Primary Structure of Murine Red Blood Cell-Type Pyruvate Kinase (PK) and Molecular Characterization of PK Deficiency Identified in the CBA Strain

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To clarify the molecular abnormality of pyruvate kinase (PK) deficiency identified in the mutant mice of CBA-Pk "/" CBA-Pk "/", we cloned murine red blood cell-type PK (R-PK) cDNA of those animals. The cDNA sequence spans 3927 bp, including an open reading frame that can encode 574 amino acids. Homology in the coding sequences between murine and human R-PK was 86.1% at nucleotide and 91.5% at amino acid levels. A homozygous missense mutation at nucleotide 1013 GGT → GAT was identified in the cDNA sequence of the mutant, causing a single amino acid substitution at no. 337 Val → 337 Arg was reported to be important for the open reading frame that can encode 574 amino acids.

Because glycolysis is obligatory for mammalian red blood cells (RBCs), which lose mitochondrial proteins by the adenosine triphosphate-dependent proteolysis during maturation of reticulocytes, several glycolytic enzyme deficiencies have been reported to be associated with hemolytic anemia. Pyruvate kinase (PK) deficiency is the most prevalent hereditary nonspherocytic hemolytic anemia caused by glycolytic enzyme defects. PK has four isoenzymes in mammals, and the RBC-type PK (R-PK) is expressed almost exclusively in mature RBCs. A unique structural profile of the R-PK is its longer amino-terminal sequence compared with that of liver-type PK (L-PK), which is transcribed from the alternative promoter of the L/R-PK gene. Both in humans and rats, the primary structure of the R-PK has been elucidated by cDNA cloning. However, the biological significance of this structure remains to be clarified.

In an accompanying report, we reported murine PK deficiency (Pkm "/" Pkm "") associated with hereditary nonspherocytic hemolytic anemia found in the CBA/N strain (hereafter CBA). Although there have been several reports describing PK variants in wild or inbred mice, none had hemolytic anemia associated with decreased PK activity. PK-deficient hemolytic anemia has been described in dogs, the Basenji breed was reported to have severe hemolytic anemia and shortened life span because of myelofibrosis. Recently, the partial cDNA sequence of canine R-PK has been reported, and a homozygous frameshift mutation because of a single base deletion was identified in the Basenji dog. The Pkm "/" Pkm "" is the first murine mutant PK locus associated with chronic hemolytic anemia, and the strain is quite useful for understanding the pathophysicsology of PK-deficient hemolytic anemia as well as for exploring new therapeutic methods for the disease in humans.

In this report, we present the structure of the murine R-PK and discuss the prominent structural homology of the R-PK observed among three species, i.e., mouse, rat, and human. The molecular abnormalities identified in the Pkm "/" Pkm "" mice are also described, and the mechanism for the compensatory hemolytic anemia found in the mutant mice will be discussed.

MATERIALS AND METHODS

Materials. Murine RNA and genomic DNA were purified from peripheral blood or bone marrow cells of the CBA strain by the standard protocols. Restriction endonuclease and DNA-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). Ni-Steel GTG agarose and SeaKem HGT agarose were obtained from FMC Bioproducts (Rockland, ME). DNA was sequenced using a DNA sequencing system (model 373A; Applied Biosystems, Foster City, CA). The computer software DNASIS (Hitachi Software Engineering, Yokohama, Japan) was used to analyze the nucleotide and protein homology and to predict protein secondary structure and molecular weight of murine R-PK.

Amplification and sequencing of the murine R-PK cDNA. The oligonucleotides used in this study are listed in Table 1. We chose the oligonucleotide primers whose sequences were highly conserved between rat and human R-PK cDNAs; LA-1, ML-3, and ML-4 were used in this study.
designed from rat R-PK, and LA-2, ML-1, and ML-2 were from human R-PK. A total of 10 μg of mouse bone marrow cell RNA was reverse-transcribed in a 20-μL mixture consisting of 50 mmol/L Tris/HCl (pH 8.3), 40 mmol/L KCl, 8 mmol/L MgCl₂, 0.5 mmol/L dideoxynucleotide triphosphate (dNTP) with 25 pmol each of primer ML-1 and ML-2, and 1.25 mmol/L Tris/HCl (pH 8.3), 40 mmol/L KCl, 8 mmol/L MgCl₂, 0.5 mmol/L dNTP with 25 pmol each of primer pairs, LA-1 and -2, and 2.5 U DNA polymerase (Takara Shuzo). The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 seconds, at 60°C for 30 seconds, and at 72°C for 120 seconds in a GeneAmp PCR System 9600.

For confirmation of nucleotide change detected in the R-PK. The oligo (dT)₁₇-primed cDNA was first amplified by long PCR with the primers, LA-1 and -2, which recognized the sequence corresponding to the 3'-untranslated region and the exon 4 region of the R-PK cDNA, respectively. By using ML-1 and -2, the cDNA sequence corresponding to the exon 3 and 4 was obtained. The 9'-end sequence was reverse-transcribed in a 20-μL mixture with ML-2. A poly (A) tail was added by terminal deoxynucleotidyl transferase to the 3' end of the cDNA. The cDNA was amplified by the rapid amplification of cDNA ends (RACE) method using ML-3 as a single gene-specific primer. The PCR product was amplified with three primers, ML-3, adaptor-oligo (dT)₁₇, and adaptor, and was introduced into a plasmid vector as described. For colony hybridization, ML-4 was labeled at its 5' end with digoxigenin and used as a probe. Positive colonies were detected by chemiluminescence (DIG nucleic acid detection kit; Boehringer Mannheim Biochemica, Mannheim, Germany). The amplified R-PK cDNAs were subcloned into the PCI vector using a TA cloning kit (In-Vitrogen, San Diego, CA) or pBluescript (Strategene, La Jolla, CA). DNA sequencing was done by the dideoxy chain termination method with fluorescent primers. To confirm the nucleotide change detected in the Pk-1⁴⁰ locus, the murine L/R-PK gene was amplified by the same reaction mixture as described above with 25 pmol each of the primers ML-5 and ML-6. The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 seconds, at 60°C for 10 seconds, and at 72°C for 10 seconds in a GeneAmp PCR System 9600. The amplified DNA was digested by BstPI to test if the recognition site was destroyed by the mutation.

Polyacrylamide gel electrophoresis (PAGE) of mouse liver extracts to separate PK isoforms. Protein extracts prepared from mouse liver were homogenized in PK sample buffer containing 10 mmol/L Tris/HCl (pH 7.5), 100 mmol/L KCl, 2 mmol/L 2-mercaptoethanol, 10 mmol/L l-cysteinamide acid, and 10 mmol/L EDTA.

PK activity assay, polyacrylamide gel electrophoresis and staining for PK activity were performed as described previously. Antibody neutralization and immunoblot analysis of murine liver and erythrocyte PK. Polyclonal antisera was raised against the rat L-PK and used to characterize the isozone expression in the mouse liver. The liver PK activity was neutralized by mixing with anti-L-PK antibody, followed by overnight incubation at 4°C. The mixture was then centrifuged at 14,000 g at 4°C for 15 minutes, and the PK activity in supernatants was measured.

Erythrocyte PK was precipitated with 280 g/L ammonium sulfate, in which both the R- and M2-PK were precipitated. A total of 100 μg of liver extract and 60 μg of concentrated erythrocyte lysates were separated in 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred onto nylon membranes (Hybond N, Amersham, UK) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, 20% methanol (pH 8.3) at 30 V, at 4°C, overnight in a Mini trans-blot electrophoretic transfer cell (BioRad, Hercules, CA). After detection by chemiluminescence using a Western-light Protein Detection Kit (Tropix, Bedford, MA), the membrane was incubated with anti-rat L-PK antibody.

### Results

Primary structure of the murine R-PK deduced by the cDNA sequence analysis. Murine R-PK cDNA sequence was amplified from bone marrow RNA of the CBA strain with oligonucleotides, which were designed from the well-conserved sequences between rat and human R-PK. The oligo (dT)₁₇-primed cDNA was first amplified by long PCR with the primers, LA-1 and -2, which recognized the sequences corresponding to the 3'-untranslated region and the exon 4 region of the R-PK cDNA, respectively. By using ML-1 and -2, the cDNA sequence corresponding to the exon 3 and 4 was obtained. The 9'-end sequence was reverse-transcribed in a 20-μL mixture with ML-2. A poly (A) tail was added by terminal deoxynucleotidyl transferase to the 3' end of the cDNA. The cDNA was amplified by the rapid amplification of cDNA ends (RACE) method using ML-3 as a single gene-specific primer. The PCR product was amplified with three primers, ML-3, adaptor-oligo (dT)₁₇, and adaptor, and was introduced into a plasmid vector as described. For colony hybridization, ML-4 was labeled at its 5' end with digoxigenin and used as a probe. Positive colonies were detected by chemiluminescence (DIG nucleic acid detection kit; Boehringer Mannheim Biochemica, Mannheim, Germany). The amplified R-PK cDNAs were subcloned into the PCI vector using a TA cloning kit (In-Vitrogen, San Diego, CA) or pBluescript (Strategene, La Jolla, CA). DNA sequencing was done by the dideoxy chain termination method with fluorescent primers. To confirm the nucleotide change detected in the Pk-1⁴⁰ locus, the murine L/R-PK gene was amplified by the same reaction mixture as described above with 25 pmol each of the primers ML-5 and ML-6. The reaction mixture was subjected to 30 cycles of amplification at 94°C for 20 seconds, at 60°C for 10 seconds, and at 72°C for 10 seconds in a GeneAmp PCR System 9600. The amplified DNA was digested by BstPI to test if the recognition site was destroyed by the mutation.

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sequence, and it was confirmed by PCRrestriction fragment-length polymorphism analysis that the mutant was homozygous with this mutation (data not shown).

The amino acid change almost completely diminished the L/R-PK activity. To investigate the effects of this amino acid change on the PK activity, the liver extracts prepared from both +/+ (wild-type) and Pk-1+/− mice were separated in a thin-layer polyacrylamide gel and stained for PK activity (Fig 2). Because the R-PK activity of the mutant separated in a thin-layer polyacrylamide gel and stained for PK activity, the liver extracts prepared from both +/+ (wild-type) and Pk-1+/− mice were separated in a thin-layer polyacrylamide gel and stained for PK activity (Fig 2). Because the R-PK activity of the mutant was too low to be stained in the gel and the mutation was identified in the region that commonly encoded both the L- and R-PK, we analyzed the L-PK. In the affected mouse liver, only the M2-PK was detected, whereas the normal CBA mouse liver expressed all the PK isoforms.

Unidirectional shows the putative recognition sequences for PKC, and the asterisk (*) depicts the Ser residue necessary as a target of phosphorylation.

Fig 1. (A) Nucleotide and deduced amino acid sequences of the murine R-PK isolated from CBA mice. (B) Comparison of the N-terminal amino acid sequences of the R-PK between three species. Underlining shows the putative recognition sequences for PKC, and the asterisk (*) depicts the Ser residue necessary as a target of phosphorylation.

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samples (Fig 3). There were no differences in size or intensity of these bands between +/+ and Pk-l"/Pk-l" mice, suggesting that the amino acid substitution did not affect stability of the R- and L-PK subunit.

DISCUSSION

CBA-Pk-I"/Pk-I" was the first described PK-deficient mice strain and is associated with chronic nonspherocytic hemolytic anemia. There have been several reports in which decreased R- or L-PK activity was shown in wild mice, inbred strains, or ethylnitrosourea-treated mice. However, because these mice showed no hematologic abnormalities and the primary structure of the murine R-PK had not been elucidated, the structure-function relationships of these PK variants were not discussed. The murine R-PK structure was determined in this study; therefore "comparative" molecular analysis of reported mutant mice may provide useful information concerning PK gene expression. Both the R- and L-PK were encoded in the single WR-PK gene, and the first and second exons are specifically transcribed as the R- and L-PK mRNAs, respectively. The deduced amino acid sequence of the murine R-PK can encode 574 amino acids, as shown previously in both rats and humans. Homology analysis of the R-PK-specific amino acid sequences at the amino termini disclosed that there was a conserved region that included a putative PKC phosphorylation site at 19Ser residue (18Arg or Lys-19Ser-20Gln-21Lys or Arg). It is known that both the L- and R-PK subunits are phosphorylated by cyclic adenosine monophosphate-dependent protein kinase and that the affinity to the substrate, phosphoenolpyruvate, is decreased by phosphorylation. Previously, the PKC in RBCs was reported to phosphorylate several membrane-associated proteins such as band 4.1, 4.9, glucose transporter, and adducin, and several lines of evidence have shown that PKC as well as other protein kinases play an important role in regulation of membrane stability and Ca" transport. Whether the R-PK might be a physiological substrate for PKC remains to be investigated.

The missense mutation identified in the Pk-l"/Pk-l" mouse caused a single amino acid substitution at the active site of PK; the substitution occurred at 338Gly, and the adjacent residue 337Arg was reported to be bound to the substrate phosphoenolpyruvate. The predicted protein secondary structure included formation of a short α-helix as a result of the Gly → Asp substitution, suggesting that the mutation would cause a conformational change in the substrate binding site. This seemed to be compatible with the observation that the L-PK activity was almost completely absent despite the immunologic detection of PK subunits in the mutant liver. A similar situation was observed in the molecular analysis of human PK variant PK Hong Kong. This variant was homozygous for the missense mutation at nucleotide 941ATT → ACT, which caused a single amino acid substitution at 314Leu → Thr. The 314Leu is next to the 313Lys and the 315Glu, which are essential residues for acid-base catalysis and magnesium binding activity, respectively. As a result, the R-PK activity was decreased; alternatively the M2-PK, which disappeared during normal erythroid maturation, was observed in the PK Hong Kong RBCs. It should be emphasized that these active site mutations generated distinct hematologic manifestations, ie, mild hemolytic anemia in mice and more severe in humans. As indicated in the accompanying report, erythroid progenitor cell counts were about 2.3 times higher in the bone marrow of Pk-l"/Pk-l" than +/+ mice, whereas the spleens of the mutants contained about 66 times more erythroblasts than

![Fig 3. Immunoblot analysis of murine erythrocytes and liver extracts developed with anti-L-PK. Staining of bands that migrated at positions corresponding to about 60 (open arrow) and 62 (closed arrow) kD are L- and R-PK, respectively. There were no differences in the size or intensity of these bands between +/+ (lanes 1 and 3) and Pk-"/Pk-" (lanes 2 and 4) mice.](image-url)
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those of normal mice. Although extramedullary hemopoiesis had been observed in the spleen of human PK-deficient subjects, it was likely that splenomegaly was a consequence of the sequestration of reticulocytes rather than of ectopic erythropoiesis. Splenic erythropoiesis might play a role in the compensatory hemolytic anemia of CBA-Pk-1

In conclusion, we reported here the primary structure of murine R-PK deduced from the cDNA sequence and the homozygous missense mutation of the PK gene identified in Pk-1

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