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# Molecular Basis of Impaired Pyruvate Kinase Isozyme Conversion in Erythroid Cells : A Single Amino Acid Substitution Near The Active Site and Decreased mRNA Content of The R-type PK

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**Summary :** Conversion of pyruvate kinase (PK) isozymes from $M_2$- to R-PK has been observed during erythroid cell maturation. To understand this mechanism, we analyzed the PK gene of a R-PK deficient patient, in whose erythrocytes the $M_2$-PK was persistently expressed. A point mutation, 1102 GTC→TTC was identified in the R-PK cDNA, and it caused a single amino acid substitution from 368Val→ Phe. The residue is very close to the 372nd Gln, the putative binding site of the monovalent cation ($K^+$). The impaired $K^+$ binding would cause the decreased affinity for phosphoenolpyruvate, consequently the variant PK may be extremely unstable. Although the proband's other PK allele did not have any structural change, the R-PK mRNA level in reticulocytes was decreased. These findings suggested that both the structural mutation near the active site and the decreased mRNA level of the R-PK were responsible for the disorder. 

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40, PK) is a key glycolytic enzyme and catalyses the conversion of phosphoenolpyruvate (PEP) into pyruvate. In mammals, PK has four isoenzymes : $M_1$, $M_2$, L and R (1, 2). Among these, R-type is exclusively expressed in erythrocytes. Both in rats and humans, the L-PK gene has been isolated, and the structural analysis revealed that both the L- and R-type PK were encoded in the single gene (3-5)

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and that tissue-specific promoters were used to generate the mRNAs with the different 5'-end sequences.

PK deficiency is the most common glycolytic enzyme defect associated with hereditary hemolytic anemia (6). Enzymatic characteristics such as low affinity for PEP, thermal instability, impaired response to the allosteric effector, fructose-1, 6-diphosphate (FDP) or increased inhibition by ATP were associated with the severity of the phenotype (7). Four distinct point mutations in seven unrelated families have already been identified (8-11), and all of them caused single amino acid substitutions. Although the isozyme switches from $M_2$ to R-type during normal erythroid maturation (12-14), the R-PK has rarely been observed in the erythrocyte lysates of some severe PK deficiencies (1, 15-17). As for PK Osaka, a severe PK deficiency, the partial purification of erythrocyte PK successfully demonstrated the R-PK activity, and the variant had several aberrant enzymatic characteristics (18, 19). Because no consanguinity was observed in the family, the proband may be heterozygous with mutant PK allele. Herein, we describe a point mutation and the decreased mRNA level of the R-PK, and discuss the structure-function relationship of the variant.

## MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase and T4 DNA polynucleotide kinase were purchased from Takara Shuzo (Kyoto, Japan) or New England Biolabs (Beverly, MA). Avian myeloblastosis virus (AMV) reverse transcriptase was obtained from Seikagaku Kogyo (Tokyo, Japan). Taq DNA polymerase (AmpliTaq) was purchased from Perkin Elmer Cetus (Norwalk, CT). A DNA sequencing system, Applied Biosystems Model 373A (Foster City , CA) was used for DNA sequencing . [$\gamma$ -$^{32}$P] ATP, [$\alpha$ -$^{32}$P] dCTP and Hybond N membrane were purchased from Amersham International (UK).

Oligonucleotides

The oligonucleotides used for the cDNA cloning, PCR, and allele-specific hybridization in these experiments were, R1; 5'-CTTAAAGGTGGGGCTTTGGA-3', R2; 5'-CCCAGGCCTACACTGAAAGC-3', R3; 5'-TGTGGGCTCGAGAACGTAGACT-3', R4; 5'-CTTGGGTTCAAGCTTGTAGGC-3', R5; AGGATGGACTTTGCTAAGTC-3', R6; 5'-CCACAGCTGTCCAATGATTTG-3', R7; 5'-AGACTCAAGGCATCTTAGGGCCTGCAG-AGC-3', R8; 5'-AGGTGAGCGACGGCATCATGGT-3', R9; 5'-CCCTACAGTGTGGG-TATTCACCCA-3', R10; 5'-GCCCAGAGGGACTGGTGACCCA-3', R11; 5'-TAGCTCCTC-AAACAGCTGC-3', 1102G; 5'-AAGCCTGTTGTCTGTGCCAC-3', 1102T; 5'-AAGCCTGT-TTTCTGTGCCAC-3', G1; 5'-CGACGAGCTCCGTGAGGCCTGGC-3'; G2; 5'-AGGAA-TGTGCAGCTGAGGTCAAT-3'.

Amplification and Sequencing of the R-PK mRNA and the Promoter Region of L-PK Gene

Reticulocyte RNA was purified by using guanidine isothiocyanate. Five micrograms of reticulocyte RNA was reverse transcribed using 10 picomoles of R1 primer, 10 units of human placental ribonuclease inhibitor, 20 units of AMV reverse transcriptase in 0.5mM deoxyribonucleotides (dNTP), 40mM potassium chloride (KCl), 8mM magnesium chloride

($MgCl_2$), and 0.05 M Tris / HCl , pH8.3. After 60 minutes at 42 °C, the R-PKcDNA was amplified with primers R2 and R3. To create restriction sites for Stu I in R2 and XhoI in R3, a mismatched nucleotide was introduced in those primers. The PCR was carried out in a total volume of 100µl containing the template cDNA, dNTP at 200µM each, 50 picomole each of primers, 10mM Tris / HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin and 5 units of Taq polymerase. The PCR profile for 40 cycles in a Thermal Cycler (Perkin Elmer Cetus) was at 94 °C for 1.5 min., 55 °C for 1 min., 72 °C for 3 min. The 1.8 kb R-type PKcDNAs were digested with StuI and XhoI, and then subcloned into HincII/XhoI sites of pBluescript (Stratagene, La Jolla, CA). As previously described, there is a nucleotide polymorphism in exon 12 of the human L-PK gene, and it can be demonstrated by the restriction endonuclease BspHI digestion. Because the proband was heterozygous with the polymorphic 1705th nucleotide (10), we sequenced the cDNAs with (1705 A) or without (1705 C) the BspHI site, representing both PK alleles. The sequenced region contained the whole coding region and part of the 3'-untranslated region, spanning 1777bp.

To amplify the 5'- and 3'-flanking regions of the L-PK gene, we used R4 / R5 and R6 / R7 primers. One microgram of the proband's genomic DNA was used for the PCR, and the products were subcloned after the digestion by HindIII / PstI for R4 / R5 and PvuII / PstI for R6 / R7. Since the proband may be a compound heterozygote of mutant PK allele, we sequenced eight independent clones for the 5'-end region and the 3'-end genomic clones either with or without a BspHI cleavage site in Exon 12 region. Together, all coding and the 3' untranslated sequences for the R-type PK cDNA and the 492bp 5' - and the 283 bp of the 3'-flanking region of the L-PK gene were analyzed.

Allele-specific Oligonucleotide Hybridization Detecting 1102 G→T Mutation

We amplified a 210-bp genomic DNA fragment flanking the 3' part of exon 8 and the 5' part of intron 8 of L-PK gene using primers R8 and R9. The PCR was carried out in a total volume of 100µl containing 1 µg of template DNA, under the same conditions as the cDNA amplification. The PCR profile for 30 cycles in Perkin Elmer Cetus Thermal Cycler was at 94 °C for 1 min., 55 °C for 1 min., 72 °C for 1 min. One fifth of the products were denatured in 0.04 M NaOH, and dotted by a commercial apparatus (BioDot SF, BioRad) onto Hybond N membrane. 1102G and 1102T probes were radiolabelled with [$\gamma$ -$^{32}$P] ATP in a T4 DNA polynucleotide kinase reaction. Hybridization was performed in 5x SSPE (1xSSPE is 0.18M NaCl / 10mM phosphate, pH7.4 / 1mM EDTA), 5x Denhardt's solution, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, at 63°C for 1102G, 55°C for 1102T. The final washing conditions were 67°C for 1102G, 63°C for 1102T in 6x SSC, 0.1% SDS.

Quantification of the R-PK mRNA content on reticulocytes by the PCR-mediated method

The R-type PK / G6PD mRNA ratios in the reticulocyte RNA of PK Osaka and a reticulocyte-rich control (hereditary spherocytosis) were quantified by the PCR. Two micrograms of the reticulocyte RNA were reverse-transcribed by 10 picomoles of oligo $(dT)_{17}$ primer. One fourth of the cDNAs (corresponding to 0.5µg RNA) was used for the quantitative PCR in the presence of 50 picomoles of the R-PK primers (R10 and 11), 15 picomoles of the G6PD primers (G1 and 2) and 10 µCi of [$\alpha$ -$^{32}$P] dCTP. Fifteen µl aliquots were obtained after 28, 31, 34, 37 and 40 cycles, and directly applied to the 2% agarose gel. After electrophoresis, the gel was dried and autoradiographed overnight, and the radioactivity incorporated into the R-PK and the G6PD cDNA was measured by the Bio-Image Analyzer BAS2000 (Fuji Photo Film, Japan)(Table 1).

## RESULTS AND DISCUSSION

PK deficiency was the most common glycolytic enzyme defect causing hereditary hemolytic anemia, and more than 300 cases have been reported (6, 7). We have found sixty-nine Japanese

Table 1. *The radioactivity obtained from the R-PK and G6PD RTPCR products*

| | PCR cycles | G6PDmRNA | R-PKmRNA | PK / G6PD |
|---|---|---|---|---|
| Control | 28 | 134.9 | 377.5 | 2.80 |
| | 31 | 939.3 | 2237.1 | 2.38 |
| | 34 | 2058.2 | 3744.9 | 1.82 |
| | 37 | 2605.2 | 4173.9 | 1.60 |
| | 40 | 2906.6 | 3944.2 | 1.36 |
| PK Osaka | 28 | 871.9 | 1612.2 | 1.85 |
| | 31 | 2026.4 | 2836.1 | 1.40 |
| | 34 | 2545.0 | 3181.5 | 1.25 |
| | 37 | 3881.0 | 3853.2 | 0.99 |
| | 40 | 4092.7 | 4007.1 | 0.98 |

PK deficient families, and only ten families were considered to be true homozygote due to the consanguinity. The R-PK has scarcely been detected in the erythrocyte lysates in a few cases of severe PK deficiency (1, 15-17) . We present here the molecular analysis of a severe PK deficiency, PK Osaka. The enzymatic characteristics of PK Osaka by the standardized methods (20) are represented by high Michaelis constant (Km) for the substrate, phosphoenolpyruvate (PEP), low Km for adenosine diphosphate (ADP), abnormal nucleotide specificity, more ATP inhibition than normal, more fructose-1, 6-diphosphate (FDP) required for activation, severely decreased thermostability, normal optimum pH and slow mobility in the polyacrylamide electrophoresis (19).

We analyzed the R-PK cDNAs derived both from the 1705 C and A alleles, and found that the 1705 C cDNA had a point mutation at no. 1102, GTC to TTC, resulting in a single amino acid substitution at 368th Val to Phe (V368F) (Figure 1). However, no structural change was found in either the coding region or the 5'-, 3'-flanking regions of L-PK gene of another allele (1705A). Although the variant was heterozygous with the 1102 mutation demonstrated by the allele-specific oligonucleotide hybridization (Figure 2), this fact indicated that the enzymatic characteristics of PK Osaka were represented by the single amino acid substitution. In all tissues so far examined, PK has been found as a tetramer (21), meaning that the variant PKs may be the heterotetramer of both normal and aberrant subunit or the homodimer consisting of either normal or aberrant subunit.

A crystallographic study has demonstrated a three-dimensional structure of cat muscle (M1-type) PK (22). The subunit consists of four domains, N, A, B and C. The substituted Val residue is located in the 7th β-strand of the A domain (Aβ7) deduced from the tertiary structure. The reported amino acid substitutions (T384M, Q421K, R426Q) (8, 10) also resided in the 7th or

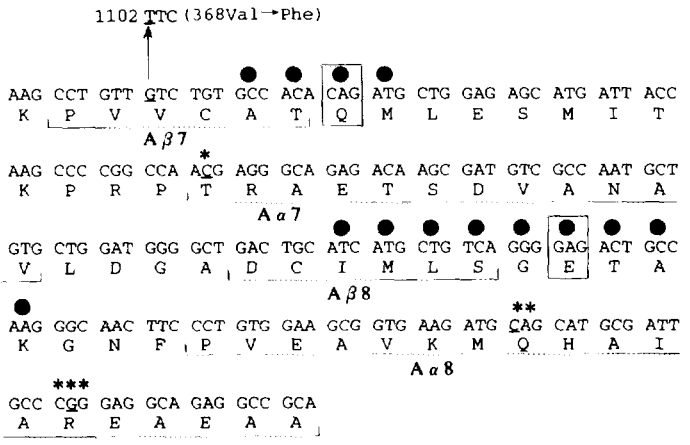


Figure 1. *Nucleotide sequencing encoding the part of A domain (Aα7, 8 and Aβ7, 8) of pyruvate kinase and the point mutations so far identified in this region.*
Open boxes indicate the 372nd Gln and 407th Glu, the putative amino acid residues for binding $K^+$. Closed circles show the amino acid residues which constitute the active site of PK. Asterisks ( *, **, ***) indicate the mutation site of PK Tokyo, Nagasaki (T384M), PK Fukushima, Maebashi (Q421K) and PK Sapporo (R426Q), respectively.

8th a-helix of A domain (Aα7 or Aα8) of PK (Figure 1). These regions are considered to be the $K^+$ binding domain of PK (23), and the $K^+$ bound enzyme has an increased affinity for PEP in the presence of $Mg^{++}$ (24). The V368F mutation might affect the interaction of $K^+$ to the 372nd Gln, a putative amino acid residue for the potassium binding.

Recently, the site-directed mutagenesis study of PK gene isolated from *Bacillus steatrothermophilus* was reported, and it revealed that the residues, 356Asp (conserved as 400Asp in human R-PK) and 444Arg (488Arg), lay at the contact between domains A (Aα7 / Aβ8) and C (Cα4 / Cβ3) by the potential salt bridge in the *R* -state. Binding of the allosteric activator may

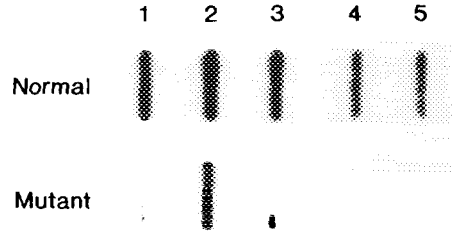


Figure 2. *Allele-specific oligonucleotide hybridization detecting 1102G→T mutation.*
A 210-bp genomic DNA fragments flanking the 3' part of the exon 8 and the 5' part of the intron 8 of L-PK gene were amplified using primers R8 and 9, and the slot-blot hybridization was performed using the allele-specific oligonucleotides, 1102G and T. The proband (1), a sister (2) and their father (3) were heterozygous with the 1102G→T mutation. Neither the mother (4) and a normal control (5) had the point mutation.

induce a reorientation of 466Trp, allowing a shift of the equilibrium to the *R* -form (25). Domain A of PK has been found to form the symmetrical eight-stranded α / β-barrel structure, and the same structure has been identified in triose phosphate isomerase, α-amylase, 2-keto-3-deoxy-6-phosphogluconate aldolase, glycolate oxidase (26). Because adjacent strands are connected by helical segments, the mutation occurring in PK Osaka might affect the conformation near Aβ8, resulting in stabilization of the variant PK in the *T*- state.

No nucleotide change was found in the cDNA or the 3'-flanking region of the L-PK gene from another allele (1705A). Neither the mutation in the 5'-untranslated region of the cDNA nor the promoter region for the R-PK transcription was detected by the examination of the 492bp of the 5' -flanking region of the L-PK gene. The titration of the R-type PK mRNA showed that the R-PK / glucose-6-phosphate dehydrogenase mRNA ratio in the proband's reticulocytes was decreased compared with the reticulocyte-rich control (Figure 3). Although the sisters and the father have the same point mutation, the R-type PK activity was much more severely affected in the sisters. These observation led us to speculate that they had inherited another mutation from the mother. These findings suggested that the variant was a compound heterozygote of the structural mutation and another mutation responsible for the low mRNA level of the R-PK. The 1705A allele might have a mutation which was responsible for the reduced transcription or the decreased stability of the R-PK mRNA.

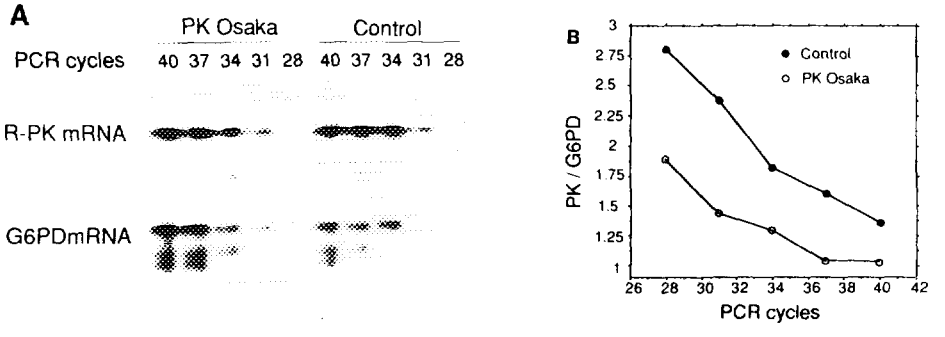


Figure 3. *The mRNA titration in the reticulocytes.*

(A) Autoradiography of the R-PK and G6PD RTPCR products. The oligo (dT) primed cDNAs synthesized from 0.5μg total reticulocyte RNA were amplified using the R-PK and G6PD primers in a single tube with [α -$^{32}$P] dCTP. Aliquots were obtained after 28, 31, 34, 37 and 40 cycle amplification and electrophoresed in a 2% agarose gel.

(B) The plot indicating the R-PK / G6PD mRNA ratios.

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