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X-Linked Liver Phosphorylase Kinase Deficiency Is Associated with Mutations in the Human Liver Phosphorylase Kinase α Subunit

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Summary

Two Dutch patients with liver phosphorylase kinase (PhK) deficiency were studied for abnormalities in the PhK liver α (α_L) subunit mRNA by reversed-transcribed–PCR (RT-PCR) and RNase protection assays. One patient, belonging to a large Dutch family that expresses X-linked liver PhK deficiency, had a C3614T mutation in the PhK α_L coding sequence. The C3614T mutation leads to replacement of proline 1205 with leucine, which changes the composition of an amino acid region, containing amino acids 1195–1214 of the PhK α_L subunit, that is highly conserved in different species. The patient showed normal levels of PhK α_L mRNA. The second patient, from an unrelated family, was found to have a TCT (bp 419–421) deletion in the PhK α_L coding sequence, resulting in a phenylalanine 141 deletion. The same deletion was found in the PhK α_L coding sequence from lymphocytes of the patient's mother, together with a normal PhK α_L coding sequence. The phenylalanine that is absent in the PhKα_L coding sequence of the second patient is a highly conserved amino acid between species. Both the C3614T mutation and the TCT (bp 419–421) deletion were not found in a panel of 80 control X chromosomes. On the basis of these results, it is postulated that the mutations found are responsible for liver PhK deficiency in the two patients investigated.

Introduction

Phosphorylase kinase (PhK; ATP:phos, phosphorylase-*b* phosphotransferase; E.C.2.7.1.38) is a key enzyme in the regulation of glycogen breakdown. After a glucagon or adrenaline stimulus, PhK is activated by cAMP-dependent protein kinase. Activated PhK, in turn, converts the inactive form of glycogen phosphorylase to the active form, thus enabling liberation of glucose-1-phosphate from glycogen. PhK consists of four different subunits present in equimolar amounts in the hexadecameric holoenzyme $(\alpha\beta\gamma\delta)_4$. The catalytic site is located on the γ subunit. The α and β subunits are subjected to phosphorylation and dephosphorylation and regulate the activity of the γ subunit. The δ subunit is a calmodulin, conferring calcium sensitivity to the enzyme (for reviews, see Pickett-Gies and Walsh 1986; Heilmeyer 1991). Isoforms of several PhK subunits, encoded by different genes or generated by differential splicing, are known. There are two isoforms of the PhK α subunit, each encoded by a separate gene, called "muscle α" (α_M) (Zander et al. 1988) and "liver α" (α_L) (Davidson et al. 1992), according to their tissue-specific expression. Both genes are located on the X chromosome and have been mapped to human Xq12-13 (Francke et al. 1989) and Xp22.2-22.1 (Davidson et al. 1992), respectively. The α_M gene encodes at least two α_M subunits, α_M and α'_M, that are generated by differential splicing (Harmann et al. 1991). The α_M subunit is expressed in fast-glycolytic muscle fibers, while the α'_M subunit is the predominating isoform in slow-oxidative and fast-oxidative glycolytic muscle fibers and in heart (Jennissen and Heilmeyer 1974; Cooper et al. 1980). The α_L gene encodes an α_L subunit but probably not an α'_L subunit. The α_L cDNA has been isolated from a rabbit liver cDNA library (Davidson et al. 1992), but the existence of an α'_L cDNA was not reported. As for the α_M subunit, tissue-specific differential splicing has been found for the β subunit, whereas only a single gene encoding this subunit is known (Harmann et al. 1991). Two separate genes are found encoding the muscle (Jones et al. 1990) and testis (Calalb et al. 1992) γ subunits. The genes for the β and muscle γ subunits have been mapped to human chromosome 16q12-q13 (Francke et al. 1989) and human chromosome 7p12-q21 (Jones et al. 1990), respectively. Chromosomal localization of the testis γ gene is unknown. The complexity of PhK subunit expression is reflected in the many forms of PhK deficiency that have been described. The most frequently occurring form is human X-linked liver PhK deficiency. In the majority of cases, this deficiency

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is also detectable in erythrocytes and leukocytes (Huijing and Fernandes 1969; Schimke et al. 1973; Lederer et al. 1975). In some cases, however, normal activity is found in blood cells (Alvarado et al. 1988; Bakker et al. 1991). Other forms of PhK deficiency that have been described are the autosomal recessive form of liver PhK deficiency (Lederer et al. 1975), combined liver and muscle PhK deficiency with an autosomal recessive mode of inheritance (Lederer et al. 1980; Bashan et al. 1981), isolated heart PhK deficiency (Mizuta et al. 1984; Servidei et al. 1988), and isolated muscle PhK deficiency (Ohtani et al. 1982; Abarbanel et al. 1986), both with unknown modes of inheritance. The deficiency of heart PhK activity is the only form that is associated with early death; the other forms have less severe symptoms and are compatible with life (for review, see van den Berg and Berger 1990). Apart from these forms of PhK deficiency found in humans, several deficiencies have been described in mouse (Lyon and Porter 1963; Cohen et al. 1976; Varsanyi et al. 1980) and rat (Malthus et al. 1980). X-linked muscle PhK deficiency of the I-strain mouse was recently shown to be caused by a single-base-pair insertion in the coding sequence of the PhK α_M subunit (Schneider et al. 1993). Human X-linked liver PhK deficiency has been mapped to Xp22 (Willems et al. 1991; Hendrickx et al. 1993), i.e., to the same region to which the gene encoding the PhK α_L subunit has been mapped (Davidson et al. 1992). Therefore, the gene encoding the PhK α_L subunit is a strong candidate gene for X-linked liver PhK deficiency. The finding of either mutations in the PhK α_L coding sequence or a diminished expression of PhK α_L subunit mRNA would enhance the candidacy of this gene. In this study we have analyzed the coding sequence from the PhK α_L subunit and investigated the PhK α_L mRNA expression in Epstein-Barr virus (EBV)–transformed lymphocytes from two unrelated Dutch patients. The first patient is a member of a family that expresses X-linked PhK deficiency, while the second patient is an isolated case of liver PhK deficiency. Two different mutations were found in the PhK α_L coding sequences of the two patients, leading to an amino acid substitution in the PhK α_L subunit of the first patient and to an amino acid deletion in the PhK α_L subunit of the second patient. The relation of these mutations to the expression of liver PhK deficiency is discussed.

Patients, Material, and Methods

Patients and Material

Lymphocytes and EBV-transformed lymphocytes were obtained from four patients (P1–P4) belonging to a large Dutch family expressing X-linked liver PhK deficiency, which has been extensively described (Huijing and Fernandes 1969; Willems et al. 1990), from a Dutch boy (P5) with liver PhK deficiency and unknown mode of inheritance, and from the mother of this patient (M5). P5 is the first child and son of nonconsanguineous parents. During pregnancy the mother suffered from diabetes. Birth was normal, but the child had a hypoglycemic event in the perinatal period. He presented, at 6 mo of age, with hepatomegaly. There was a slight delay in motor development, hypertriglyceridemia, and elevation of liver transaminases. PhK activity (measured as described by Lederer et al. 1975) was <0.1 in erythrocytes (normal values 3.2 ± 0.4), suggesting the diagnosis of liver PhK deficiency.

cDNA Sequence of the Human PhK α_L Subunit

A human liver cDNA library was constructed using a bacteriophage Lambda vector (Lambda Zap; Stratagene). This library was screened with a human PhK α_L cDNA probe PC1 (Hendrickx et al. 1993). Two overlapping clones, containing the complete coding sequence of the human PhK α_L subunit, were isolated and sequenced. The complete cDNA sequence of the human PhK α_L subunit will be published elsewhere. The human PhK α_L cDNA sequence has been submitted to the EMBL data bank. The accession number is X80497.

RT-PCR and Sequencing

Total RNA was isolated from lymphocytes and EBV-transformed lymphocytes by using RNA-SR™ (Biogenesis) according to the manufacturer's instructions. For first-strand cDNA synthesis, 10 μg of total RNA was used. The primers used in the reaction were oligo-dT or HLα3r (TCACAGTCCACGTCACCATG, complementary to nt 2282 to 2263 of human PhK α_L cDNA). First-strand cDNA synthesis was performed using reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. PCR amplification of overlapping fragments was essentially as described by Saiki et al. (1988). The primer pairs used in the PCR-reactions are as follows: HLα1f (ATCCCAAGAACCGACTAAGG; nt −119 to −100) and HLα1r (CTTTGGCCACCTGTCT; complementary to nt 295 to 280); Hlα2f (AGGCCTACCGAGCTGGAG; nt 218 to 234) and HLα2r (GTGTTTCCTCAATAAGTC; complementary to nt 1374 to 1357); HLα3f (TCCTTGCCCGCTGGTGAAATC; nt 1250 to 1269) and HLα3r (TCACAGTCCACGTCACCATG; complementary to nt 2282 to 2263); HLα4f (CAGTCCTGATAGTGATTCAG; nt 1908 to 1927) and HLα4r (GCAAACTTGATCTCATGCGG; complementary to nt 3362 to 3343); and HLα5f (GGAGCCTGAACTGCTCAGGA; nt 2789 to 2808) and HLα5r (AGAGTCCGTGAGACCAGATG; complementary to nt 3990 to 3971). The PCR products were sequenced directly by using the PUC sequencing kit (Boehringer Mannheim) according to the manufacturer's instructions.

Restriction Analysis of PCR-Amplified Genomic DNA Fragments

Genomic DNA was isolated from EBV-transformed lymphocytes of P1–P5 and from lymphocytes of P5, M5, and

40 female controls, by established methods. A 102-bp genomic DNA fragment was amplified from DNA of P1–P4 and from DNA of 40 female controls, with PCR as described above, using HLα6f (GCCACTTCTTTTATGACAGC; nt 3590 to 3609) and HLα6r CCGAATTGGGCAACAATTCC; complementary to nt 3691 to 3672) as primer pair for the reaction. The PCR products were digested with the restriction enzyme *Dde*I (Boehringer) (recognizing the sequence CTNAG) and then were analyzed on a 3% agarose (Pharmacia) gel. A second 101-bp DNA fragment was PCR-amplified from DNA of P5, M5, and 40 female controls, with HLα7f (TACAACACCGCCACCTGTGG; nt 346 to 365) and HLα7r (GTCATCTGGGCCAGGAAGAG; complementary to nt 446 to 427) as primer pair for the reaction. The PCR products were digested with *Ksp*632I (Boehringer) (recognizing the sequence CTCTTCN) and were analyzed on a 3% agarose (Pharmacia) gel.

RNase Protection Assay

Total RNA from EBV-transformed lymphocytes and normal lymphocytes was isolated as described above. In each experiment 10 μg of RNA (based on E_{260} measurements and confirmed with gel electrophoresis) was used. A PhK α_L cDNA clone of 352 bp, containing bases 747–1098 of the coding sequence of the PhK α_L subunit, was cloned into a Bluescript plasmid. Full-length antisense RNA probe was made by using T_3 RNA polymerase (2.5 U/reaction; RT Biotechnology) in a reaction mixture containing 0.25 μg of linearized probe, 40 mM Tris HCl pH 8.0, 7 mM $MgCl_2$, 25 mM NaCl, 500 μM ATP, 500 μM CTP, 500 μM GTP, 10 mM DTT, 2 mM spermidine, 0.5 U of RNAsin (Promega), and 5 μM ^{32}P-labeled UTP (600 Ci/mmol; Amersham), in a total volume of 15 μl. The mixture was incubated for 3 h at room temperature. Subsequently, 1 U of RNase-free DNase (Promega) was added, and the mixture was incubated for another 15 min at 37°C. The labeled probe was purified by extraction with an equal volume of phenol/chloroform, followed by two extractions with an equal volume of chloroform alone, and was precipitated with 2.5 vol of ethanol. The probe was redissolved in water and was diluted to 20,000 cpm/μl. The probe was used on the same day that it was prepared, and for each experiment 1 μl of diluted probe was used.

The RNase protection assays were carried out by using the RPA II™ kit (Ambion) according to the manufacturer's instructions. The protected fragments were run on a 5% polyacrylamide gel. After drying, the gel was exposed for 72–96 h to a film (X-OMAT™ AR; Kodak). The gels were then analyzed with a phosphor imager (Molecular Dynamics), and the signal was quantitated by delineating a rectangle around the signal, followed by volume integration with the Image Quant™ (Molecular Dynamics) program.

Results

Activities of PhK in EBV-Transformed Lymphocytes

PhK activity in control lymphocytes was 132.2 ± 16.4 U/mg protein (n = 4). PhK activity in control EBV-transformed lymphocytes was 92.2–119.9 U/mg protein (n = 2). Thus PhK activity in EBV-transformed lymphocytes of normal individuals was comparable to PhK activity in control lymphocytes. The PhK activities of the EBV-transformed lymphocytes of P1–P4, all belonging to a large Dutch family expressing X-linked liver PhK deficiency, vary from 4.2 to 20.9 U/mg protein and are <20% of normal. The activity in EBV-transformed lymphocytes of P5, an isolated case of liver PhK deficiency, was 13% of normal. It was likely, therefore, that all batches of EBV-transformed lymphocytes from patients with liver PhK deficiency investigated were expressing the deficient gene and could be used to search for mutations in the coding sequence of the PhK α_L subunit and to establish the effect of possible mutations on PhK α_L subunit mRNA expression.

Mutations in the Coding Sequence from the PhK α_L Subunit

The complete coding sequences of the PhK α_L subunit from P1 and P5 were sequenced after PCR-amplification of overlapping fragments from reverse-transcribed total RNA from EBV-transformed lymphocytes. The obtained PCR products were not cloned into a vector but were sequenced directly to exclude erroneous mutations generated by *Taq* polymerase during amplification. The patient sequences were compared with the cloned PhK α_L cDNA sequences and with the PhK α_L coding sequences of control EBV-transformed lymphocytes. In P1, a C3614T mutation was found, leading to an amino acid replacement of proline 1205 by leucine (fig. 1). No other deviations from the normal cDNA sequence were seen. For further analysis of the C3614T mutation, the PCR fragment harboring the mutation (nt 3362 to 3990) was amplified with RT-PCR from total RNA from EBV-transformed lymphocytes of P2–P4, all patients from the same family, followed by sequencing. We found that the mutation was also present in the PhK α_L coding sequences from P2–P4. The C3614T mutation adds a *Dde*I restriction site to the PhK α_L coding sequence (fig. 1*B*). When a genomic DNA fragment of 102 bp harboring the mutation was PCR-amplified from DNA of P1–P4 and was digested with *Dde*I (see Patients, Material, and Methods), a 76-bp and a 26-bp fragment were generated (fig. 2*A*). When the corresponding DNA sequence was amplified from a panel of 80 normal X chromosomes, the fragment was not digested by *Dde*I (results not shown).

In P5 a 3-bp deletion was found in PhK α_L cDNA amplified from total reverse-transcribed RNA from EBV-transformed lymphocytes. A TCT triplet corresponding to bp 419–421 was absent (fig. 3). This leads to deletion of phe-

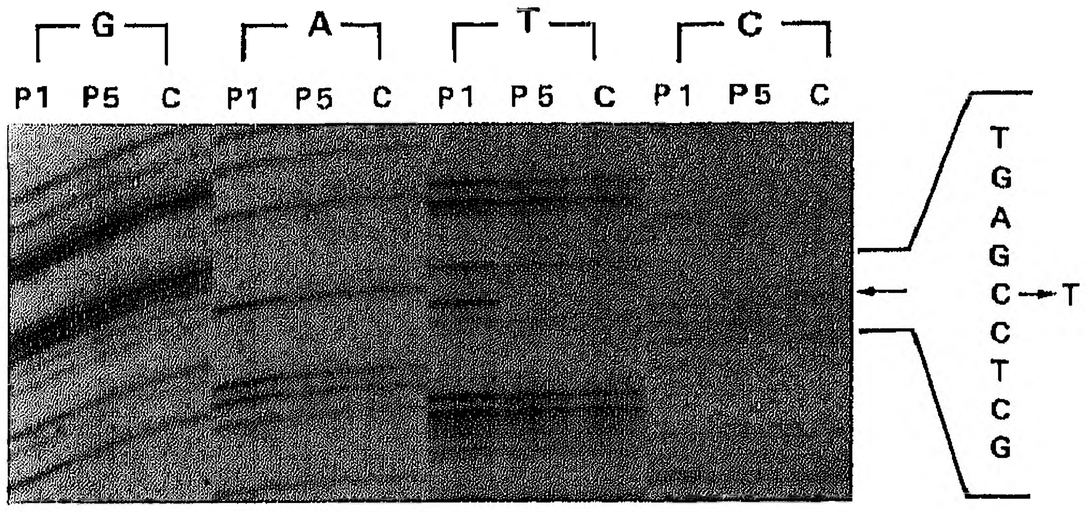


Figure 1 Partial coding sequence of the PhK α_L subunit of P1, showing the C3614T mutation (*A*) and the influence of the mutation on restriction by *Dde*I and amino acid sequence (*B*). *A*, Part of the PhK α_L coding sequences of P1, P5, and a control (lanes C). The G's of the three sequences are shown in three adjacent lanes, with the order in which they are brought onto the gel written above the lanes. The same is done for the A's, T's, and C's. The C3614T mutation (*arrow*), visible in the sequence of P1, was not present in the PhK α_L coding sequence of P5. *B*, Influence of the C3614T mutation on digestion by *Dde*I and amino acid sequence. The amino acid sequences are presented above and below the cDNA sequences of the control and of P1, respectively. The *Dde*I restriction site (CTNAG), generated by the mutation, is underlined in the P1 cDNA sequence. The C3614T mutation in the sequence of P1 is marked with an asterisk (*).

nylalanine 141. No other deviations from the normal PhK α_L sequence were detected. The TCT (bp 419–421) deletion was also found in the PhK α_L coding sequence from lymphocytes of the same patient and from lymphocytes of the mother, where both a normal sequence and a sequence lacking the TCT triplet were found (fig. 3). The intensity of the signal in lymphocytes of the mother was the same for the normal and mutated sequences. Because of the TCT deletion, a *Ksp*632I restriction site is lost from the PhK α_L coding sequence (fig. 3*B*). After PCR amplification of a genomic DNA fragment of 101 bp, comprising bp 419–421 of the PhK α_L coding sequence, followed by digestion by *Ksp*632I (see Patients, Material, and Methods), two fragments, one of 79 bp and one of 22 bp, will normally be generated. When DNA of 40 female controls was used as template for the PCR reaction, all 101-bp fragments were digested by *Ksp*632I. When the corresponding fragment from DNA of P5 was treated with *Ksp*632I, no digestion of the fragment was seen. Amplified DNA of the mother showed a normal digested and a mutated fragment (fig. 2*B*).

Expression of PhK α_L Subunit mRNA in EBV-Transformed Lymphocytes

Expression of the PhK α_L mRNA was examined with RNase protection assays, using a ^{32}P-labeled antisense RNA probe of 352 bp. The probe was chosen in a region that contained no known splicing variant and in such a way that neither the C3614T mutation nor the TCT (bp 419–421) deletion was located within the probe region. The relative amount of PhK α_L mRNA was measured as described above (see Patients, Material, and Methods). The expression of PhK α_L mRNA in P1–P4 varied from 76% to 96% of expression in control EBV-transformed lymphocytes (fig. 4). The mRNA expression for the PhK α_L sub-

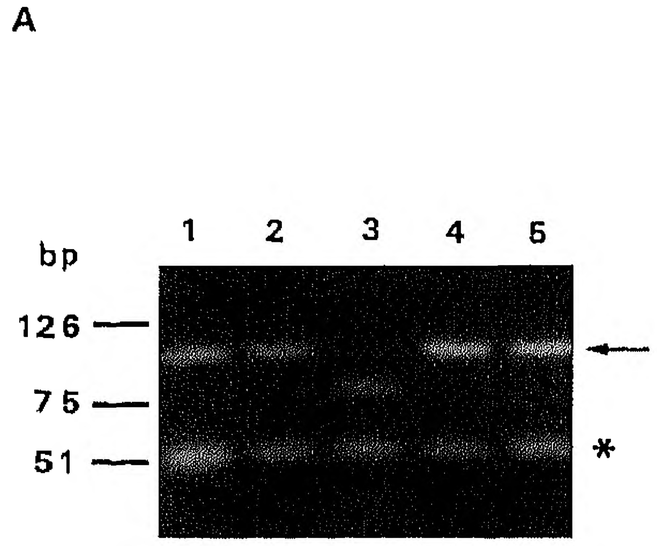
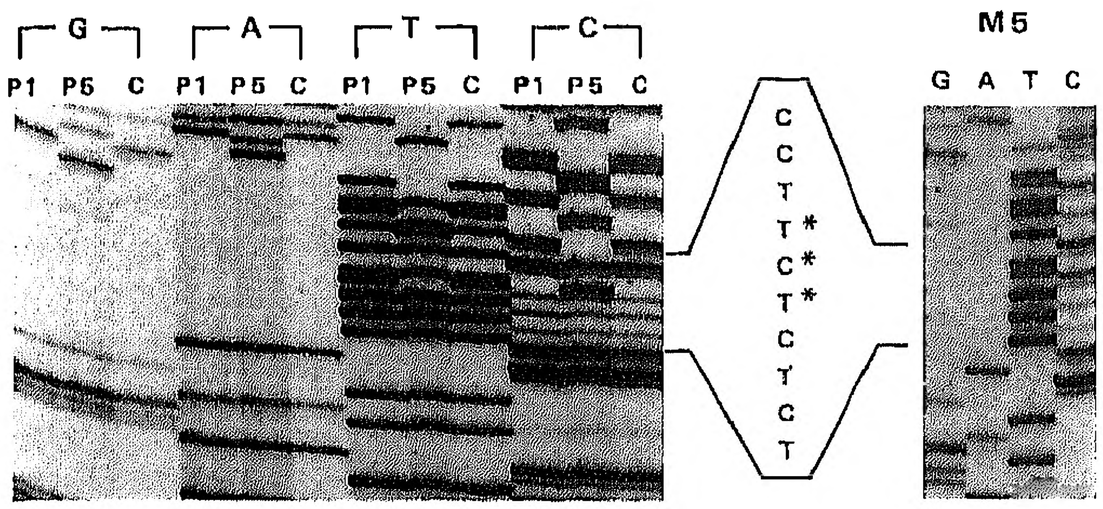


Figure 2 PCR-amplification of PhK α_L DNA fragments, followed by digestion with *Dde*I (*A*) or *Ksp*632I (*B*) and analysis on agarose gels. *A*, *Dde*I cutting of a PCR-amplified 102-bp fragment (described in Patients, Material, and Methods) from DNA of P1 harboring the C3614T mutation (lane 3). The corresponding DNA fragment is not digested by *Dde*I if control DNA is used as a template (*arrow;* lanes 2, 4, and 5) and has the same length as the untreated PCR fragment (lane 1). The band marked with an asterisk (*) is a nonspecific PCR product that is also seen in the negative control. *B*, *Ksp*632I restriction site present in a PCR-amplified PhK α_L fragment (described in Patients, Material, and Methods) of control DNA (lane 4) but not found in the corresponding DNA fragment of P5 (lane 2). In the mother, two fragments are seen (lane 3), a normal fragment (*arrow*) and a fragment that contains the TCT deletion. The untreated PCR product is shown in lane 1.



B

		Q	V	D	A	T	S	L	F	L	L	F
control	400	CAG	GTG	GAT	GCC	ACC	TCT	CTC TTC		CTC	CTG	TTC
								** *				
P5	400	CAG	GTG	GAT	GCC	ACC	TCT	C	TC	CTC	CTG	TTC
		Q	V	D	A	T	S	L		L	L	F

Figure 3 Partial coding sequence of the PhK α_1 subunit of P5, showing a TCT deletion (*A*) and influence of the mutation on restriction digestion by *Ksp*632I and on amino acid sequence (*B*). *A*, RT-PCR–amplified PhK α_1 cDNA sequences from EBV-transformed lymphocytes of P5, P1, a control (lanes C) and the mother of P5 (M5), showing a TCT (bp 419–421) deletion from the sequence of P5 and from one of the two coding sequences of the mother. The sequences of P1, P5, and the control are brought onto the gel in the same way as described in fig. 1 and are shown in the left-hand panel. The sequence of the mother is shown in the right-hand panel. *B*, Influence of the TCT (bp 419–421) deletion from the PhK α_1 coding sequence of P5 on digestion by *Ksp*632I and on amino acid sequence. The amino acid sequences corresponding to the cDNA sequences of the control and of P5 are written above and below the cDNA sequences, respectively. The *Ksp*632I restriction site is underlined in the control sequence. The bases marked with an asterisk (*) are deleted in the sequence of P5.

unit in EBV-transformed lymphocytes of P5 was 81% of normal.

Discussion

Two different mutations in the human PhK α_1 subunit coding sequence were found in two unrelated kindreds. P1 belongs to a Dutch family expressing X-linked liver PhK deficiency in several generations. The PhK α_1 coding sequence of P1 shows a C3614T point mutation, which leads to exchange of proline 1205 for leucine (fig. 1). The mutation was also present in the PhK α_1 coding sequences of three affected family members of P1. Proline 1205 is completely conserved between species and lies in a highly conserved amino acid region of the α subunits, in the middle of a group of seven amino acids that are conserved in the human and rabbit liver α protein sequences and in the human, rabbit, and mouse muscle α subunits (fig. 5*A*). Furthermore, this region shows amino acid homology with amino acids 1054–1079 of the rabbit muscle β subunit (46% identity and 19% conservative substitutions; Kilimann et al. 1988), and proline 1205 (amino acid 1059 of the β subunit) is conserved in the rabbit muscle β sequence (fig. 5*A*). No other mutations in the entire coding sequence of EBV-transformed lymphocyte PhK α_1 have been found. Furthermore, the mutation was not detected in DNA of

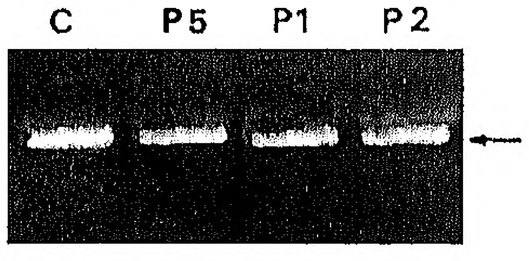


Figure 4 RNase protection assay of the PhK α_1 subunit in EBV-transformed lymphocytes from three patients with liver PhK deficiency. The RNase protection assay was performed as described in Patients, Material, and Methods. The amounts of the 352-bp protected fragment in EBV-transformed lymphocytes of the patients (*arrow*; lanes 2–4) are comparable to the amount found in control EBV-transformed lymphocytes (lane 1).

A

HLα	1195	G	I	C	H	F	F	Y	D	S	A	P	S	G	A	Y	G	T	M	T	Y
RLα	1195	G	I	C	H	F	F	Y	D	S	A	P	S	G	A	Y	G	T	M	T	Y
HMα	1183	G	I	C	T	L	L	Y	D	S	A	P	S	G	R	F	G	T	M	T	Y
RMα	1197	G	I	C	T	L	L	Y	D	S	A	P	S	G	R	F	G	T	M	T	Y
MMα	1201	G	I	C	T	L	L	Y	D	S	A	P	S	G	R	F	G	T	M	T	Y
RMβ	1049	D	D	M	T	S	F	Y	N	T	P	P	L	G	K	R	G	T	C	S	Y

B

HLα	132	H	L	Q	V	D	A	T	S	L	F	L	L	F	L	A	Q
RLα	132	H	L	Q	V	D	A	T	S	L	F	L	L	F	L	A	Q
HMα	132	H	L	Q	L	D	A	T	S	V	Y	L	L	F	L	A	Q
RMα	132	H	L	Q	L	D	A	T	S	V	Y	L	L	F	L	A	Q
MMα	132	H	L	Q	L	D	A	T	S	V	Y	L	L	F	L	A	Q
RMβ	169	H	L	Q	M	D	A	I	S	L	Y	L	L	T	L	A	Q

Figure 5 Amino acid sequence similarities of the two regions of the human PhK α_1 coding sequence containing the mutations found in P1 (*A*) and P5 (*B*). *A*, Similarity comparison of the region containing proline 1205. Proline 1205, which has been exchanged for a leucine in the PhK α_1 coding sequence of P1, is a highly conserved amino acid that is present in all PhK α subunits sequenced so far. The region comprising the mutation is also homologous to amino acids 1054–1079 of the rabbit muscle β sequence, and the proline is conserved in this rabbit muscle β (RMβ) sequence. *B*, Similarity comparison of the region containing phenylalanine 141. Phenylalanine 141, which is deleted in the PhK α_1 coding sequence of P5, is conserved between human liver α (HLα) and rabbit liver α (RLα) and is substituted by a tyrosine in human muscle α (HMα), rabbit muscle α (RMα), mouse muscle α (MMα), and rabbit muscle β (RMβ), which is a conservative substitution. The amino acid sequence surrounding the HLα phenylalanine 141 is highly conserved in all the sequences mentioned, including the RMβ sequence. HLα = human liver α; RLα = rabbit liver α (Davidson et al. 1992); HMα = human muscle α (Wöllrich et al. 1993); RMα = rabbit muscle α (Zander et al. 1988); MMα = mouse muscle α (Schneider et al. 1993); and RMβ = rabbit muscle β (Kilimann et al. 1988).

40 female controls, indicating that the mutation is not a common polymorphism. Another possible reason for the deficiency might be a diminished expression of PhK α_L mRNA, caused by a mutation either in the promoter region of the gene or in the 5′ UTR of the mRNA. We found that there is a normal level of PhK α_L mRNA expression in EBV-transformed lymphocytes of P1–P4, as was determined with RNase protection assays (fig. 4). This was found both when the results were based on quantitation of total RNA by measuring E_{260} values and when a human actin antisense mRNA probe was used as a control for estimation of the expression level of PhK α_L mRNA (results not shown). This indicates that there is a normal transcription of the PhK α_L gene in EBV-transformed lymphocytes of P1–P4. Thus it is highly probable that the C3614T point mutation in the human liver PhK α_L subunit causes the defect in this family with X-linked PhK deficiency.

In P5, who is suffering from liver PhK deficiency with unknown mode of inheritance, a TCT triplet was missing in the PhK α_L coding sequence from EBV-transformed lymphocytes, giving rise to a phenylalanine 141 deletion. The same deletion was found in the PhK α_L coding sequence from lymphocytes of the patient and from lymphocytes of the mother, in the latter together with a normal coding sequence (fig. 3). This implies that the mother is a carrier for the mutation. The mutation was not found in DNA from a panel of 80 normal X chromosomes, again indicating that the deletion is not commonly found in the normal population. Phenylalanine 141 lies in an amino acid region that is highly conserved in human and rabbit liver α protein sequences and in human, mouse, and rabbit muscle α protein sequences (fig. 5*B*). The function of this region is unknown. The region is also highly homologous with amino acids 168–189 of the rabbit PhK β subunit (59% identity and 27% conservative substitutions; Kilimann et al. 1988). The PhK α_L mRNA expression in EBV-transformed lymphocytes of P5 was normal, as estimated with RNase protection assays (fig. 4). This result correlates with the finding that in lymphocytes of the mother the signals of the mutated and the normal PCR-amplified sequences were of equal strength (fig. 3*A*). It is very likely therefore that the TCT deletion is responsible for the liver PhK deficiency in this patient, i.e., that he is suffering from the X-linked form of liver PhK deficiency.

Knowledge of the mutations in the PhK α_L coding sequences of two kindreds expressing liver PhK deficiency enables both unambiguous diagnosis of future patients belonging to these kindreds and carrier detection. Genetic testing of family members is greatly facilitated by the fact that both mutations give rise to an altered restriction pattern of the PhK α_L coding sequence (fig. 2). In conclusion, our results strongly indicate that the X-linked form of liver PhK deficiency in two unrelated kindreds is caused by two different single mutations in the coding sequences of the PhK α_L subunit. It is suggestive, therefore, that X-linked liver PhK deficiency will be molecularly heterogeneous. The search for mutations in additional families will be hampered because of the large size of the mRNA that exceeds 4,500 nt. However, if carrier detection is sought in other families in which liver Phk deficiency is diagnosed, it will be necessary to establish the underlying genetic defect in each of these families.

Acknowledgments

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