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Autosomal glycogenosis of liver and muscle due to phosphorylase kinase deficiency is caused by mutations in the phosphorylase kinase β subunit (PHKB)

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Glycogen storage disease due to phosphorylase kinase deficiency occurs in several variants that differ in mode of inheritance and tissue-specificity. This heterogeneity is suspected to be largely due to mutations affecting different subunits and isoforms of phosphorylase kinase. The gene of the ubiquitously expressed β subunit, PHKB, was a candidate for involvement in autosomally transmitted phosphorylase kinase deficiency of liver and muscle. To identify such mutations, the complete PHKB coding sequence was amplified by RT-PCR of RNA isolated from blood samples of patients and analyzed by direct sequencing of PCR products. The characterization of mutations was complemented by PCR of genomic DNA. In one female and four male patients, we identified five independent nonsense mutations (Y418ter; R428ter; Y974H+E975ter; Q656ter in two cases), one single-base insertion in codon N421, one splice-site mutation affecting exon 31, and a large deletion involving the loss of exon 8. Although these severe translation-disrupting mutations occur in constitutively expressed sequences of the only known β subunit gene of phosphorylase kinase, PHKB, they are associated with a surprisingly mild clinical phenotype, affecting virtually only the liver, and relatively high residual enzyme activity of ~10%.

INTRODUCTION

Deficiency of phosphorylase kinase (Phk), a regulatory protein kinase in glycogen metabolism, is responsible for one quarter of all cases of glycogen storage disease and occurs with a frequency of ~1 in 100 000 births. Phk deficiency occurs in several variants that differ in mode of inheritance (X-linked or autosomal), tissue-involvement (liver, muscle, liver and muscle, liver and kidney, heart) or clinical course (1,2). Patients affected with glycogenosis of the liver due to Phk deficiency present as infants with hepatomegaly, retardation of growth and motor development, and a characteristic rounded face (‘dollface’). Hypoglycemia and elevated transaminases, triglycerides and cholesterol are often observed. Typically, patients improve with age and are often asymptomatic as adults.

The heterogeneity of Phk deficiency is thought to be primarily due to the fact that Phk is composed of four different subunits, each of which has several isoforms or splicing variants. Mutations have recently been characterized in the genes encoding the muscle and liver isoforms of the regulatory α subunit (PHKA1 and PHKA2) (2–8) and the testis/liver isoform of the catalytic γ subunit (PHKG2) (9) which explain X-linked muscle-specific, X-linked liver-specific and autosomal liver-specific Phk deficiency, respectively. Only one gene, PHKB, is known to encode the regulatory β subunit. Thirty exons of this gene are expressed in all tissues investigated (liver, muscle, brain and heart) whereas three exons are subject to tissue-specific differential splicing (10–12). PHKB is located on human chromosome 16q12-q13 (13) and was therefore a candidate for involvement in another variant of Phk deficiency, autosomal-recessive Phk deficiency affecting both liver and muscle.

In the present study, we searched the PHKB coding sequence of several patients with this form of glycogen storage disease, using a strategy based primarily on RT-PCR of RNA isolated from full blood and complemented by PCR of genomic DNA. We report here the first mutations identified in the PHKB gene. Five independent nonsense mutations, a single-base insertion, a splice-site mutation and an extensive intragenic deletion demonstrate that mutations in PHKB cause Phk deficiency of liver and muscle.

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abundance of the mRNA (ref. 2 and references therein), we suspected that this might also be the case for both mutant alleles in this family, so that they would be detectable together in the patient but missed against the background of the higher levels of mRNA from the normal alleles in the parents. We therefore amplified exons 14 and 31 from genomic DNA of both parents and could indeed identify, with signal intensities similar to the normal alleles, the Y418ter mutation in the father and a Y974H plus E975ter double mutation in the mother.

In patient 3, RT-PCR revealed an apparently homozygous deletion of 16 nucleotides after codon Q964, leading to a frameshift and translation termination after one additional codon (Fig. 2). As the deletion corresponds precisely to the beginning of exon 31, we suspected a splice-site mutation and amplified this exon from the patient’s genomic DNA. Indeed, the last residue of intron 30 was found to be mutated from a G to a T (Fig. 2), destroying the 3′-splice site consensus and leading to the use of an AG dinucleotide 16 residues downstream as an abnormal 3′ splice site.

In patient 4 a heterozygous nonsense mutation was identified in codon R428 (exon 14, Fig. 2). By RT-PCR, however, we were unable to directly detect a second mutation in his PHKB coding sequence. Since the mutant and the normal sequence signals in codon 428 are of comparable intensity, it was evident that a second allele is present and expressed in similar abundance as the R428ter allele in this patient. Then, we noted that amplification of interval βm30/m31 yielded an additional product of smaller molecular size and minor abundance (~10% of the normal-sized product, not shown), only from RNA of patient 4 but none of eight other individuals analyzed. This smaller RT-PCR product was sequenced and found to represent an RNA lacking exon 21. We therefore suspected that the splicing behavior of exon 21 might be affected in patient 4, and amplified and sequenced this exon and its vicinity from his genomic DNA. We found it heterozygous for a C to T transition that converts Q656 into a stop codon (Fig. 2).

As Q656 is the last codon of exon 21, this suggested that the single-base exchange three nucleotides upstream of the splice site might be both, a nonsense and a splice-site mutation that causes, in a fraction of transcripts, the skipping of exon 21 but perhaps, in the majority of transcripts, the retention of intron 21. As intron 21 is long (>2 kb), the RNA retaining it would not be amplified with significant efficiency in our standard RT-PCR, and the Q656ter allele would not be seen in the sequence of the normal length PCR product. To test this hypothesis, we amplified RNA from patient 4 and five control individuals with primers βm30 in exon 19 and β23 at the beginning of intron 21 (Table 1 and Fig. 2, bottom). RT-PCR products of the expected size of 289 nt, representing an RNA molecule containing exons 19, 20 and 21 as well as the 5′ end of intron 21, were obtained from patient 4 but also from all five controls (not shown), indicating that intron 21 splicing is generally ‘leaky’. Sequencing showed that in patient 4 the majority of the aberrantly spliced RNA represented the mutant Q656ter allele whereas a control was homogeneous for the normal Q656 sequence. Moreover, an additional intron 21 RT-PCR product ~200 nt longer was obtained only from patient 4 but none of the controls, with an abundance of ~25% of the main band. It was sequenced and found to contain intron 20 (length, 204 nt) in addition to intron 21, and to represent only the Q656ter allele. In conclusion, the Q656ter mutation in patient 4 interferes with normal RNA splicing around exon 21 (a weak signal from the Q656ter allele can in fact be seen in the sequence of the

### Table 1. Primers for amplification and sequencing of human PHKB

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position (5′ end)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR amplification primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>βm23</td>
<td>-12</td>
<td>5′-GAC CGG AGC GCG ATG G-3′</td>
</tr>
<tr>
<td>βm24</td>
<td>137</td>
<td>5′-CAG AGA GTT TCA TTA TCA TG-3′</td>
</tr>
<tr>
<td>βm16</td>
<td>78</td>
<td>5′-CTC AGT TTA TGA ACC TCT TA-3′</td>
</tr>
<tr>
<td>βm29</td>
<td>916</td>
<td>5′-CTA ATG CAG GAT AAC TGA TG-3′</td>
</tr>
<tr>
<td>βm5</td>
<td>844</td>
<td>5′-CTG TTA CCC AGA GAA TCA AG-3′</td>
</tr>
<tr>
<td>βm15</td>
<td>1797</td>
<td>5′-CTT TAT GTG ATC ATC TAT CAG CA-3′</td>
</tr>
<tr>
<td>βm30</td>
<td>1718</td>
<td>5′-GAC GTT GTT TTT TTA CCC GA-3′</td>
</tr>
<tr>
<td>βm31</td>
<td>2301</td>
<td>5′-CTC AAT GTG ATC AGA AAC GG-3′</td>
</tr>
<tr>
<td>βm25</td>
<td>2223</td>
<td>5′-CAT CCT GCT GGG TAT ACT GC-3′</td>
</tr>
<tr>
<td>βm26</td>
<td>2567</td>
<td>5′-TGC ATG ACC AGT TCT TGC GC-3′</td>
</tr>
<tr>
<td>βm32</td>
<td>2483</td>
<td>5′-CAA GAG TGA TCC AAA ACA TCA-3′</td>
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<tr>
<td>βm4</td>
<td>3377</td>
<td>5′-GTT CGT ATA TTA ATG GCT GC-3′</td>
</tr>
</tbody>
</table>

Additional sequencing primers | | |
| βm35 | 539 | 5′-AGA AGA TAA TAA GAT GAC ACT GC-3′ |
| βm1 | 570 | 5′-CCA GAT CTA CCA AAC TG-3′ |
| βm22 | 1455 | 5′-ATT GGA AAA CCT CAT GCT C-3′ |
| βm37 | 1379 | 5′-GTC CTA CCA ATC ACA TGT ATC CT-3′ |
| βm18 | 3003 | 5′-CTC TAC AAC GAT CTG TCT G-3′ |
| βm36 | 2935 | 5′-TCT CTC TCC TGT TTA ACG AC-3′ |

Exon amplification primers | | |
| βm33 | exon 8 -92 | 5′-CCA AAT TGC TGG GAT TAC AG-3′ |
| βm34 | exon 8 +85 | 5′-GTT CCA ATC TAA TGG TGT AG-3′ |
| βi18 | exon 14 -91 | 5′-TAC CCA GTC GTG TGT ATC ATA-3′ |
| βi19 | exon 14 +85 | 5′-AAC ATG AAT ACC AGC AAA GGC-3′ |
| βi22 | exon 21 -112 | 5′-CCT CCT TAT TAG GTG GTG GA-3′ |
| βi23 | exon 21 +30 | 5′-ATA GAT GCA TAC TGA AAA TCA G-3′ |
| βi20 | exon 31 -107 | 5′-AGC TGA AAA TGT TCT CCC AAG-3′ |
| βi21 | exon 31 +78 | 5′-AGG CAG TTT CCC TTA TAC TAG-3′ |

### RESULTS

**Point mutations in the PHKB gene**

RNA was purified from deep-frozen blood samples, reverse-transcribed, amplified by PCR in several overlapping segments covering the complete coding sequence (Table 1), and analyzed by direct sequencing as previously described (8).

Patient 1 was found to be heterozygous for two PHKB mutations. A stretch of seven A residues in exon 14 is extended by an additional A resulting in a frameshift in codon 421 and translation termination at the following triplet, and a C to T transition converts glutamine 656 in exon 21 to a stop codon (Fig. 1). RNA and genomic DNA from the patient’s parents were also analyzed by PCR and sequencing in both regions, confirming the mutations and demonstrating that the frameshift mutation had been inherited from the father and the nonsense mutation from the mother.

Patient 2 was found to be heterozygous for three different mutations: two nonsense mutations in codons Y418 (exon 14) and E975 (exon 31) and a Y974H missense mutation (Fig. 1). Surprisingly, RT-PCR analysis of RNA from both parents did not yield significant sequence signals for any of these mutations. As translation-terminating mutations often result in reduced
Figure 1. PHKB mutations in patients 1 and 2. The left column shows sequencing recordings of patient RT-PCR products around the paternal and maternal mutations. All mutations are heterozygous, and asterisks mark the mutated positions where the signals of the two alleles are superimposed. Arrowheads indicate the direction of reading (left to right: sense; right to left: antisense). In the right column the normal partial sequences are given, and codons whose meaning is modified in the mutant alleles are indicated below. Mutated nucleotides are highlighted by bold print and shading. The paternal mutation of P1 is the insertion of an additional A into a tract of seven A residues. When read on the complementary strand as shown here, the superposition of two sequences out of register by one residue therefore becomes apparent at the other end of the T tract.

standard RT-PCR product) and instead enhances the formation of three aberrant splicing products retaining intron 21, retaining both introns 20 and 21, or excluding exon 21, respectively.

Remarkably, an identical Q656ter mutation had been found in patient 1 but could be detected in the sequence of the standard RT-PCR product (Fig. 1). It remains to be explained why the splicing of intron 21 should be impaired by this mutation in patient 4 but not in patient 1. Patient 4 may carry an additional mutation in his Q656ter allele, e.g. near the beginning or the end of intron 21, which would independently affect the splicing of this intron or at least render the splicing mechanism more sensitive to the Q656ter mutation. Alternatively, patient 1 may possess a sequence peculiarity that stabilizes the correct splicing of intron 21 against the Q656ter mutation. The Q656ter alleles of patients 1 and 4 are clearly distinct, as the genomic DNA of the mother of patient 1 is homozygous for an ATTA tetranucleotide deletion near the end of intron 20 (bottom of Fig. 2) whereas patient 4 is homozygous for the published sequence containing this tetranucleotide. It remains unresolved whether the ATTA deletion itself has an effect on intron 21 splicing or whether there are other sequence differences between the two individuals in regions not analyzed. None were found, in patient 4 or the mother of patient 1, in exon 21 and its immediate vicinity [92 nt upstream to 8 nt downstream (sequence at the bottom of Fig. 2)]. As the Q656ter mutation is clearly a deleterious mutation, either with or without an additional capacity as a splice-site mutation, we did not pursue this question further.

Of the nine sequence abnormalities described above, three occur in exon 14, two identical but apparently independent Q656ter mutations and the ATTA polymorphism lie in or near exon 21, and three (counting both base replacements of the maternal double mutation of patient 2) are in exon 31. The identification of more PHKB mutations may clarify whether these sequence regions are indeed hypermutable.

Restriction site polymorphism in intron 20

The ATTA deletion near the end of intron 20 abolishes an AsnI restriction site. To investigate whether it is polymorphic, the genomic DNA of additional, unrelated individuals was analyzed by PCR of the β22/23 interval (sequence at the bottom of Fig. 2), AsnI digestion, and agarose gel electrophoresis of the resultant
Figure 2. *PHKB* mutations in patients 3 and 4. In patient 3, 16 nt are missing in the mRNA, due to a homozygous splice-site mutation of the last nucleotide of intron 30. Patient 4 is heterozygous for two nonsense mutations, the second of which is detectable only in the genomic DNA (see text). At the bottom, the sequence of exon 21 and its vicinity is shown to illustrate the locations of the Q656ter mutations of patients 1 and 4 and of the ATTA polymorphism (both marked by bold print and shading; the exon sequence is in capital letters). PCR primers $\beta_i22$ (upstream) and $\beta_i23$ (downstream) are underlined.

DNA fragments (not shown). Of 31 chromosomes analyzed, 20 (65%) were positive for ATTA/AanI and 11 (35%) were negative, identifying this structural feature as the first polymorphic marker in the *PHKB* gene at 16q12-q13.

Deletion of exon 8 in a region rich in repetitive genomic elements

Patient 5 was expected to have a homozygous mutation because his parents are consanguineous. Indeed, a homogeneous amplification product of reduced length was obtained by RT-PCR with primers $\beta_m16/m29$ (Fig. 3a). Its sequence revealed the absence of exon 8 (not shown), resulting in a frameshift and truncation of the reading frame after only 19% of its normal length. An unaffected brother and two unrelated controls yielded normal-sized RT-PCR products whereas products of both sizes could be amplified from RNA of both parents, indicating heterozygosity (Fig. 3a). We then tried to amplify exon 8 and its immediate vicinity from the patient’s genomic DNA with primers $\beta_m33/m34$ but obtained no product, in contrast to DNA from eight control individuals (Fig. 3b, additional controls not shown).

To obtain more information on the extent and nature of the deletion of exon 8 in the genomic DNA of patient 5, we then sequenced the 6 knt genomic *Hind*III fragment containing exon 8 (subclone $\beta_2$-H1; ref. 10), finding that exon 8 lies within a cluster of retroposons (Fig. 3c). There are two Alu elements, 1 and 2 knt downstream of exon 8, and an inverted Alu half-element lies immediately upstream of the exon. This constellation is flanked by two LINE-1 elements, both in orientation opposite to that of *PHKB* and both apparently extending beyond the borders of subclone $\beta_2$-H1. The upstream LINE-1 begins immediately adjacent to the Alu half-element and is cut off at nt 742 of the LINE-1 reference sequence by the *Hind*III site, beyond which it
may continue for up to 5500 nt. The downstream LINE-1 sets in at the right-hand HindIII site with nt 1882 and is truncated at nt 3653 of the LINE-1 reference sequence. Thus, exon 8 is surrounded by long stretches of potentially recombinogenic repetitive DNA elements that might be responsible for the deletion. Next, we investigated whether this constellation of repetitive elements found in clone β2-H1 is indeed representative for the normal gene structure. Two pairs of primers were designed to amplify sequence intervals extending from the unique sequence region into the upstream or downstream LINE-1 elements (βi34/m34 and βi32/i33, respectively, Fig. 3c). PCR products of expected sizes and terminal sequences were obtained from all six control individuals but not from patient 5, confirming that clone β2-H1 represents the normal structure surrounding exon 8 of the PHKB gene and that this structure is disrupted in patient 5. Several other sequence intervals were probed by PCR (Fig. 3c), and all yielded specific products (confirmed by sequencing) from control individuals but not from patient 5. Hence, the deletion in patient 5 extends over at least 6 knt between the positions of primers βi41 and βi43.

**DISCUSSION**

The five independent nonsense mutations, one single-base insertion, one splice-site mutation and one large intragenic deletion identified here demonstrate that mutations in the PHKB gene cause autosomal-recessive glycosgenosis due to Phk deficiency of both liver and muscle. Patients 2, 4 and 5 are exceptionally well-characterized published cases where Phk deficiency had been determined in muscle biopsies as well as in liver or erythrocytes (see Materials and Methods). PHKB therefore was our first candidate gene for the mutation search, which proved successful in all three cases. In normal diagnostic practice, however, Phk activity measurements in muscle and liver are rarely carried out. Symptoms of liver involvement predominate, and the presence or absence of clinical muscle symptoms is inconclusive (9) so that muscle is rarely biopsied. In fact, even liver biopsies are often avoided and activity determinations only performed on erythrocytes. Family history is also seldom so suggestive of autosomal-recessive inheritance as in cases 2 and 5 (Materials and Methods). Therefore, it is often difficult to predict whether a case of liver glycosgenosis due to Phk deficiency is of the X-linked liver-specific (mutations in PHKA2), the autosomal liver-specific (mutations in PHKG2) or of the autosomal liver-and-muscle type (mutations in PHKB).

Patients 1 and 3 are examples who had no muscle symptoms and whose muscle was therefore not analyzed. In patient 1, who is male, we first searched the X-chromosomal PHKA2 gene and then PHKG2, candidate genes in which mutations do not affect Phk activity in muscle, before finally finding the mutations in PHKB. As patient 3 is female, autosomal inheritance was suspected from the beginning but again, PHKG2 was analyzed first. Liver glycosgenosis due to Phk deficiency is generally benign and tends to improve clinically with increasing age. This is also observed for the older patients with mutations in PHKB (see Methods). Indeed, it appears that cases with PHKB mutations are even more mildly affected than PHKA2 and PHKG2 cases, with relatively high residual Phk activities around 10% in affected tissues, and transaminases and triglycerides not or only moderately enhanced. Impairment of muscle function by this type of Phk deficiency may be so mild that it is never noted (patients 1 and 3). However, it remains to be seen whether more significant muscle symptoms will develop in the long run, as some patients with muscle-specific Phk deficiency have developed complaints only as adults. Also in glycosogenosis type III (debranching enzyme deficiency) it is observed that ‘although the enzyme defect is expressed in both liver and muscle in most patients, clinical myopathy is not common and often manifests in adult life, long after the liver symptoms have remitted’ (14).
There are five known genes for Phk subunits (not counting the subunit 6 which is calmodulin), and the PHKB gene is the fourth in which mutations have been identified. The fifth, PHKG1, encodes the muscle isoform of the catalytic γ subunit and is a candidate for autosomal muscle-specific Phk deficiency. Thus, the genes associated with most, and the most frequent, forms of Phk deficiency are now known. However, there remain unresolved questions in the molecular genetics of Phk. In particular, we still do not understand the molecular basis of the very rare and clinically most severe forms, hepato-renal (15,16) and heart-specific (17–19) Phk deficiency. The results of the present study emphasize this. PHKB is the only known gene of the β subunit, and all mutations described here are in exons that are believed to be expressed in all tissues. These mutations therefore met our expectations insofar as they cause Phk deficiency in liver as well as in muscle. However, it is unclear why they do not also affect the kidney or the heart. It is also striking that although all these mutations are severe translation-terminating mutations that lead to truncated protein products unlikely to have residual function, patients mostly have notable residual Phk activities around 10% in the affected tissues, liver and muscle, and even higher values in erythrocytes, leukocytes or fibroblasts. In contrast, a patient with a mutation terminating the PHKA1 gene product after 90% of its normal length (3) had only 0.3% residual activity in muscle, in spite of the existence of a second isoform gene for the α subunit. This may be explained by the existence of another, yet unidentified β subunit gene. It is also possible that the β subunit can be replaced in the holoenzyme by the partially homologous α subunit but not vice versa, or that a partially stable and active complex can be formed without β but not without α. Indeed, partial denaturation of Phk with LiBr, or partial proteolysis, is known to produce an αγδ complex that is catalytically active (20,21).

**MATERIALS AND METHODS**

**Laboratory methods**

Purification of RNA and genomic DNA from deep-frozen blood samples was performed according to conventional procedures. RT-PCR and direct cycle-sequencing are described in detail in ref. 8. All mutations were confirmed from two or more independent RNA or DNA preparations. The complete PHKB coding sequence was analyzed in patients 1, 2, 4 and 5, and ~50% of the coding sequence was analyzed in patient 3, without finding other sequence abnormalities.

**Patients**

Patient 1 (FB.M, male), the only child of unrelated, healthy German parents, was admitted at the age of 22 months because of an extended abdomen due to hepatomegaly. Transaminases and triglycerides were slightly elevated. Phk activity in erythrocytes was markedly decreased (12% residual activity) while the parents’ erythrocyte Phk activities were in the heterozygote range (mother: 56%; father: 40% of normal). Glycogen in erythrocytes was high (10 mg/dL; normal range: 0–10 mg/dL) and the phosphorylase a/a+b ratio in leukocytes very low (0.03; normal range 0.4–0.5). At present (age 4 years), the child presents with body height at the 10th and weight at the 50th percentile, hepatomegaly, and a tendency to develop hypoglycemic symptoms after several hours of fasting or physical activity. There are no clinical indications of muscle involvement. Also the mother reports that she has hypoglycemic symptoms upon physical exercise that are countered by carbohydrate intake.

Investigations of patients 2, 4 and 5 have been published before, demonstrating Phk deficiency in liver or erythrocytes as well as in muscle and suggesting an autosomal-recessive mode of inheritance in cases 2 and 5. Patient 2 (L.H.O, male; refs 22,23) and his sister (also affected) are the children of unaffected, unrelated Norwegian parents. They came to medical attention as infants because of hepatomegaly; glucagon response by both was normal. Residual Phk activities were 18% of normal in hemolysates of both, 5% in liver of the sister (but the phosphorylase a/a+b ratio, 0.5, was surprisingly normal), and 0–13% (depending on pH) in muscle of patient 2 (a/a+b ratio: 0.07). Today, at age ~25, both individuals are fully capable of everyday physical activities, but tend to develop hypoglycemic symptoms upon activity or fasting which are ameliorated by carbohydrate intake. Hepatomegaly has receded, clinical muscle symptoms have never been noted.

Patient 3 (PB.A, female), now 6 years old, is the daughter of healthy Dutch parents and has one brother and one sister, both unaffected. Although she is apparently homozygous for her splice-site mutation, the parents are reportedly unrelated but originate from the same geographical region. She came to medical attention because of an extended abdomen due to hepatomegaly but is free of any symptoms of hypoglycemia or muscle involvement. Transaminases were found slightly enhanced on occasion but mostly normal, triglycerides and cholesterol were normal. Phk activity was undetectable in liver and 12% of controls in erythrocytes. Remarkably, a nearly normal phosphorylase a/a+b ratio, 0.43, was determined in erythrocytes.

The parents of patient 4 (K.M.D, male, British; refs 24,25) are healthy and unrelated and there is one healthy half-brother. The patient, now 15 years old, was referred at age 5 years because of abdominal extension (noted since early infancy) and muscle weakness. He presented with height on the 25th percentile, a doll-like face, thin extremities, hepatomegaly, and reduced muscle power and bulk. He was never found to be hypoglycemic, and the glucagon response was normal. Laboratory findings included normal creatine kinase, slightly raised aspartate aminotransferase, slightly fibrosed and enhanced glycogen (8.8 g%; normal <5 g%) in a liver biopsy, and reduced Phk activity both in erythrocytes (13% of normal) and in muscle (9%). Muscle glycogen was high (1.35 g%; normal, 0.34) and the a/a+b ratio very low (0.01).

Patient 5 (M.H.H, male, Israeli-Arab; refs 26,27) has two healthy brothers and two affected sisters. His healthy parents are distantly related. A protuberant abdomen had been noted since 18 months of age. Upon admission at age 4 years, severe hepatomegaly, a doll-face, mild generalized muscular hypotonia, height below the third percentile and weight in the 20th percentile were observed. There was no history of hypoglycemic symptoms, and blood glucose levels and a glucagon test were normal. In a liver biopsy, strongly elevated glycogen (17 g%; controls, 1–5 g%) undetectable phosphorylase a, and Phk activity reduced to 20% of normal were found. A muscle biopsy also demonstrated glycogenosis (1.9 g%; controls, 0.3–1 g%) and Phk deficiency (25% residual activity). At present, neither the patient (age 21) nor his elder sisters display any notable symptoms (no hepatomegaly, normal height, no impairment of everyday physical activity).
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REFERENCES


