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Autosomal Recessive Phosphorylase Kinase Deficiency in Liver, Caused by Mutations in the Gene Encoding the β Subunit (*PHKB*)

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Summary

The association of autosomal recessive phosphorylase kinase deficiency in liver of a 3 ½-year-old female child with mutations in the gene encoding the common part of the β subunit of phosphorylase kinase is reported. The proband had a severe deficiency of phosphorylase kinase in liver, while the phosphorylase kinase activity in erythrocytes was only slightly diminished. She had no symptoms of muscle involvement. The complete coding sequences of the liver γ subunit and of the β subunit of phosphorylase kinase of the proband were analyzed for the presence of mutations, by either reverse-transcribed PCR or SSCP analysis. Three deviations from the normal sequence were found in the region encoding the common part of the β subunit of phosphorylase kinase—namely, a 1827G→A (W609X) transition, a 2309A→G (Y770C) transition, and a deletion of nucleotides 2896–2911—whereas no mutations were detected in the sequence encoding the liver γ subunit of phosphorylase kinase. The 1827G→A mutation and the deletion both result in the formation of early stop codons. Investigation of DNA showed that the deletion is caused by a splice-acceptor site mutation (IVS30^{-1},g→t). Family analysis revealed that the 1827G→A and IVS30^{-1},g→t substitutions are located on different parental chromosomes and that compound heterozygosity for these mutations segregates with the disease. The 2309A→G mutation was detected in 2%–3% of the normal population. Thus, it is concluded that the deficiency of phosphorylase kinase in this proband is caused by compound heterozygosity for the 1827G→A and the IVS30^{-1},g→t mutations and that the 2309A→G mutation is a polymorphism. This implies that a defect in the sequence encoding the common part of the β subunit of phosphorylase kinase may present as liver phosphorylase kinase deficiency.

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Introduction

Phosphorylase kinase (PhK; ATP:phosphorylase b phosphotransferase; E.C.2.7.1.38) plays an essential role in glycogen breakdown. It catalyses the activation of phosphorylase by phosphorylation. Activated phosphorylase, in turn, catalyses the liberation of glucose-1-phosphate from glycogen. PhK consists of four different subunits—α, β, γ, and δ—which assemble to form the holoenzyme $(\alpha\beta\gamma\delta)_4$. The activity of the catalytic site–containing γ subunit is modified by the phosphorylation state of the α and β subunits, whereas the δ subunit, which is a calmodulin, confers calcium sensitivity to the enzyme (for reviews, see Pickett-Gies and Walsh 1986; Heilmeyer 1991). From molecular biological studies and from isolation and characterization of isoenzymes of PhK, it has become known that there are two separate genes encoding isoforms of the α subunit of PhK (Zander et al. 1988; Davidson et al. 1992), a single gene encoding several isoforms of the β subunit of PhK (Harmann et al. 1991), two genes that encode isoforms of the γ subunit of PhK (Hanks 1989; Jones et al. 1990; Maichele et al. 1996), and at least three genes encoding the δ subunit of PhK (Scambler et al. 1987).

Tissue-specific expression has been described for the genes encoding the isoforms of the α and γ subunits of PhK, whereas no tissue specificity has yet been found for the expression of the δ subunit–encoding genes. It has been hypothesized that the δ subunit of PhK may be provided by each of the calmodulin-encoding genes (Bender et al. 1988). Tissue-specific variation of amino acid areas of the β subunit of PhK is generated by differential splicing of the product of a single gene (Harmann et al. 1991).

The isoforms of the subunits, which form the constituents of the muscle isoenzyme of PhK, have been called "muscle α" (α_M), "muscle β," "muscle γ," (γ_M), and "muscle δ." The gene encoding the α_M subunit is located on human chromosome Xq12-q13 (Francke et al. 1989), the β subunit lies on human chromosome 16q12-q13 (Francke et al. 1989), and the γ_M-encoding gene has been mapped to human chromosome 7p12-q12 (Jones et al. 1990). The isoenzyme isolated from human liver (I. E. T. van den Berg, H. Van Faasen, E. A. C. M. Van Beurden, H. E. M. Malingré, W. H. Lamers, M. Bollen, and R.

Berger, unpublished data) consists of the liver α (α_L) subunit, which maps to human chromosome Xp22 (Davidson et al. 1992), the β subunit, the testis or liver γ (γ_{TL}) subunit, which is located on chromosome 16p11-p12 (Whitmore et al. 1994), and a δ subunit. The γ subunit of the liver isoenzyme was initially called "PhK-γT," because its mRNA is highly abundant in testis tissue (Hanks 1989). The subunit has been renamed "γ_{TL}" as a result of the finding that defects in the gene encoding this isoform of the γ subunit can cause autosomal recessive deficiency of PhK in liver (Maichele et al. 1996).

Deficiency of PhK may be caused by a defect in each of the genes encoding the isoforms of the α, β, and γ subunits of PhK (reviewed in van den Berg and Berger 1990). In line with the hypothesis that the δ subunit can be supplied by different genes, it is expected that a defect in one of these genes will not lead to deficiency of PhK. The tissue-specific expression and the chromosomal location of the affected gene will be reflected in the phenotype and the mode of inheritance of the consequent deficiency of PhK. Thus, on the basis of the tissues that are involved and on the basis of the mode of inheritance of the various forms of PhK deficiency, candidate genes can be assigned.

In this study, the genetic defect causing deficiency of PhK in liver of a young female patient was elucidated. Likely candidates for this form of PhK deficiency are the gene encoding the γ_{TL} subunit of PhK (Maichele et al. 1996) and the nonmuscle region of the gene encoding the β subunit of PhK. Both these candidates are located on autosomes and are expressed in liver but not in muscle. In the patient described here, no mutations were detected in these two candidate sequences. Instead, two mutations that lead to the formation of early stop codons were detected in the region of the β gene, which is expressed in liver and in muscle. This is, to our knowledge, the first patient described with a phenotype of liver PhK deficiency that is associated with mutations in the gene encoding the β subunit of PhK.

Patients, Material, and Methods

Case History

The proband, a girl 3 ½ years old at the time of mutation analysis, was born after an uneventful pregnancy, as the third child of nonconsanguineous parents. The parents and the two elder siblings were healthy, and there was no history of metabolic liver disease in the family. Recently, a fourth, female child was born.

At the age of 23 mo, the proband was referred to a paediatrician because of hepatomegaly. She had a history of hypoglycemia after prolonged fasting. Clinical examination showed a girl with a doll facies and a distended abdomen, which were due to hepatomegaly. Her length was at the 10th percentile, and her weight was at the 90th percentile. There was no hypotonia or muscle weakness. Serum analysis revealed elevated transaminases, LDH, and triglycerides. These findings were indicative of a glycogen-storage disease with a mild clinical presentation. Measurement of the activity of debranching enzyme (deficient in glycogen-storage disease type III) in erythrocytes yielded high values (table 1), whereas the activity of PhK was slightly diminished but well above the values found for patients with X-linked liver PhK deficiency. Repeated measurement of PhK activity in erythrocytes from a second blood sample yielded a normal value (table 1).

A liver biopsy showed storage of glycogen without fibrosis. Measurement of PhK in liver revealed a strongly diminished activity of PhK (7% of the lowest control value) in liver of the proband (table 1). Because of the absence of muscle involvement, a muscle biopsy was not consented to by the parents. It was concluded that the patient suffered from glycogen-storage disease in the liver and that the disease was due to deficiency of liver PhK. PhK activity in erythrocytes of the newborn sister was slightly diminished (table 1).

Isolation of RNA and DNA

Total RNA was isolated from lymphocytes of the patient, the parents, and a control, by use of Tripure™ (Boehringer Mannheim) according to the manufacturer's instructions. DNA was isolated from peripheral blood lymphocytes of the patient, the parents, the sister, the brother, and 40 control persons, by established methods. DNA of the newborn sister was isolated from cord blood by use of established methods.

Isolation of cDNA Clones Containing Sequences Encoding the β Subunit of PhK

A human liver cDNA library was constructed by cloning the liver cDNAs into the polylinker of a bacteriophage lambda vector (Lambda Zap; Stratagene) with EcoRI. The library was screened with a ^{32}P-labeled β probe from rabbit skeletal muscle, βPhK-D1N (Kilimann et al. 1988), which was provided by Dr. M. W. Kilimann (Institut für Physiologische Chemie, Ruhr-Universität, Bochum). Five positive clones were isolated by this method. The clone with the largest insert (PhKβ12), was sequenced completely. Sequencing was performed by use of Sequenase™ version 2.0 (US Biochemical) according to the manufacturer's instructions. The selected cDNA clone consisted of 3,015 nt, of which nt 1–2040 were highly similar to nt 1249–3279 of the β sequence from rabbit skeletal muscle (Kilimann et al. 1988), with the exception of nt 1088–1178 of the human liver cDNA clone, which encoded the alternatively spliced nonmuscle region of the β subunit of PhK (Harmann et al. 1991). This cDNA sequence has been submitted to the EMBL data bank and has accession number X84897.

Table 1

Measurement of Activities of Debranching Enzyme and Phosphorylase Kinase in Erythrocytes and of Phosphorylase Kinase in Liver of Proband

	Activities in[a]		
	Erythrocytes[b]		
	Debranching Enzyme (nmol/24 h/mg Hb)	Phosphorylase Kinase (nmol/min²/mg Hb)	Liver Phosphorylase Kinase (nmol/min²/mg protein)
Proband	88, 76	4.2, 6.9	25
Newborn		3.7, 4.6	
Controls	10–45 (n = 40)	6–18 (n = 30)	350–1050 (n = 6)
X-linked PhK deficiency		0–2.7 (n = 21)	

[a] n = Number of subjects on which range is based.
[b] Two numbers for a given entry denotes that measurements were taken from two different blood samples.

Reverse Transcriptase–PCR (RT-PCR) and Sequencing of the Coding Region of the Human γ_{TL} Subunit of PhK

Approximately 5 μg of total RNA of the patient and a control was transcribed to cDNA with oligo-dT as a primer, by use of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. PCR amplification was essentially as described by Saiki et al. (1988). A 1,413-nt fragment was amplified by PCR using the primers CCTTCAGGATGACGCTG (87-102 of PhK-γT [Hanks 1989]) and ATGGCTCCCTTCCACCC (complementary to nt 1499–1483 of PhK-γT [Hanks 1989]) and was subcloned into the TA vector (Invitrogen). Several clones were sequenced by the AmpliCycle™ sequencing kit (Perkin-Elmer) and sequence-derived primers, according to the manufacturer's instructions.

RT-PCR and Mutation Analysis of Part of the Coding Region of the β Subunit of PhK

PCR amplification of nt 79–2057 of the isolated cDNA sequence PhKβ12 was as described by Saiki et al. (1988), with the following primer pairs: (1) HLBF (GGGGACAAGCACTTTATATCATCG, nt 79–102 of the PhKβ12 clone) and HLBR (TGTTTGCCCTGCACTAAAAC, complementary to nt 1177–1158 of the PhKβ12 clone) and (2) HLBFA (ACCCACCCACGAAATTCTTC, nt 917–936 of the PhKβ12 clone) and HLBRA (ACAGAGCTTCCTACACCTTC, complementary to nt 2057–2038 of the PhKβ12 clone). The PCR products were sequenced directly as described above, by use of sequence-derived primers.

Sequencing of DNA Fragments

DNA of the patient, her family, and a control was amplified as described above, by the following primer pairs: HLB-20F and HLB-20R, HLB-25F and HLB-25Rm, and HLB-31F and HLB-31R. The primers are listed in table 2. The PCR products were sequenced as described above, with HLB-20F, HLB-25F, and HLB-30R used as sequencing primers.

SSCP Analysis of the Gene Encoding the Human ß Subunit of PhK

PCR amplification of exons 1–14 and of the fragments containing mutations was performed as described above, with primers based on the genomic sequence of the β subunit (Wüllrich-Schmoll and Kilimann 1996). Nonradioactive detection of SSCPs was performed by the PhastSystem™ (Pharmacia-Biotech). The PCR products were diluted 1:1 in a formamide loading buffer (98% formamide, 2% glycerol, 0.05% bromophenol blue, and 0.05% xylene cyanol) and were heated at 95°C for 4 min. The samples were loaded onto 20% homogenous phastgels (Pharmacia-Biotech), and electrophoresis was performed at 4°C or 15°C. Running time of the samples was determined by following the instructions of the manufacturer. DNA was visualized by silver staining.

Analysis of Amplified Created Restriction Sites in Genomic DNA Fragments

A 119-nt fragment was amplified from DNA of the patient, her family, and a control, by primer HLB-20F and primer HLB-20Rm with a single mismatch complementary to nt 1829 of the coding sequence (table 2). The fragments were digested with *Mbo*II. A 155-nt fragment was amplified from DNA of the patient, her family, and 40 controls, by primer HLB-25F and primer HLB-25Rm with a mismatch complementary to nt 2310 (table 2). The PCR products were digested with *Xba*I. A 147-nt fragment was amplified from DNA of the patient, her family, and a control, with the primers HLB-31Fa and HLB-31Rm, which contain a mismatch complementary to nt 2897 of the coding sequence (table 2). The PCR products were digested with *Alu*I. The restriction

Table 2

List of Primers Used for Amplification of DNA Fragments from Gene Encoding β Subunit of PhK

Oligonucleotide	Exon	Sequence[a]	Position[b]	Size (bp)
HLB-20Rm		AGTGGACGTCCATGCATTCT	1847	264
HLB-20F	20	ACTCATGGTTGAGCATCCTG	−59	119
HLB-20R		TCCACCACATACTAAGAAGG	+112	
HLB-25F	25	AACTCTAGTGAAGCACAGTG	−140	155
HLB-25Rm		TTTGGCTGCCAGCTCTTCTC	2329	
HLB-31F	31	TGAGAACCAGAGCATAACGG	−53	215
HLB-31R		TCACGGAGCAGAGAGAAGGC	+54	
HLB-31Fa	31	GCTGAAAATGTTCTCCAAGG	−127	147
HLB-31Rm		ATATCTGACAGGGTTGGTAG	2915	

[a] Nucleotides that are mutated to generate an amplified created restriction site in combination with either the normal or mutated sequence are underlined.

[b] Derived from the PHKB-gene, GenBank/EMBL accession numbers X84909–X84938 (Wüllrich-Schmoll and Kilimann 1996).

fragments were analyzed on a 6% polyacrylamide sequencing gel without urea. The fragments were visualized by staining the gel with ethidium bromide for 10 min.

Sequence Notation

The first nucleotide of the coding sequence is defined as nt 1. The numbers of the nucleotides of the intron sequences are either related to the g of the splice-donor site (+1) or to the g of the splice-acceptor site (−1). The starting methionine of the coding sequence is defined as amino acid 1.

Results

The complete coding sequence of the γ_{TL} subunit of PhK was amplified, by RT-PCR, from lymphocyte mRNA of the patient, as described in Patients, Material, and Methods. The amplified product was cloned, and several independent clones were sequenced completely. No deviations from the normal sequence were found in the coding region of the γ_{TL} subunit of the patient.

Since no mutations could be detected in the sequence encoding the γ_{TL} subunit of this patient, the second likely candidate, which is the nonmuscle alternatively spliced region of the coding sequence of the β subunit of PhK, was analyzed for the presence of deviations from the control sequence. Part of the coding sequence of the β subunit of PhK was amplified from patient and control lymphocyte mRNA by RT-PCR, by use of primer sequences derived from the PhKβ12 clone (see Patients, Materials and Methods). The alternatively spliced nonmuscle sequence corresponds to nt 1010–1100 of the amplified fragment. No mutation was detected in this 91-nt region of the β sequence of the proband. However, on sequence analysis of the remaining part of this β sequence, it was found that the amplification product of the patient contained three deviations from the normal sequence: (1) a G→A mutation at nt 500 of the amplified fragment, changing the codon for a tryptophan to a stop codon (fig. 1); (2) an A→G mutation at nt 982 of the amplified fragment (not shown), which leads to substitution of tyrosine by cysteine; and (3) a deletion comprising nt 1565–1580 or nt 1569–1584 of the amplified fragment (fig. 2). The patient was heterozygous for each of the three mutations.

While these investigations were in progress, the complete genomic structure of the human gene encoding the β subunit of PhK was reported (Wüllrich-Schmoll and Kilimann 1996). Comparison of the reported sequence with the sequence of the PhKβ12 clone showed that the PhKβ12 clone contained nt 1274–3306 of the (nonmuscle) coding sequence and nt 3307–4277 of the 3′ UTR of the human β mRNA. The mutations detected in the β fragment of the patient were found to be a 1827G→A (W609X) transition, a 2309A→G (Y770C) mutation, and a deletion either of nt 2892–2907 or of nt 2896–2911 of the coding sequence of the β subunit. No discrimination can be made between these two possibilities by sequencing the cDNA, because a CTAG stretch both precedes the deletion and forms the end of the deletion (fig. 2*B*). However, since nt 2896–2911 are the first 16 nt of exon 31 and end with the splice-acceptor consensus dinucleotide AG, it is highly probable that the deletion in the mRNA of the patient is caused by a splice-acceptor site mutation in intron 30, resulting in the use of an alternative splice-acceptor site in exon 31. The 1827G→A mutation may lead to the formation of a truncated protein, which lacks 485 C-terminal amino acids (fig. 1*B*). Alternatively, the amount of the 1827G→A containing mRNA may be strongly diminished, because of unstability of the mRNA. Translation of the mRNA harboring the 16-nt deletion may yield a truncated pro-

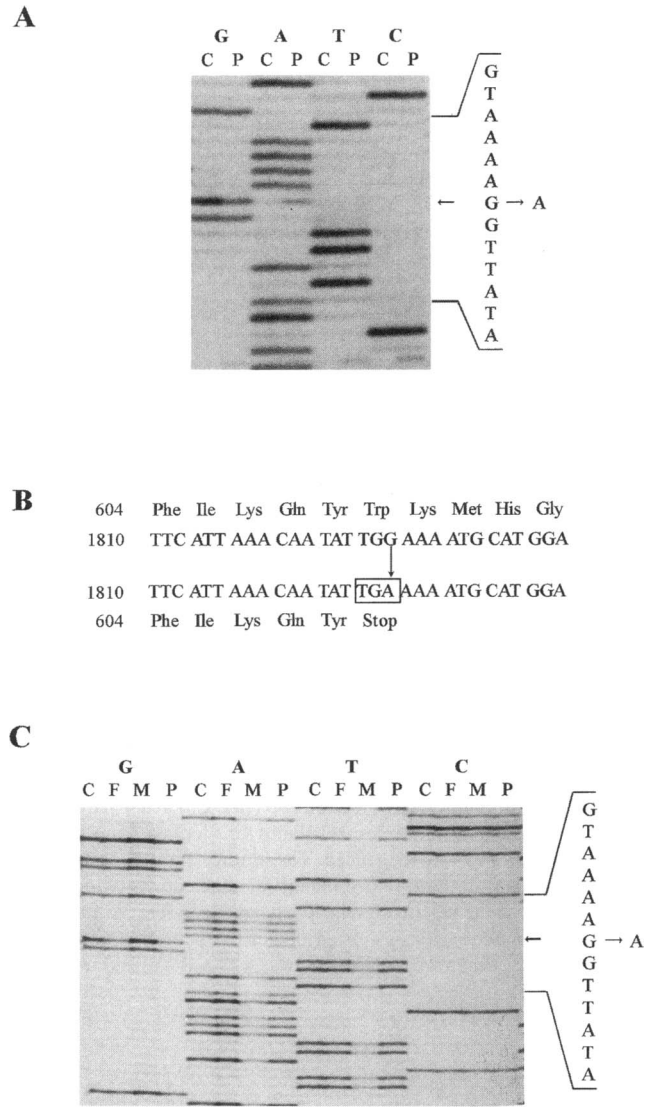

Figure 1 Sequence analysis of part of the coding sequence of the β subunit of PhK harboring the 1827G→A (W609X) mutation. *A*, Partial coding sequence of the β subunit of the proband (lanes P) and a control (lanes C), derived from cDNA. The cDNA was amplified as described in Patients, Material, and Methods. The G's of the two sequences are applied to the gel in adjacent lanes. The same was done for the A's, T's, and C's. The proband is heterozygous for the 1827G→A (W609X) mutation (*arrow*). *B*, Influence of the 1827G→A (W609X) mutation on the amino acid sequence of the β subunit. The 1827G→A (W609X) transition changes the codon for tryptophan to a stop codon (*arrow*). *C*, Partial coding sequence of the β subunit of the proband (lanes P), her parents (lanes F [father] and lanes M [mother]), and a control (lanes C), amplified from genomic DNA. The samples are applied to the gel as described under *A*. The father and the proband are heterozygous for the 1827G→A (W609X) mutation, whereas the mother has the normal sequence.

tein of 966 amino acids, of which the C-terminal isoleucine is introduced by the frameshift (fig. 2*B*), or may lead to rapid breakdown of the resulting mRNA.

The 5′ coding region of the β subunit of the proband was screened for the presence of additional mutations, by amplification of exons 1–14 by PCR, from DNA of the proband and a control, as described in Patients, Material, and Methods, followed by SSCP analysis of the amplified fragments. No deviations from the normal electrophoresis pattern were detected in exons 1–14 of the gene encoding the β subunit of PhK of the proband (results not shown).

The DNA fragments harboring the respective mutations identified in the coding region of the β subunit of the proband were amplified from genomic DNA of the proband, her parents, and a control and were sequenced directly. The 1827G→A (W609X) transition was found in DNA of the patient and in DNA of the father but not in DNA of the mother (fig. 1*C*). The proband and her parents were all found to be heterozygous for the 2309A→G (Y770C) mutation (not shown). The conclusion that the 16-nt deletion is caused by an acceptor-splice site mutation was confirmed by the detection of a g→t transversion at the downstream boundary of intron 30 of the β gene (IVS30^{-1},g→t), in the DNA fragments of the proband and her mother but not in DNA of the father (fig. 2*C*). Each of the three mutations described here was detectable by SSCP analysis (not shown).

Primers were designed in order to create restriction sites in combination with either the normal or the mutated sequence. The 1827G→A (W609X) mutation is detected by amplification and digestion of a 119-nt product surrounding the mutation. One mismatch primer together with the mutation creates a restriction site for *Mbo*II. The fragment harboring the mutation is digested by *Mbo*II, whereas the fragment containing the normal sequence remains undigested. The 2309A→G (Y770C) mutation is detected by amplification of a 155-nt DNA fragment surrounding the mutation, by a mismatch primer that adds a *Xba*I restriction site to the normal sequence but not to the mutated sequence. Treatment of the DNA fragments with *Xba*I results in digestion of the fragment containing the normal sequence but leaves the mutated fragment undigested. The IVS30^{-1},g→t mutation is identified by amplification of a 147-nt DNA fragment surrounding the mutation, by a mismatch primer that creates an *Alu*I restriction site in the normal sequence but not in the mutated sequence. Incubation with *Alu*I results in digestion of the fragment containing the normal sequence, whereas the fragment harboring the mutated sequence is not digested.

Restriction analysis of the three respective DNA fragments of the proband and her parents, by the appropriate restriction enzymes, yielded results that were in agreement with the sequence data (fig. 3). The newborn sister of the proband was heterozygous for each of the three mutations and has, therefore, a genotype that, with respect to the β gene of PhK, is identical to that in the proband (fig. 3). An elder sister of the proband was found to be heterozygous for the 1827G→A (W609X)

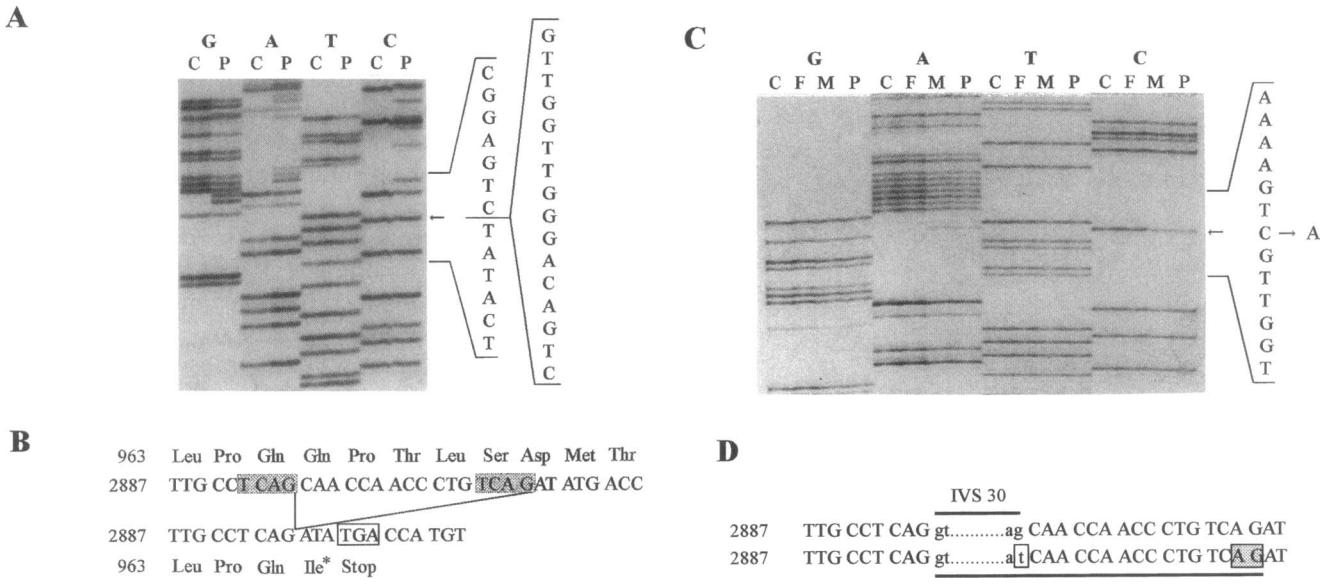

Figure 2 Sequence analysis of part of the coding sequence of the β subunit of PhK harboring the deletion of nt 2896–2911, and analysis of the corresponding DNA sequence. *A*, Partial coding sequence of the β subunit of PhK derived from cDNA of the proband (lanes P) and a control (lanes C). The complementary strand was sequenced. The samples are applied to the gel as described in figure 1. The starting point of the deletion is indicated by the arrow. The starting 4 nt of the deletion are equal to the 4 nt flanking the deletion, so that the exact location of the deletion cannot be determined solely on the basis of analysis of the coding sequence of the β subunit. The deleted sequence is shown on the right-hand side of the figure. *B*, Influence of the deletion of nt 2896–2911 on the amino acid sequence of the β subunit. The 4-nt repeat is marked by a gray-shaded box. The extent of the deletion is indicated by the unbroken lines. Because of the frameshift, the glutamine is substituted by an isoleucine (marked with an asterisk [*]), whereas the second codon turns into a stop codon (*boxed*). *C*, Partial sequence of the β subunit of the proband (lanes P), her parents (lanes F [father] and lanes M [mother]), and a control (lanes C), derived from genomic DNA. The samples are applied to the gel as described in figure 1. The complementary strand was sequenced. The mother and the proband are both heterozygous for the IVS30^{-1},g→t mutation (*arrow*). *D*, Influence of the IVS30^{-1},g→t mutation on splicing. The nucleotides belonging to the intron sequence are shown in lowercase letters, and the nucleotides of the coding sequence are shown in uppercase letters. A line above the upper normal sequence indicates the normally spliced-out intron sequence. The sequence that is spliced out in the patient is underlined. The IVS30^{-1} nucleotide in patient DNA is surrounded by a box. The alternative splice-acceptor dinucleotide AG, which is utilized in patient DNA, is denoted by a gray-shaded box.

transition, whereas the 2309A→G (Y770C) substitution and the IVS30^{-1},g→t splice-acceptor site mutation were not found in her DNA. Thus, she has inherited the 1827G→A (W609X) mutation from her father, but not the 2309A→G (Y770C) mutation. The only mutation detected in DNA of the elder brother of the proband was the 2309A→G (Y770C) mutation, for which he was heterozygous. These findings were confirmed by either sequencing or SSCP analysis (not shown). The mode of inheritance of the mutations in the family of the proband is illustrated in figure 4.

To investigate the distribution of the 2309A→G (Y770C) transition in the normal population, a 155-nt DNA fragment surrounding this mutation was amplified from DNA of 40 controls, by use of a mismatch primer that adds a *Xba*I restriction site to the normal sequence, as described above. Both restriction analysis of the amplified fragments by *Xba*I and SSCP analysis showed that two of the normal controls were heterozygous for the 2309A→G (Y770C) mutation, indicating that this mutation may be found in 2%-3% of the normal population.

Discussion

The proband presented here is a 3 ½-year-old female child who was found to suffer from an autosomal recessive form of PhK deficiency due to mutations in the gene encoding the common part of the β subunit of PhK. She has a severe deficiency of PhK in liver, whereas the activity of PhK in erythrocytes is only slightly diminished. Symptoms of muscle involvement are completely absent. A similar clinical presentation has been described for the X-linked form of liver PhK deficiency, which is caused by mutations in the gene encoding the α_L subunit of PhK, but association of this clinical phenotype with mutations in the gene encoding the common part of the β subunit of PhK has not been reported before.

Initially, the γ_{TL} gene and the nonmuscle part of the β gene of PhK were considered to be the most likely candidate sequences, since both these regions are located on autosomes and are expressed in liver but not in muscle. However, extensive investigations of the two above-mentioned candidate sequences of the proband did not yield any positive results. In contrast, three mutations

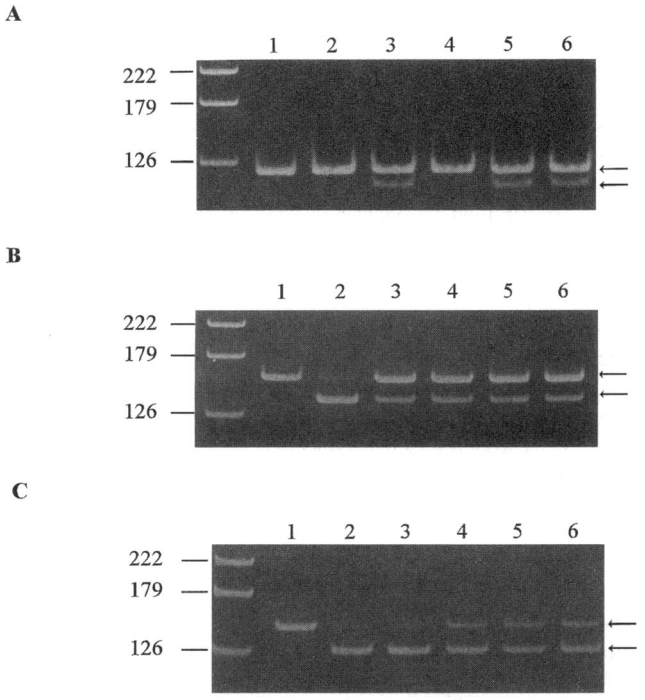

Figure 3 Analysis of DNA fragments that harbor the 1827G→A (W609X), the 2309A→G (Y770C), and the IVS30^{-1},g→t mutations, amplified from DNA of the proband, her parents, the newborn sister, and a control. *A, Mbo*II digestion of DNA fragments harboring the 1827G→A (W609X) mutation. DNA of a control (lane 2), the father (lane 3), the mother (lane 4), the proband (lane 5), and the newborn (lane 6) was amplified as described in Patients, Material, and Methods. Digestion of the amplified DNA fragment results in the formation of two fragments, of 110 and 9 nt, when the mutation is present. The proband, her mother, and the newborn sister show a band of 119 nt (*upper arrow*), which is the same length as that of the untreated PCR product (lane 1), and a band of 110 nt (*lower arrow*), indicating that they are heterozygous for the nonsense mutation. The control and the father are homozygous for the normal sequence. The band of 9 nt cannot be detected by this method. *B, Xba*I digestion of DNA fragments harboring the 2309A→G (Y770C) mutation. The samples are treated and applied to the gel, as described above for panel *A*. Digestion of the amplified DNA fragment results in the formation of two fragments, of 132 and 23 nt, when the normal sequence is present. The proband, her parents, and the newborn sister show a band of 155 nt (*upper arrow*), which is the same length as that of the untreated PCR product (lane 1), and a band of 132 nt (*lower arrow*), indicating that they are heterozygous for the missense mutation. The control is homozygous for the normal sequence. The band of 23 nt cannot be detected by this method. *C, Alu*I digestion of DNA fragments harboring the IVS30^{-1},g→t mutation. The samples are treated and applied to the gel as described above for panel *A*. Digestion of the amplified DNA fragment results in the formation of two fragments, of 126 and 20 nt, in the case of a normal sequence. The proband, her father, and the newborn sister show a band of 146 nt (*upper arrow*), which is the same length as that of the untreated PCR product, and a band of 126 nt (*lower arrow*), indicating that they are heterozygous for the splice-site mutation. The band of 20 nt cannot be detected by this method.

were found in the region of the β gene that is not subject to tissue-specific alternative splicing. Since two of these mutations, the 1827G→A (W609X) transition and the IVS30^{-1},g→t transversion, will lead either to the formation of truncated β subunits or to the production of unstable mRNA, it is highly probable that these mutations are disease-causing mutations.

Several additional lines of evidence indicate that the 1827G→A and IVS30^{-1},g→t substitutions are responsible for the PhK deficiency in this patient and that the 2309A→G (Y770C) mutation is a polymorphism. First, family analysis shows that the 1827G→A (W609X) and the IVS30^{-1},g→t mutations are located on different chromosomes of the patient (see fig. 4). Second, the presence of only one of these two mutations in members of the family of the proband is associated with a normal phenotype. Third, the 1827G→A (W609X) transition and the 2309A→G (Y770C) substitution are located on different chromosomes of the father, but he never experienced any symptoms of liver disease, which implies that one of these mutations is not disease causing. Finally, the 2309A→G (Y770C) mutation is detected in 2%-3% of the control population, and it is highly probable, therefore, that this mutation is a polymorphism. Thus, both on the basis of these arguments and on the basis of the absence of mutations in the gene encoding the γ_{TL} subunit of PhK, it is concluded that the proband suffers from PhK deficiency due to compound heterozygosity for a 1827G→A (W609X) transition and a IVS30^{-1},g→t splice-acceptor site mutation in the gene encoding the β subunit of PhK. This implies that the newborn sister of the proband, who, with respect to the β gene, has the same genotype as the proband, will also be affected by the disease. This diagnosis is supported by the observation that the newborn sister has a slightly diminished activity of PhK in erythrocytes (table 1) and has experienced an episode of hypoglycemia in the 2d mo after birth.

The 1827G→A (W609X) and IVS30^{-1},g→t mutations will, theoretically, affect both the liver and the muscle isoenzyme of PhK and are also expected to influence PhK activity in erythrocytes. However, the proband has the clinical phenotype of liver PhK deficiency with only slightly diminished activity of PhK in erythrocytes. To classify the phenotype associated with these mutations, measurement of the activity of the muscle isoenzyme is needed. Unfortunately, muscle tissue was not available for measurement of PhK activity. The absence of symptoms of muscle involvement in the proband does not exclude deficient activity of PhK in muscle, because muscle symptoms may develop later in life, as has been reported for several patients suffering from isolated muscle PhK deficiency (Abarbanel et al. 1986; Wehner et al. 1994). The high residual activity of PhK found in erythrocytes of the proband may be the result of the formation of a relatively instable but active αγδ complex. Chan and Graves (1982) have shown that αγδ complexes are active in vitro. The formed αγδ complex might be more stable in erythrocyte lysates than in liver homogenates, so that in vitro activity of the mutated PhK is detectable in erythrocyte lysate but not in liver homogenate. It is

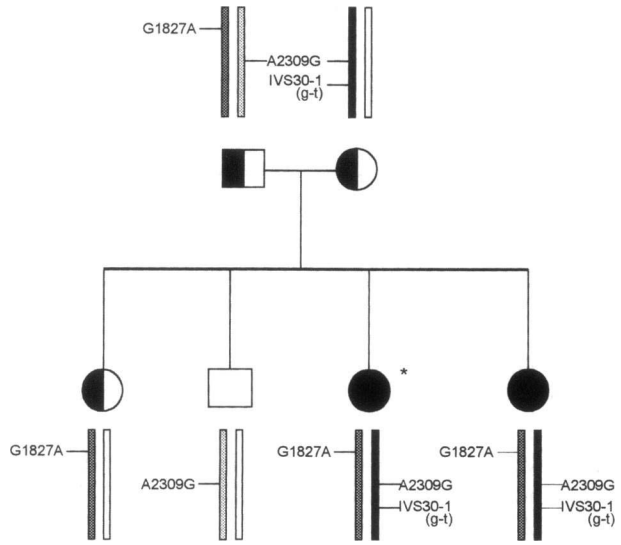

Figure 4 Pattern of inheritance of the 1827G→A (W609X), the 2309A→G (Y770C), and the IVS30^{-1},g→t mutations in the family of the proband. The alleles are presented by bars, with a specific grade of shading for each individual allele. The proband is marked by an asterisk (*). The elder sister and brother are shown on the left of the proband, and the newborn sister is shown on the right. The father has the 1827G→A (W609X) mutation and the 2309A→G (Y770C) mutation in different alleles. The mother has the 2309A→G (Y770C) mutation and the IVS30^{-1},g→t mutation in the same allele.

likely, however, that regulation of in vivo activity of PhK will be impaired by the absence of the β subunits or by the presence of severely mutated β subunits.

In conclusion, the proband described here is, to our knowledge, the first patient reported to suffer from PhK deficiency due to mutations in the region of the gene encoding the common part of the β subunit of PhK. The resulting clinical phenotype in this proband is that of autosomal recessive deficiency of liver PhK. Characterization of the phenotype commonly associated with PhK deficiency due to mutations in the gene encoding the β subunit awaits identification of additional patients.

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