Identical Point Mutations of the R-type Pyruvate Kinase (PK) cDNA Found in Unrelated PK Variants Associated With Hereditary Hemolytic Anemia

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We cloned and sequenced the cDNAs for R-type pyruvate kinase (R-PK) from three homozygous PK patients. A point mutation (1261AGC to ATG) identified in the R-PK cDNA of PK Nagasaki was identical with that previously identified in an unrelated family, PK Tokyo. The mutation has also been identified in the PK L gene of a Lebanese family, PK Beirut, by Neubauer et al. (Blood 77:1871, 1991). These results suggest either that the point mutation is very old and that the same mutation occurs sporadically in the same hot spot in unrelated families. A point mutation, 1261AGC to AAG, was detected in both PK Fukushima and PK Maebashi; this mutation causes a single amino acid substitution, from Gln1261 to Lys, in R-PK. Consequently, the hydrophobicity properties near the mutated site are drastically changed. A silent mutation (1261AGG to CGG) was also identified in those variants. The screening of these three point mutations showed only the silent mutation in a normal individual as well as in individuals with PK variants. These results indicate that the multiplicity of the mutant PK allele is smaller than expected, and that the silent mutation is a polymorphic change commonly distributed in the Japanese population.

Materials and Methods

Patients. Case reports, laboratory data, and the enzymatic properties of PK Nagasaki, PK Fukushima, and PK Maebashi have been reported previously. Because of the consanguineous marriage of the parents, these patients are considered to be homozygous for the mutant PK alleles. Clinical histories and kinetic studies of compound heterozygotes of the PK variant, PK Osaka, PK Beppu, PK Niigata, and PK Nichinan have been described.

Materials. Restriction endonucleases and modifying enzymes were purchased from Takara Shuzo (Japan) and New England Biolabs (Beverly, MA). Taq DNA polymerase (AmpliTaq) was obtained from Cetus (Norwalk, CT). Modifying T7 DNA polymerase (Sequenase; United Biochemicals, Cleveland, OH) was used for DNA sequencing. α32P dCTP and γ32P ATP were purchased from Amersham (UK). The computer software, DNASIS (Hitachi Software Engineering, Japan), was used for analysis of the hydrophobicity properties of the variant protein based on the theory of Hopp and Woods.

Oligonucleotides. Oligonucleotides used for cDNA cloning have been described previously.9 L10 (5'-GTCTTACAACATTTGA-CATCC-3') and L12 (5'-TAGTCCCTAAAAAGACGTG-3') were used for amplifying the exon 12 region. 1261C (5'-GTGAAGATGCAGCATGC-3') and 1261A (5'-GTGAAAGATGACGATGC-3') were allele-specific oligonucleotides (ASOs) which detected a single nucleotide change at no. 1261 of R-PK cDNA.

cDNA synthesis, amplification, and sequencing of R-type PK mRNA. Purification of reticulocyte RNA from patients, PK Nagasaki, PK Fukushima, and PK Maebashi, cDNA synthesis, and amplification of the R-PK cDNA were performed as described previously. Briefly, the R-PK cDNA were amplified with four sets of primers, and subcoded into pBluescript (Stratagene, La Jolla, CA). Whole coding and 55 bp 3' noncoding sequences were sequenced by the dyeoxy method.

ASO hybridization for detecting point mutations in genomic DNA from other PK variants. To detect the mutations identified in PK Nagasaki, PK Fukushima, and PK Maebashi in the PK L gene of other PK variants, we used ASO hybridization technique. Because both 1261AGC to ATG and 1261AGC to AAG mutations are located in exon 9 of human L-PK gene (our unpublished results, January 1990), we amplified a 330-bp genomic DNA fragment that flanked whole exon 9 sequences using primers L10 and L12 under conditions previously described. To detect the 1261C to T mutation, either ASOWT or ASOPK primers were used, and 1261C and 1261A were used for 1261C to A mutation. Hybridization was performed in 5X SSPE (1X SSPE is 0.18 mol/L NaCl, 10 mmol/L phosphate pH 7.4, 1 mmol/L EDTA), 5X Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), 100 μg/mL denatured salmon sperm DNA, at 57°C for ASOWT, 54°C for ASOPK, 55°C for 1261C, and 52°C for 1261A. The final washing conditions were 60°C for ASOWT, 57°C for ASOPK, 58°C for 1261C, and 56°C for 1261A in 6X SSC.
Detection of the 1705A to C silent mutation in the Japanese population. To test whether the silent mutation, 1705A to C, is present in other PK variants and normal subjects, we amplified 0.5 μg of the genomic DNA from a normal subject and PK variants by polymerase chain reaction (PCR) with primers L21 and L22 under the conditions previously described. One fifth of the products were digested with BspHI (NEB) according to the manufacturer’s recommended conditions and electrophoresed in a composite gel of 3% NuSieve agarose (FMC Bioproducts, Rockland, ME) and 1% HGT agarose (FMC Bioproducts).

RESULTS

Identification of the same mutation as PK Tokyo in the R-PK cDNA of PK Nagasaki. Sequencing of the R-type PK cDNA from PK Nagasaki showed that the variant had the same point mutation as PK Tokyo, 1151ACG to ATG. This mutation causes a single amino acid substitution at Thr421 to Met, which causes thermal instability of the PK. This point mutation was also identical with that in the PK L gene of a Lebanese PK-deficient patient, PK Beirut, reported by Neubauer et al.

Single amino acid substitution, Gln421 to Lys, caused by a point mutation, 1264C to A, identified in the R-type PK cDNA in both PK Fukushima and PK Maebashi. Sequencing of the R-PK cDNA from PK Fukushima demonstrated a point mutation, 1264CAG to AAAG (Fig 1), which caused a single amino acid substitution, Gln421 to Lys. Although the site was not located in the active site, which has been proposed from the tertiary structure deduced from cat muscle PK, this substitution causes a diverse change of the net charge in the eighth α helix of the A domain of PK. The hydrophobicity properties of the R-type PK near the mutated site are drastically changed by this substitution (Fig 2). The R-PK cDNA of PK Maebashi also had the same point mutation.

A silent point mutation, 1705AGG to CGG, was detected in the R-type PK cDNA of PK Fukushima and PK Maebashi. A point mutation, 1705AGG to CGG, was also detected in the R-type PK cDNA of both PK Fukushima and PK Maebashi. This mutation does not change an amino acid residue (Ser). We evaluated the existence of this nucleotide change in the genomic DNA from normal subjects and PK variants by amplifying exon 12 of the PK L gene, and by BspHI digestion of the products. Because the point mutation altered the recognition sequence of this restriction endonuclease, from TCGCGA to TCGCGC, the mutation was detected by the disappearance of the BspHI cutting site in the PCR products. Figure 3 shows the agarose electrophoresis of the BspHI digest of PCR samples. This mutation was found in the PK L gene of both PK-deficient patients and a normal individual, and 8 PK alleles of 18 have this mutation. Both PK Tokyo and PK Nagasaki, the variants with 1151C to T mutation, are homozygous for adenine at no. 1705, and in contrast, both PK Fukushima and PK Maebashi, those with 1264C to A mutation, are homozygous for cytosine at the polymorphic base. Among four other compound heterozygous PK variants, PK Nichinan and PK Beppu are homozygous for 1705A, and the others, PK Niigata and PK Osaka, are heterozygous for the nucleotide at 1705.

ASO hybridization for screening the point mutations in other Japanese PK families. Because both 1151C to T and 1264C to A mutation were identified in unrelated families, we screened these mutations in other PK variants, who showed slow-electrophoretic mobility or thermal instability in their enzymatic properties. All four PK variants examined are considered to be compound heterozygotes. ASO hybridization demonstrated that PK Nagasaki was homozygous for the point mutation as well as PK Tokyo (Fig 4), and that both PK Fukushima and PK Maebashi were homozygous for the 1264C to A mutation. Among other PK variants tested, none had these point mutations.
**DISCUSSION**

We report here two point mutations in three homozygous PK variants, and a polymorphic nucleotide change found in the Japanese population. The PK variants that were considered unique have proven to be identical from our present results; the point mutation found in PK Nagasaki is identical with those previously detected in another Japanese family with PK variant, PK Tokyo8 and a Lebanese PK variant, PK Beirut, reported by Neubauer et al.9 When the biochemical characters of these three variants were compared,9,11 the parameters such as thermal stability, electrophoretic mobility, optimum pH, ATP inhibition, and FDP (Fructose-1,6-diphosphate) activation were almost identical; however, there were some discrepancies in nucleotide specificity between the three variants and in the Michaelis constant for ADP and phosphoenolpyruvate between our data11 and that of Neubauer et al.5 These discrepancies may be caused by the mutant enzymes being unstable during biochemical characterization. Similar discrepancies have been described in the biochemical and genetic analyses of the glucose-6-phosphate dehydrogenase deficiency, one of the major genetic defects which causes CNSHA.18

The fact that the mutation has been found in three unrelated families of different races means either that this mutation occurred long before racial divergence between Lebanese and Japanese, or that it occurred sporadically in the same hot spot in the human L-PK gene in unrelated families. We are inclined to favor the former explanation, because PK Tokyo and PK Nagasaki seem to be derived from a common evolutional origin judging from the fact that both of them are homozygous for the adenine at no. 1705 of the R-PK cDNA, which are polymorphic nucle-
otides in the Japanese population. A similar speculation can be applied to individuals with PK Fukushima and PK Maebashi, who are homozygous for cytosine at no. 1705. These results indicate that these mutations occurred in a common ancestor. Further information about DNA polymorphisms of the PK gene will provide insights into the origin of these PK variants.

The point mutation found in PK Fukushima causes a single amino acid substitution, Gln48 to Lys. The hydrophobicity properties are drastically changed by this substitution (Fig 2). As a result, stability of the patient's PK is severely impaired. It should be noted that both the mutation identified in PK Tokyo, "C to T, and this mutation reside in exon 9 of the human PK L gene. This observation suggests that the exon 9-encoded region may be functionally important for PK activity. The relationship between structure and function of PK will be further clarified by structural studies on PK variants.

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