K+ efflux was increased to 142% to 145% of normal controls and not inhibited by furosemide, as previously observed in HbSC disease RBCs.

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MATERIALS AND METHODS

Clinical features of the Amish PK-deficient subjects. AY and JY are siblings, being brother and sister and members of the STY, Sr. family (Fig 1). STY, Sr, is a distant consanguineous relative of SIY and AB who are believed to be the ancestral pair of the Mifflin County Pennsylvania Amish red blood cell (RBC) PK-deficient gene. Family history showed JY to be one of 10 Old Order Amish siblings of the STY, Sr, pedigree. Two sisters had been affected with the Amish RBC PK-deficient hemolysis, one of whom died. A subsequent sister born later, JY, likewise was found to have this RBC disorder.

JY was initially seen at age 1 month, having been icteric at 48 hours of life with deepening jaundice during the subsequent 5 days. As the icterus gradually faded, he evidenced increasing pallor and was referred to the Hematology Center, Harrisburg Hospital. He was transfused with packed RBCs sufficient to maintain the hematocrit at 26%, and such transfusions were maintained every eighth week. The pretransfusion hematologic values were: RBCs, 1.56 × 1012/μL, hemoglobin (Hb), 5.1 g/dL, hematocrit (Hct), 18.5%, mean cell volume (MCV), 112 fl, reticulocytes, 15.4%. Blood film showed 58 nucleated RBCs/100 white blood cells (WBCs), macrocytosis, polychromasia, and marked anisocytosis. Total bilirubin was 2.6 mg/dL, and lactate dehydrogenase (LDH) 1,250 U. RBC PK activity was 0.1 U/1012 RBCs. At 6 months of age, his spleen became palpable 5 cm below the left costal margin. Transfusion therapy was continued until 17 months of age, when splenectomy was performed. Splenic red pulp preparations showed reticuloendothelial cell, RBCs, and reticulocyte phagocytosis and increased reticulocytes. Subsequent examinations have shown normal findings and no transfusion has been required. Current examinations at age 31 years have shown

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Taq DNA polymerase. The amplified DNA fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) by the restriction enzyme chain reaction (PCR) was performed in a total volume of 100 µL, Hb 10 g/dL, Hct 29.6%, MCV 116 fl, and echinocytes 26%. In the blood film, macrocytes, polychromasia, and echinocytes were noted. LDH was 278 U.

**Materials.** Blood samples from the probands were obtained under informed consent. High molecular weight genomic DNAs were purified by the standard protocol.12 Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs (Beverly, MA). Taq DNA polymerase (AmpliTaq) was obtained from Perkin Elmer Cetus (Norwalk, CT). DNA was sequenced using a DNA sequencing system (Model 373A; Applied Biosystems, Foster City, CA). The computer software, DNANIS (Hitachi Software Engineering, Yokohama, Japan), was used to analyze the hydrophobicity properties of the variant protein based on the theory of Hopp and Woods.15

*Hematologic data and cellular studies about PK-deficient RBCs.** Routine hematologic methods were used to determine the Hb level, Hct, RBC indices, and reticulocyte count. Whole RBC deformability and RBC sodium (Na+) and potassium (K') concentrations were determined as previously described.13 The ouabain-insensitive component of K'-efflux was measured as described previously.13

**Amplification and sequencing of the human L-PK gene.** Oligonucleotides used for the amplification of the exons of human L-PK gene are listed in the Table 1. L1 (5'CCCGGAGGCCAGGCGCAAACTGGT-3') and L2 (5'ACAAAATCTGATCATGACATT-3') were used for amplifying the 3' end sequences of exon 10. Polymerase chain reaction (PCR) was performed in a total volume of 100 µL containing 1 µg of the template DNA, deoxynucleotides at 200 mmol/L each, a pair of primers, 10 mmol/L Tris/HC1 (pH 8.3), 50 mmol/L KCI, 1.5 to 3 mmol/L MgCl2, 0.001% gelatin, and 5 U of Taq DNA polymerase. The amplified DNA fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) by the restriction sites within the DNA or primers. Exons 1, 3 through 12, and the adjacent intron sequences were sequenced by the dideoxy chain termination method with fluorescent primers.

**RESULTS**

**Cellular studies of the PK-deficient RBCs.** Hematologic characteristics of the subjects studied and the rheologic properties of PK-deficient RBCs are listed in Table 2. Because both JY and AY had been splenectomized, the anemia was not severe on the examination. The MCV in PK deficiency was increased, and it seemed to be reflected on the high reticulocyte counts, which were usually observed in PK deficiency after splenectomy. It should be noted that the PK-deficient RBCs showed increased maximum deformability as well as increased deformability at isotonic conditions.

To elucidate further the hydration status and surface area-volume ratio of PK-deficient RBCs, we measured the density distribution and cation content of these cells (Fig 2, Table 3). There is a significant shift to lower density in PK-deficient RBC populations as seen in Fig 2. Table 3 shows that the potassium content of PK-deficient cells is similar to that of controls, whereas the potassium and total cation contents are significantly increased to 117% to 121% of normal controls.

To investigate the nature of the abnormal cation regulation in PK-deficient RBCs further, we determined the ouabain-insensitive K' efflux in the presence or absence of 1.0 mmol/L furosemide, an inhibitor of Cl− transport and chloride-dependent K' flux. Table 4 shows that PK-deficient cells had a markedly increased K' efflux. The first-order rate constant for K' efflux of the PK-deficient cells was 0.034 to 0.035 h−1, compared with 0.024 for control cells (142% to 145% increase). The addition of furosemide reduced the rate constant for K' efflux of normal cells, but did not decrease that of the PK-deficient cells.

**Identification of a point mutation in the L-PK gene of the Amish PK variant.** Sequencing of the L-PK gene from the Amish PK variant showed that the variant had a single nucleotide change at the 3'-end nucleotide of exon 10 of human L-PK gene, corresponding to no. 1436 of human R-PK cDNA, 1436 CCG → CAC. This missense mutation caused a single amino acid substitution; 479 Arg → His (R479H), which subsequently decreased the hydrophobicity properties near the mutated site (Fig 3). The predicted secondary structure by the theory of Chou and Fasman showed that the core of α-helix might be formed by this amino acid change (data not shown). This Arg residue is well conserved during evolution, including chicken M1-, cat M1-, rat R-, and human M2-type PK (Fig 4).16-19

**Demonstration of the point mutation by the Hpa II digestion of the genomic PCR products.** To confirm this nucleotide change in genomic DNAs of the probands, the 3' end

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**Table 1. Oligonucleotides for PCR of Genomic PK**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Product (bp)</th>
<th>Restriction Sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5'-CTAAAGCTTCTGAGGACAGGTCGACG-3'</td>
<td>5'-GAGGGTCTGAGAAGATCG-3'</td>
<td>374</td>
<td>HindIII/Sty I</td>
</tr>
<tr>
<td>III</td>
<td>5'-GCGAGATCTACAGGCGAAGAGTCGCC-3'</td>
<td>5'-ATACAAATATGGCTGTGACC-3'</td>
<td>260</td>
<td>Sac I/Pst I</td>
</tr>
<tr>
<td>IV-V</td>
<td>5'-GCCGCTTCTGAGAAGATCGACGG-3'</td>
<td>5'-TCCACTTCTGAGTGTACGAG-3'</td>
<td>599</td>
<td>Eco RI/Apa I</td>
</tr>
<tr>
<td>VI</td>
<td>5'-GACGACCGGTGGAGGATGATGACG-3'</td>
<td>5'-CACGCGAGAGGATGATGACG-3'</td>
<td>272</td>
<td>Sac I/Bcl I</td>
</tr>
<tr>
<td>VII-VIII</td>
<td>5'-GTCAGCCCACTTCTGAGGTCGACG-3'</td>
<td>5'-CCTATGAGTGGATATTCAACCC-3'</td>
<td>628</td>
<td>Pvu II/Pst I</td>
</tr>
<tr>
<td>IX-X</td>
<td>5'-CGGCTCAGAGAAGAGTAATCGGCG-3'</td>
<td>5'-CAAGATGATGATGATGACGACATCC-3'</td>
<td>531</td>
<td>Kpn I/Bam HI</td>
</tr>
<tr>
<td>XI</td>
<td>5'-GATTACGGTCACCTTCTGAGG-3'</td>
<td>5'-CTTACCCAGAATATGGAGG-3'</td>
<td>281</td>
<td>Eco RI/Bam HI</td>
</tr>
<tr>
<td>XII</td>
<td>5'-CCACGCTTCTGACATGATGATGATG-3'</td>
<td>5'-AAGGATGATGATGATGACGACG-3'</td>
<td>468</td>
<td>Pvu II/Pst I</td>
</tr>
</tbody>
</table>

The primers used in sequencing the entire coding region for the R-type PK are shown. To create appropriate restriction sites, the underlined nucleotides are added or substituted to the primers.
Table 2. Hematologic and Rheologic Data on the Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>MCV (fL)</th>
<th>Reticulocytes (%)</th>
<th>Max Dl</th>
<th>DI 290</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY</td>
<td>29</td>
<td>F</td>
<td>11.0</td>
<td>37.3</td>
<td>116.1</td>
<td>18.5</td>
<td>111</td>
<td>112</td>
</tr>
<tr>
<td>JY</td>
<td>31</td>
<td>M</td>
<td>9.3</td>
<td>27.3</td>
<td>115.8</td>
<td>25.4</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

Maximum deformability index (Max Dl) was attained by osmotic gradient ektacytometry. DI 290 is the deformability index of RBCs suspended in a buffer the osmolarity of which is 290 mOsm/kg.

Maximum region of exon 10 was amplified by PCR and examined the Hpa II cleavage site, that should be abolished by the nucleotide change. A 201-bp fragment flanking the 3' end of exon 10 was amplified by PCR. When the Hpa II site is intact, the band becomes four fragments, 132, 39, 28, and 2 bp, after Hpa II digestion. As shown in Fig 5, one of the Hpa II sites was abolished in the PCR products of the probands, meaning the homozygous mutation in the probands' L-PK gene.

DISCUSSION

Here we report a molecular abnormality in the Amish PK deficiency. The probands had suffered from severe hemolytic anemia, and required transfusion every 8 weeks. After splenectomy the anemia was compensated, with a Hb level of about 9 to 10 g/dL. Cellular studies of the PK-deficient RBCs showed increased K+ content and a markedly expanded ouabain-insensitive K+ efflux. The pathophysiology of PK-deficient erythrocytes has been studied. Keitt has shown that PK-deficient erythrocytes were almost entirely dependent on mitochondrial oxidative phosphorylation rather than on glycolysis to maintain adenosine triphosphate (ATP) levels. A rapid decrease of ATP levels because of decreased oxidative phosphorylation during maturation of erythrocytes allows massive losses of potassium and water, resulting in the formation of spiculated erythrocytes, ie, echinocytes. These cells usually have decreased deformability, and are easily sequestered by the reticuloendothelial system. Spleenectomy may prevent reticulocytes and young erythrocytes from being trapped by the reticuloendothelial system, resulting in improvement of the anemia in most cases. The hematologic data of the Amish PK deficiency showed post-splenectomy reticulocytosis, which was usually observed in PK deficiency, and the large, lower density cells with increased deformability may reflect mainly the increased number of reticulocytes. Despite the increased K+ content, these cells are prone to lose K+ and water rapidly because of impaired ATP availability, and become dehydrated and en-

Table 3. Cation Content of PK-Deficient RBCs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patient</th>
<th>Control</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+ mEq/L RBCs</td>
<td>AY</td>
<td>JY</td>
<td>11.9 ± 3.30</td>
</tr>
<tr>
<td>K+ mEq/L RBCs</td>
<td>102.0</td>
<td>84.6 ± 2.04</td>
<td>.025</td>
</tr>
<tr>
<td>Na+ + K+ mEq/L RBCs</td>
<td>113.9</td>
<td>96.4 ± 1.39</td>
<td>.005</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additive</th>
<th>Normal RBCs</th>
<th>PK-Deficient RBCs</th>
<th>( \Delta [\text{K}^+] ) Concentration/mL RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.024</td>
<td>0.034</td>
<td>0.035</td>
</tr>
<tr>
<td>1.0 mmol/L furosemide</td>
<td>0.014</td>
<td>0.037</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Cells were incubated for 2 hours at 37°C in 10 mmol/L HEPES (pH 7.5), 5 mmol/L glucose, 0.2 mmol/L ouabain, and NaCl to bring the final osmolarity to 290 mOsm/kg, in the presence or absence of the indicated additive. Values shown are the negative \( \Delta \) of the calculated intracellular K+ concentration as a function of time.
Fig 3. Hydrophobicity profiles near the mutated site. The dotted line indicates the hydrophobicity properties of normal R-PK from no. 471 to no. 490 amino acid residues, and the solid line shows the altered hydrophobic properties of the Amish PK variant. The arrows show the position of the 479th amino acid residue, which is substituted from Arg to His, and the shift of the hydrophobicity profile caused by the amino acid substitution.

trapped in the splenic environment. This hypothesis is supported by the finding of entrapped reticulocytes in the splenic red pulp of the patient’s spleen. It should be noted that the potassium efflux of the Amish PK-deficient erythrocytes was increased up to 142% to 145% of normal controls. Activation of Na⁺-independent K⁺/Cl⁻ cotransport seemed unlikely in PK deficiency, because the addition of 1.0 mmol/L furosemide (an inhibitor of K⁺/Cl⁻ cotransport) to the medium did not reduce the rate constant of K⁺ efflux. The K⁺ efflux may be increased by a mechanism similar to that observed in HbSC RBCs. The reported enzymatic characteristics of the identical variant PK were a low Michaelis constant (Km) for PEP, low Km for adenosine diphosphate, narrow nucleotide specificity, less ATP inhibition than normal, more fructose-1,6-diphosphate (FDP) required for 50% activation, thermal instability, normal optimum pH, and normal migration in polyacrylamide gel electrophoresis. It should be noted that lower response to the allosteric effector, FDP, and thermal instability were unfavorable characteristics of this variant, which may be responsible for the phenotype.

There are four mammalian PK isozymes: type-M1, M2, L, and R. The M-type PK (M-PK) gene encodes both M1 and M2 isozymes. In contrast, L and R isozymes are encoded by the L-PK gene. Both the M- and L-PK gene of rats and humans have 12 exons. Alternative splicing produces two distinct mRNAs, which contain either exon 9 (M1) or 10 (M2) of the M-PK gene. Exons 1 and 2 of the L-PK gene are specifically transcribed to the R- and L-PK mRNA by the tissue-specific promoter. It is of interest that among four isoenzymes only M1-type is not allosterically regulated. Because M1 and M2 isozymes differ only in the exon 9-10 region, exon 10 should encode the amino acid residues responsible for the allosteric property. Comparison of exon 10 between the M- and L-PK gene disclosed that there was an extensive homology, indicating that the exon 10-encoding region of L-PK gene also contained the allosteric structure (Fig 4). It seemed to be rational that the variant showed lower response to the allosteric effector, because the missense mutation identified in exon 10 would cause the drastic change of hydrophobicity and secondary structure. The amino acid substitution (R479H) resides in the third α-helix of the C domain of PK subunit, deduced from the tertiary structure of cat muscle PK. The C domain is considered to be important for the intersubunit contact as well as the allosteric regulation. Because PK has kinetic activity only as either dimer or tetramer, the structural change may interfere with tetramer formation, resulting in a drastic loss of activity.

The identification of the molecular lesion of erythrocyte PK deficiency in the Mifflin County Pennsylvania Amish enables us to detect the mutation by a simple procedure, PCR and restriction endonuclease digestion. Thus, it can be applied to genetic counseling or the prenatal diagnosis of the Amish PK-deficient hemolytic anemia, which has been known as a most severe form of PK deficiency. As only the Geauga County Ohio Amish are related to the Mifflin County Pennsylvania Amish deme by an ancestral genetic member (ie, AB), no other demes of Amish within the United States are believed to exist sharing this missense mutation or the erythrocyte PK deficiency hemolytic anemia.

Fig 4. The comparison of the amino acid sequence of the C domain of PK, encoded by exon 9 (M1) or 10 (M2, R). Open boxes surrounded by solid lines indicate amino acid residues, which are conserved in all species examined. Boxes with dotted lines show the amino acids, which are only found in the allosterically regulated isozymes, M2 and R (L). The arrow points the Arg residue, which is substituted to His in the Amish PK variant. The numbers indicate the amino acid corresponding to the Arg residues.
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Fig 5. Hpa II digestion of the PCR products flanking the 3' end of exon 10 from the probands and a normal control. Because there are three Hpa II recognition sites in the amplified DNA, the PCR product of normal control is digested into four fragments: 132, 39, 28, and 2 bp. When the 1436 G to A mutation is present, the 201-bp products are digested into three fragments: 171, 28, and 2 bp. M, pBR322 digested with Msp I; 1, normal control; 2, the Amish PK-deficient subject JV; 3, the Amish PK-deficient subject AV.

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