Am. J. Hum. Genet. 64:1541–1549, 1999

Complete Genomic Structure and Mutational Spectrum of PHKA2 in Patients with X-Linked Liver Glycogenosis Type I and II

Jan Hendrickx,[1] Philip Lee,[2] James P. Keating,[3] Dietbrand Carton,[4] Imdadali B. Sardharwalla,[5] Mendel Tuchman,[6] Christiane Baussan,[7] and Patrick J. Willems[1]

[1]Department of Medical Genetics, University of Antwerp, Antwerp; [2]Institute of Child Health, Great Ormond Street Hospital, London; [3]Department of Pediatrics, St. Louis Children's Hospital and Washington University School of Medicine; [4]Kinderkliniek Hooft, University Hospital Gent, Gent; [5]Willink Biochemical Genetics Unit, Royal Manchester's Children's Hospital; [6]Departments of Pediatrics and Laboratory Medicine and Pathology, University of Minnesota, Minneapolis; and [7]Biochimie 1, Centre Hospitalier de Bicêtre, Bicêtre, France

Summary

X-linked liver glycogenosis (XLG) is probably the most frequent glycogen-storage disease. XLG can be divided into two subtypes: XLG I, with a deficiency in phosphorylase kinase (PHK) activity in peripheral blood cells and liver; and XLG II, with normal in vitro PHK activity in peripheral blood cells and with variable activity in liver. Both types of XLG are caused by mutations in the same gene, PHKA2, that encodes the regulatory α subunit of PHK. To facilitate mutation analysis in PHKA2, we determined its genomic structure. The gene consists of 33 exons, spanning $\geq$65 kb. By SSCP analysis of the different PHKA2 exons, we identified five new XLG I mutations, one new XLG II mutation, and one mutation present in both a patient with XLG I and a patient with XLG II, bringing the total to 19 XLG I and 12 XLG II mutations. Most XLG I mutations probably lead to truncation or disruption of the PHKA2 protein. In contrast, all XLG II mutations are missense mutations or small in-frame deletions and insertions. These results suggest that the biochemical differences between XLG I and XLG II might be due to the different nature of the disease-causing mutations in PHKA2. XLG I mutations may lead to absence of the α subunit, which causes an unstable PHK holoenzyme and deficient enzyme activity, whereas XLG II mutations may lead to in vivo deregulation of PHK, which might be difficult to demonstrate in vitro.

Received December 12, 1997; accepted for publication April 14, 1999; electronically published May 3, 1999.

Address for correspondence and reprints: Dr. J. Hendrickx, Department of Physiology, University of Antwerp-RUCA, Groenenborgerlaan 171, 2020 Antwerp, Belgium. E-mail: jan.hendrickx@ruca.ua.ac.be

0002-9297/99/6406-0007$02.00

Introduction

Protein kinases are very important enzymes controlling numerous physiological processes by transferring phosphate from ATP to different protein substrates. Phosphorylase kinase (PHK), the prototype of protein kinases, plays a regulatory role in a cascade of enzymatic reactions controlling glycogen breakdown. PHK consists of four different subunits: α, β, γ, and δ. The γ subunit harbors the catalytic activity and is regulated by phosphorylation of the α and β subunits and by interaction of Ca^{2+} with the δ subunit, which is calmodulin (see Hendrickx and Willems 1996). Several isoforms exist for all of these different subunits. Some of these isoforms are encoded by different genes, whereas others result from differential splicing of the same gene (Hendrickx and Willems 1996). Two genes encoding the α subunit (Wüllrich et al. 1993; Hirono et al. 1995), one gene encoding the β subunit (Wüllrich-Schmoll and Kilimann 1996), two genes encoding the γ subunit (Hanks 1989; Wehner and Kilimann 1995), and three calmodulin genes (Wawrzynczak and Perham 1984; Sen Gupta et al. 1987; Fischer et al. 1988) have been isolated. Most of these genes have already been implicated in different glycogen-storage disorders.

X-linked liver glycogenosis (XLG [MIM 306000]) is probably the most common inherited disorder of glycogen metabolism (Huijing and Fernandes 1969). XLG is caused by a genetic deficiency of PHK. It is a relatively mild disease, which is mainly characterized by hepatomegaly and growth retardation during childhood (Huijing and Fernandes 1969; Schimke et al. 1973; Lederer et al. 1975; Willems et al. 1990). Two types, XLG I and XLG II, have been described. Both types of patients show very similar clinical symptoms. However, patients with XLG I show a clear in vitro enzyme deficiency of PHK in erythrocytes, leukocytes, and liver (Huijing and Fernandes 1969), whereas XLG II patients show normal or even elevated in vitro PHK enzyme activity in erythrocytes and leukocytes and varying activity in liver (Hendrickx et al. 1994). Both types of XLG have been shown

to be caused by mutations in the PHKA2 gene, which encodes the liver α subunit of PHK (Hendrickx et al. 1995, 1996, 1998; Hirono et al. 1995; van den Bergh et al. 1995; Burwinkel et al. 1996, 1998). The XLG I mutations include four nonsense mutations (Hendrickx et al. 1995; Burwinkel et al. 1998), two frameshift mutations (Burwinkel et al. 1998), one splice-site mutation (Hendrickx et al. 1995), three missense mutations (van den Bergh et al. 1995; Burwinkel et al. 1998), and three in-frame deletions (van den Bergh et al. 1995; Burwinkel et al. 1998), whereas the XLG II mutations include eight missense mutations (Hirono et al. 1995; Burwinkel et al. 1996, 1998; Hendrickx et al. 1996, 1998), a deletion of one amino acid, and an insertion of two amino acids (Hendrickx et al. 1996). The different nature of XLG I and XLG II mutations has previously led to the hypothesis that XLG I mutations disrupt the PHKA2 protein, whereas XLG II mutations might affect the regulation of the PHK enzyme (Hendrickx et al. 1996).

We have previously determined the partial genomic structure and the intron-exon boundaries of the PHKA2 gene (Hendrickx et al. 1995). In the present study, the complete genomic structure was determined, providing primer sequences to perform mutation analysis using exon-by-exon scanning, and a series of XLG I and XLG II mutations was identified.

Patients and Methods

Patients

Mutation analysis was performed in 10 unrelated patients (table 1). All patients are male and show hepatomegaly, delayed growth in childhood, and elevation of serum transaminases. The PHK activity in liver and erythrocytes is shown in table 1. Patient 5 belongs to one of the families involved in the genetic localization of the XLG I gene (Hendrickx et al. 1993). Clinical and biochemical data on patient 8 (Keating et al. 1985) and patient 9 (Tuchman et al. 1986) have been published previously. Patient 7 belongs to the group 2 (i.e., XLG II) patients, described by Maire et al. (1991), who have a normal erythrocyte PHK activity when the latter is measured with exogenous phosphorylase as a substrate but who have a deficient activity when it is measured with endogenous phosphorylase.

PCR

PCR was performed in a 25-μl reaction volume containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X100, 1 mM of each primer (table 2), and 0.4 mM of each dNTP, by use of 0.2 U *Taq* DNA polymerase (Eurogentec). Detailed PCR conditions are available.

Subcloning of Genomic Clones

Approximately 1 μg cosmid or P1 clone was diluted to 250 μl in 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA and was sonicated to obtain fragments of an average length of 500 bp. To generate blunt-ended fragments, T4 DNA polymerase was used. The fragments were precipitated with ethanol and were treated with 30 U T4 DNA polymerase for 20 min at room temperature in a 50-μl reaction volume containing 50 mM Tris-HCl (pH 8.0), 5mM $MgCl_2$, 50 mM DTT, 5 μg BSA/ml, and 0.1 mM of each dNTP. Subsequently, the fragments were ligated into the *Sma*I restriction site of pUC18 by use of the SureClone ligation kit (Pharmacia). Alternatively, genomic clones were digested with *Sau*3A and were ligated into the *Bam*HI site of pUC18 by use of the Ready To Go ligation kit (Pharmacia).

Table 1

Patients Included in Mutation Analysis

Patient	Type of XLG	PHK Activity[a] (% of the Control Mean)		Descent	Mutation
		Erythrocytes	Liver		
1	I	5.6	ND	United Kingdom	Q1169X
2	I	8.7	ND	United Kingdom	fs100
3	I	6.9	ND	Belgium	fs1119
4	I	10.0	6.2	United Kingdom	fs226
5	I	27.2	ND	United Kingdom	E1125K
6	II	177.0	ND	United Kingdom	ΔK189–T190
7	II	123.0	ND	France	R186H
8	II	ND	80.0	United States	R186C
9	I	10.0	10.0	United States	R295H
10	II	83.0	ND	United Kingdom	R295H

[a] All enzyme assays have been performed with rabbit muscle phosphorylase as a substrate; ND = not determined.

Table 2

PHKA2 Exons and Primers Used for SSCP Analysis

Number	Exon Size (bp)[a]	Position	Intron Size (kb)	Primer[b]		Fragment Size (bp)	Annealing Temperature (°C)
				Forward	Reverse		
1	204	−171–78	⩾5.0	cggcctgggagcctcggggag	gagaatgagttacatgagaag	159	48
2	159	79–237	1.5	cagccgagatacagctgtcag	cagcggagatacgctgtcag	234	55
3	48	238–285	1.1	gggccgtgttgctgattctgc	tttatcctctgactttctgac	121	50
4	169	286–454	2.5	cccccataactatgcagagcg	actccttatacaatgggctcc	246	55
5	82	455–537	3.6	gcaacatggcttcataagctc	ggaacatctgaaaagactacc	156	55
6	80	538–618	1.4	ctctttctcccctctgattcc	gcagaaggaagaacaaacagg	151	55
7	99	619–717	2.3	aaaaggaacaccagatattgg	cttccctgctctctgaaatgc	238	55
8	147	718–864	1.3	gcctattacgcatctccagg	catcgacacaggacagaagg	238	55
9	54	865–918	1.6	ctctgagctcatcgaatgtac	ttagagatgaaacagttgagg	138	50
10	123	919–1041	2.5	tgtatgtattctgacatcacc	attatcaacgtaacacccagc	204	55
11	96	1042–1137	4.2	ccttcctgccatggatgatgg	tagataagcggtgcaaagggc	169	55
12	108	1138–1245	2.8	tttatcctctgactttctgac	cctgaacctccaattacatgg	203	55
13	79	1246–1324	.6	atgkgcagtagacacgctcac	ttcatagtgataatgctcctc	134	55
14	135	1325–1459	.8	gacaagagtcatgagctaacg	caaatctagaaccgagatgcc	240	48
15	110	1460–1569	.7	aaccgtatttcctctaactcc	aaaagatgctcgttggcctgc	186	55
16	145	1570–1714	.2	tccaggtaaagaaagggtggc	tttgtaagagcccaaccaccc	253	55
17	79	1715–1793	2.7	ctgcccagactaaactaattc	taagaaaggtcagtgctactc	168	55
18	170	1794–1963	1.5	tccacacactacctgtttccc	cagcgaactacccanaaaagc	234	55
19	174	1964–2137	12.0	ctacagcttggtttcattggc	agaaggtattttaacaggcag	260	55
20	89	2138–2226	1.8	gtgttgttcttcctcctc	gccattgtagaaacaccatac	195	48
21	134	2227–2360	.7	atgcctagccgatctccctgc	cctaagccattctgttcatgg	237	52
22	157	2361–2517	.9	tttctgggtttttctacctc	gagattggaagtggaagagg	252	55
23	80	2518–2597	.2	gttgtctgcctctcacccttc	gcaaacctgaggtccccaagg	149	55
24	79	2598–2676	.1	tgttccatggagggcgaggag	tccactctgaggaaggctgtg	200	55
25	130	2677–2806	.6	ccctgtctctgaaaccctatc	tgcgcggagctgcaggagctg	204	55
26	102	2807–2908	.7	gatttaaacggcagcatttag	agtgctggttctaatgtctgc	369	52
27	119	2909–3027	.9	gctgtagagttcctgagaacc	ctgtcgctttctggggtaagc	233	50
28	30	3028–3057	1.5	gcgtctgctcaaagctgg	actttccacattcaagcc	141	48
29	54	3058–3111	1.7	ttggtgtcattgctgtca	gggacggacacaataatc	140	52
30	171	3112–3282	2.0	ggctgcgtggcttctgaggcc	gctgtgtgtttgcttgcctg	304	62
31	54	3283–3336	.8	gaatctgactgaaatgcgctc	cttattgtgaacccacagagg	139	55
32	201	3337–3537	.6	tggtggttgtctgcctgtccc	ccagcmaacccagctccctgg	261	55
33	896	3538–4433	...	gtggggctccgcttattgtcc	tcatgtttccaggtgagaccc	234	57

[a] The discrepancy between the length of exons 20 and 21 in this report and that in the study by Hendrickx et al. (1995) is due to errors in the 1995 study.

[b] Except for the forward primer of exon 1, which is located in the 5′ UTR, and the reverse primer of exon 33, which is located in the 3′ UTR, all primers are located in PHKA2 introns.

Hybridizations

Probes were radioactively labeled with α-[^{32}P]dCTP by use of the T7 QuickPrime kit (Pharmacia). Hybridizations were performed overnight in 10 × Denhardt's, 10% (w/v) dextran sulfate, 0.1% (w/v) SDS, and 3 × SSC at 65°C. Washing was performed in 1 × SSC, 0.1% (w/v) SDS for 30–45 min at 65°C.

SSCP Analysis

Radioactive PCR was performed, as described above, in the presence of 1 μCi α-[^{32}P]dCTP. Annealing temperatures were optimized for the different primer sets (table 2). SSCP analysis was performed on a 0.5 × Mutation Detection Enhancement (MDE) gel (Baker) in 0.6 × Tris-borate EDTA (TBE) containing 10% (v/v) glycerol at room temperature and on a 0.5 × MDE gel in 0.6 × TBE without glycerol at 4°C. Electrophoresis was performed overnight at 400 V.

Sequence Analysis

PCR products were ligated into either pUC18, by use of the SureClone ligation kit (Pharmacia), or pT-Adv, by use of the AdvanTage PCR cloning kit (Clontech), prior to being sequenced. Alternatively, PCR products were purified from agarose gels by use of the Sephaglas BandPrep kit (Pharmacia) and were sequenced directly. Sequence reactions were performed with the PRISM ready-reaction dye-terminator kit (Perkin-Elmer) and were analyzed on an ABI 373 automated sequencer.

To exclude the possibility of PCR artifacts, all mutations have been confirmed by either PCR or modified PCR on genomic DNA from the patient, followed by

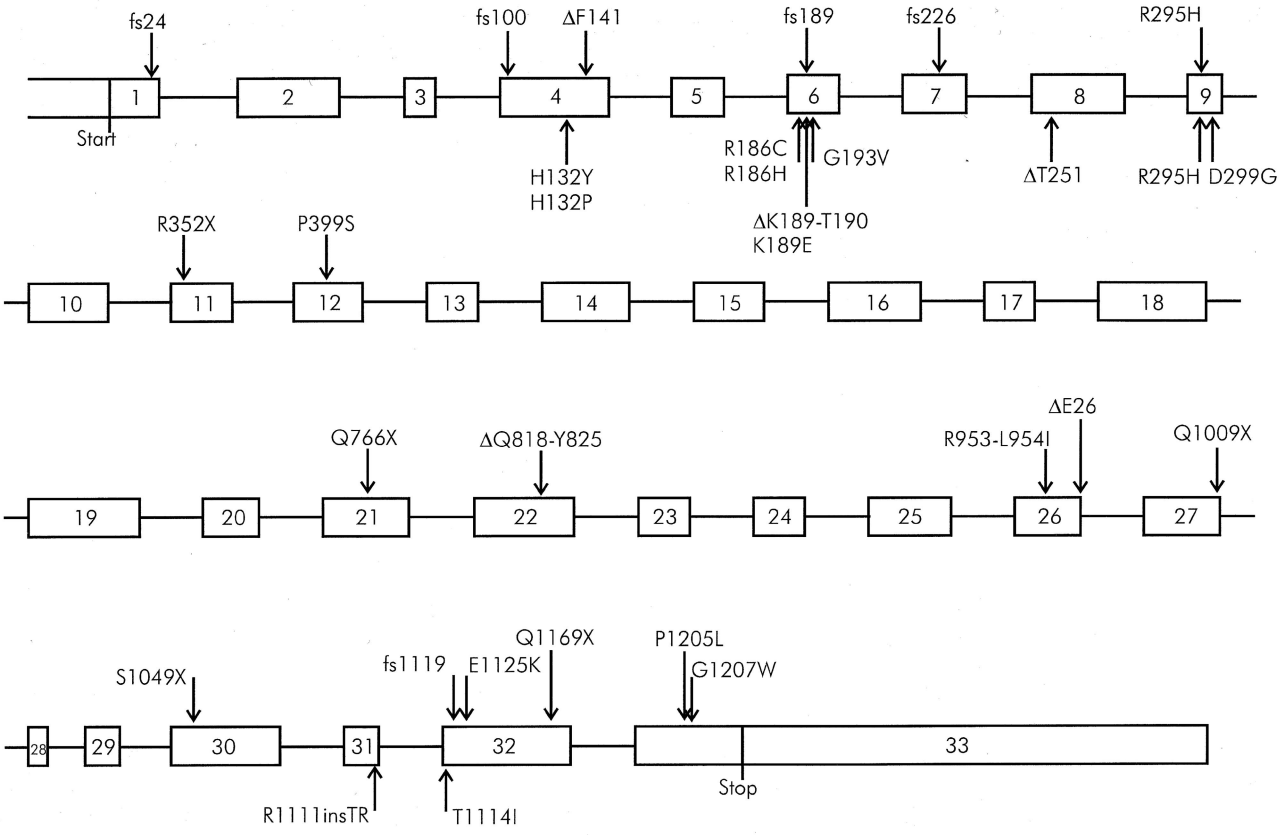

Figure 1 Genomic structure of PHKA2 gene. Exons are indicated by boxes, introns by lines. Exons are to scale; introns are not. The position of XLG I mutations is indicated above the gene, and the position of XLG II mutations is indicated below the gene. The start codon in exon 1 and the stop codon in exon 33 are also indicated.

restriction digestion and/or electrophoresis. Specific confirmation methods and primer sequences are available on request.

Results

Isolation of Genomic Clones

Seven cosmids and two P1 clones containing different parts of the PHKA2 gene were isolated. Cosmids JH3 and JH4 were isolated by screening of a cosmid library constructed from a 49, XXXXY lymphoblastoid cell line (Blonden et al. 1989) with the PC1 clone as described elsewhere (Hendrickx et al. 1993). JH3 has an insert of ~44 kb, JH4 an insert of ~36 kb. Both clones are located in the 3′ end of the gene. Cosmid A159 was isolated from an ICRF cosmid bank and was kindly provided by Dr. Van de Vosse (Department of Genetics, University of Leiden). A159 has an insert of ~38 kb and is located in the 5′ region of the gene. Additionally, an ICRF P1 library was screened with a small PCR product containing the first 260 nucleotides of the PHKA2 cDNA, yielding two positive P1 clones, O166 and P1426. Since none of the isolated cosmid or P1 clones contained the first exon of PHKA2, an ICRF X-specific cosmid bank was screened once more with a PCR product containing only exon 1. This yielded four additional cosmid clones—F0552, I122, I102 and J0726—of which only the first was used further.

Genomic Structure of the PHKA2 Gene

Two approaches were used to determine the genomic structure of the PHKA2 gene. First, the genomic clones JH3, JH4, A159, F0552, O166, and P1426 were fragmented either by sonication or by *Sau*3A restriction digestion, and the fragments were ligated into either the *Sma*I or the *Bam*HI site of pUC18, respectively. Subsequently, the different subclones were screened with the PHKA2 cDNA, and positive subclones were sequenced. Alternatively, exon-exon PCR was performed with PCR primers located in adjacent exons, followed by sequencing of the PCR fragments to determine the boundaries of the intervening intron.

The genomic organization of the PHKA2 gene is shown in figure 1. The gene contains 33 exons, spanning

Table 3

PHKA2 Mutations in XLG I and XLG II

cDNA Change	Protein Change	Exon (E) or Intron (I)	Type of Mutation	Type of XLG	Reference
70insT	fs24	E1	Insertion, 1 bp	I	Burwinkel et al. (1998)
298delG	fs100	E3	Deletion, 1 bp	I	Present study
394C→T	H132Y	E4	Missense	II	Burwinkel et al. (1996)
395A→C	H132P	E4	Missense	II	Burwinkel et al. (1996)
419del3	ΔF141	E4	Deletion, 3 bp	I	Van den Bergh et al. (1995)
556C→T	R186C	E6	Missense	II	Hendrickx et al. (1996); present study
557G→A	R186H	E6	Missense	II	Burwinkel et al. (1996); Hendrickx et al. (1998); present study
567del22	fs189	E6	Deletion, 22 bp	I	Burwinkel et al. (1998)
567A→G	K189E	E6	Missense	II	Burwinkel et al. (1998)
565del6	ΔK189–T190	E6	Deletion, 6 bp	II	Present study
578G→T	G193V	E6	Missense	II	Hirono et al. (1995)
674del13	fs226	E7	Deletion, 13 bp	I	Present study
750del3	ΔT251	E8	Deletion, 3 bp	II	Hendrickx et al. (1996)
884G→A	R295H	E9	Missense	I/II	Present study
896A→G	D299G	E9	Missense	II	Burwinkel et al. (1996)
1054C→T	R352X	E11	Nonsense	I	Burwinkel et al. (1998)
1196C→T	P399S	E12	Missense	I	Burwinkel et al. (1998)
2296C→T	Q766X	E19	Nonsense	I	Hendrickx et al. (1995)
2452del24	ΔQ818–Y825	E22	Deletion, 24 bp	I	Burwinkel et al. (1998)
2858del 3	N953–L954I	E26	Deletion, 3 bp	I	Burwinkel et al. (1998)
2908+1G→T	ΔE26	I26	Splice site	I	Hendrickx et al. (1995)
3025C→T	Q1009X	E27	Nonsense	I	Hendrickx et al. (1995)
3146C→A	S1049X	E30	Nonsense	I	Hendrickx et al. (1995)
3331ins6	R1111insTR	E31	Insertion, 6 bp	II	Hendrickx et al. (1996)
3341C→T	T1114I	E32	Missense	II	Hendrickx et al. (1996)
3355delAA	fs1119	E32	Deletion, 2 bp	I	Present study
3373G→A	E1125K	E32	Missense	I	Present study
3505C→T	Q1169X	E32	Nonsense	I	Present study
3614C→T	P1205L	E33	Missense	I	Van den Bergh et al. (1995)
3619G→T	G1207W	E33	Missense	I	Burwinkel et al. (1998)

⩾65 kb. Clone F0552 contains only exon 1, clone A159 exons 2–18, clone O166 exons 2–11, clone P1426 exons 2–28, clone JH4 exons 10–29, and clone JH3 exons 10–33. Exon 1 contains 171 bp from the 5′ UTR (Hirono et al. 1995), the start codon, and the first 78 bp of the open reading frame (ORF). Exon 33 contains base pairs 3538–3705 of the ORF and all 728 bp of the 3′ UTR. The remaining 31 exons vary in size from 30 bp (exon 28) to 201 bp (exon 32) (table 2).

Except for introns 1 and 19, intron lengths have been estimated by either exon-exon PCR, comparison of overlapping cosmid subclones, or sequencing (table 2). As can be deduced from the estimated insert length (38 kb) of clone A159 and the genomic length of exons 2–18, intron 1 must be ⩾5 kb. The length of intron 19 was estimated to be ~12 kb, as can be deduced from the estimated length of clone JH4 (36 kb), which contains exons 10–29. All intron-exon boundaries follow the gt 5′–donor and ag 3′–acceptor rule and are in good agreement with the consensus for intron-donor and -acceptor sequences in general (Shapiro and Senapathy 1987). Exon and adjacent intron sequences have been submitted to GenBank (accession numbers AF044540–AF044572).

Mutation Analysis in the PHKA2 Gene

The availability of the genomic structure and the sequence of the intron-exon boundaries facilitated mutation analysis using exon-by-exon scanning. On the basis of the intron-exon boundaries, PCR primer pairs flanking each exon were designed (table 2). By use of these primers, the different PHKA2 exons of patients with either XLG I or XLG II were amplified, and the PCR products were analyzed, by SSCP analysis, for the presence of mutations. This yielded seven novel mutations in eight unrelated families and two known mutations in additional families (table 3).

Five novel XLG I mutations—including one nonsense, three frameshift, and one missense mutation—were identified. The first mutation, identified in patient 1, is a nonsense mutation changing the CAG codon (glutamine) at amino acid position 1169 into a TAG stop codon (Q1169X). Both the mother and sister of patient 1 are

heterozygous carriers of the Q1169X mutation. Three frameshift mutations have been identified. The first is caused by deletion of guanine at nucleotide position 298 of the ORF, leading to both a frameshift at amino acid position 100 (fs100) and a premature stop codon 48 codons downstream. This mutation was identified both in patient 2, an isolated patient from the United Kingdom, and in his mother. The second frameshift, identified in patient 3, an isolated Belgian patient, is caused by the deletion of two adenine residues at nucleotide positions 3355 and 3356, which results in both a different reading frame from amino acid 1119 (fs1119) and a premature stop codon 83 codons downstream. A third frameshift is caused by deletion of 13 nucleotides (positions 675–687). This leads to a frameshift starting from amino acid 226 (fs226), resulting in an in-frame stop codon 44 codons downstream. This mutation was identified in patient 4, an isolated English patient, and in his mother. The fifth XLG I mutation was identified in patient 5, who belongs to one of the families used in the linkage mapping of the XLG I gene (family 4 in Hendrickx et al. 1993). This mutation was a missense mutation that changes the GAA codon (glutamic acid) at amino acid position 1125 into an AAA lysine codon (E1125K).

One novel XLG II mutation was identified. This mutation, a deletion of two codons (six nucleotides) that encode lysine and threonine at amino acids positions 189 and 190 (ΔK189–T190), was found in patient 6, an isolated English patient, and in his mother. Additionally, two previously described XLG II mutations, both of which change the arginine codon at amino acid position 186, were shown to be present in additional patients with XLG II. The R186H mutation (Burwinkel et al. 1996; Hendrickx et al. 1998) was found in patient 7, who belongs to a family of French descent that contains three patients in three generations. This patient was included in an earlier study, by Maire et al. (1991). The R186C mutation (Hendrickx et al. 1996) was found in patient 8, who belongs to an American glycogenosis family described by Keating et al. (1985).

Finally, a missense mutation that replaces the CGC codon for arginine at amino acid position 295 with a CAC histidine codon (R295H) was identified in both a patient with XLG I and a patient with XLG II. The patient with XLG I (patient 9) was an isolated American patient with a pronounced PHK deficiency in both liver and erythrocytes (both had 10% of the control mean) and with an unusually severe phenotype (Tuchman et al. 1986). The patient with XLG II (patient 10) was an isolated patient from the United Kingdom who had normal erythrocyte PHK activity (83% of the control mean).

All missense mutations were shown to be absent in a panel of 50 healthy female controls, suggesting that they are disease-causing mutations and not common polymorphisms. All mutations have been confirmed by either PCR or modified PCR on genomic DNA of the patients, followed by restriction digestion and/or electrophoresis of the PCR product, excluding the possibility of PCR artifacts.

Discussion

To facilitate mutation analysis in the PHKA2 gene of patients with XLG, we determined its genomic structure. The PHKA2 gene has an estimated length of ⩾65 kb and consists of 33 exons. Exon-by-exon mutation analysis of PHKA2 was performed by SSCP analysis, followed by sequencing of aberrant PCR fragments. This led to the identification of seven new mutations and two previously described mutations in additional patients. Together with the PHKA2 mutations reported in the literature, 19 mutations have now been found in 18 patients with XLG I (2 different mutations, the substitution of proline 399 by serine and of asparagine 953 and leucine 954 by isoleucine, were present in one patient, but it is not known which one causes XLG), and 12 mutations have been found in 16 patients with XLG II (fig. 1 and table 3).

It is intriguing that mutations in the same gene, PHKA2, lead to two different biochemical phenotypes: (i) XLG I, with pronounced enzyme deficiency, and (ii) XLG II, with normal PHK enzymatic activity in erythrocytes and leukocytes and with variable activity in liver. Previously, we have suggested a correlation between the nature of the mutation and the biochemical phenotype: XLG I mutations might be disruptive and lead to reduced α subunit and PHK, whereas XLG II mutations might lead to normal amounts of mutant α subunit causing dysregulation of PHK in vivo (Hendrickx et al. 1996). Since the in vitro assay of PHK measures only the activity of the catalytic γ subunit of PHK and not that of the regulated holoenzyme, XLG II mutations might not cause PHK deficiency in vitro. Twelve of the 19 XLG I mutations identified so far are nonsense (R352X, Q766X, Q1009X, S1049X, and Q1169X), splice site (ΔE26), or frameshift (fs24, fs100, fs189, fs226, and fs1119) mutations or large deletions (ΔQ818–Y825). These mutations most likely result in a truncated or disrupted α subunit, which is probably nonfunctional and degrades quickly. In that case, the PHK deficiency in patients with XLG I could be explained by the fact that a PHK holoenzyme lacking the α subunit ($\beta\gamma\delta$ complex) is unstable. I-strain mice that have an X-linked muscle PHK deficiency due to a frameshift mutation in the muscle α-subunit gene (Schneider et al. 1993) show absence of the α but also of the β and γ subunits, as well as a reduction of the δ subunit in muscle (Cohen et al. 1976; Picton et al. 1983). Absence of the muscle α subunit obviously leads to absence or reduction of the other PHK subunits. Disruptive mutations in PHKA2 similarly may

Table 4

PHK Enzyme Activities in XLG II

Mutation	Patient	Reference for		Activity in PHK (% of Control Mean)		
		Mutation	Patient	Erythrocytes	Leukocytes	Liver
H132Y		Burwinkel et al. (1996)	Hendrickx et al. (1994)	128	82	16
H132P		Burwinkel et al. (1996)	Lozano et al. (1994)	175		30
R186C		Hendrickx et al. (1996)	Hendrickx et al. (1994)	164		
R186C	8	Present study	Keating et al. (1985)			80
R186H		Burwinkel et al. (1996)	Bakker et al. (1991)	173	57	7
R186H		Hendrickx et al. (1998)	Gitzelmann (1957)	96		16
R186H	7	Present study	Maire et al. (1991)	122		
R186H		Burwinkel et al. (1996)	Maire et al. (1991)	156		
K189E		Burwinkel et al. (1998)	Burwinkel et al. (1998)	400		10
ΔK189–T190	6	Present study	Present study	177		
G193V		Hirono et al. (1995)	Hirono et al. (1995)	200		
ΔT251		Hendrickx et al. (1996)	Hendrickx et al. (1996)	142		
R295H	10	Present study	Present study	83		
D299G		Burwinkel et al. (1996)	Burwinkel et al. (1996)	160		30
R1111insTR		Hendrickx et al. (1996)	Hendrickx et al. (1994)	259	93	
T1114I		Hendrickx et al. (1996)	Hendrickx et al. (1994)	548	77	132

cause reduced liver α subunit and, consequently, reduced liver PHK.

In contrast to XLG I mutations, all XLG II mutations identified so far are missense mutations (H132P, H132Y, R186C, R186H, K189E, G193V, R295H, D299G, and T1114I), small deletions (ΔK189–T190 and ΔT251) or small insertions (R1111insTR), presumably imposing only a minor influence on the primary protein structure of the α subunit. As we have suggested previously (Hendrickx et al. 1996), several of these XLG II mutations disrupt potential phosphorylation sites (the arginine 186–to–glycine 193 region, the threonine 251 residue, and the arginine 1111–to–threonine 1114 region) that may be involved in the regulation of PHK by phosphorylation of the α subunit. Therefore, mutations in these regions might disturb the activation of PHK. Consequently, XLG II patients would show a normal amount of PHK, which, however, could not be activated by phosphorylation. The region containing arginine 186 to glycine 193 seems to be especially important. Five different XLG II mutations, identified in nine unrelated patients, are clustered in these eight amino acids. Moreover, these amino acids are completely conserved in all known α subunits (Zander et al. 1988; Davidson et al. 1992; Schneider et al. 1993; Wüllrich et al. 1993) and in the *Caenorhabditis elegans* α-subunit homologue (Sulston et al. 1992) and are very well conserved in the β subunit (Kilimann et al. 1988; Wüllrich-Schmoll and Kilimann 1996).

For some of the XLG mutations, it is unclear whether the proposed genotype-phenotype correlation holds. First, seven XLG I mutations—including small deletions involving amino acid F141 and amino acids N953 and L954 and missense mutations that substitute R295, P399, E1125, P1205, and G1207—do not result in a truncated α subunit. Second, three XLG II mutation sites (the histidine 132, arginine 295, and aspartic acid 299 residues) do not resemble any known functional protein domains or motifs. Possibly, the residues mutated in XLG I may be important for either the structure of the α subunit or holoenzyme assembly, whereas the residues mutated in XLG II may also have a regulatory function. However, this will remain speculative until more information is available about both the regulation of PHK and the position of the respective residues in either the folded α subunit or the assembled holoenzyme. Third, the presence of the R295H mutation in both a patient with XLG I and a patient with XLG II might be difficult to reconcile with the proposed genotype-phenotype correlation.

It is noteworthy that the PHK-enzyme assays in these two patients have been performed in different labs. This suggests that the specific conditions of the enzyme assay may determine whether PHK is deficient in vitro—that is, whether a patient is classified as having XLG I or XLG II. This is corroborated by the fact that, when PHK is determined by an alternative assay method that measures the activation of endogenous phosphorylase, patients with XLG II do show a PHK-enzyme deficiency (Maire et al. 1991; Hendrickx et al. 1994). In this context, it is also noteworthy that liver PHK shows a significant variability in patients with XLG II (table 4). Whereas liver PHK is deficient in most XLG II patients, two patients showed normal activity. Most of these PHK measurements have been performed in different labs, probably under slightly different reaction conditions. These minor differences in reaction conditions may be responsible for the variability. Alternatively, since PHK

is part of a cascade of enzymatic reactions, each of which is influenced by a variety of factors, individual differences in one of these factors may also result in a varying outcome of the enzyme assay, especially if the mutation influences the regulation of the enzyme. As a consequence, the classification of patients as having either XLG I or XLG II, which is based on the in vitro PHK activity, may not be correct in all cases. It would be interesting to analyze the different enzyme-assay methods used by different laboratories, to find an explanation for the variation in PHK activity in XLG II and to develop an assay method that does detect a PHK deficiency in XLG II. Additionally, the identification of more XLG I mutations and XLG II mutations, which is facilitated by the genomic structure of PHKA2, may significantly extend our knowledge of the structural elements and factors involved in the regulation of PHK. This and an improved and standardized enzyme assay seem essential to confirm and possibly to adjust the genotype-phenotype–correlation hypothesis.

Acknowledgments

We are grateful to all the families with XLG, for their cooperation; to Dr. E. Van de Vosse, from the Department of Genetics, University of Leiden, Leiden, the Netherlands, for cosmid clone A159; and to Dr. G. Carlson, from the School of Biological Sciences, University of Missouri, and Prof. J. Fernandes, from the University of Gröningen, Gröningen, the Netherlands, for critical review of the manuscript. This work was supported by a concerted action from the University of Antwerp (to P.J.W.).

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov (for exon and intron sequences [AF044540–AFO44572])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for XLG [MIM 306000])

References

Bakker HD, Taminiau JAJM, Van Den Berg JET, Berger R (1991) Hepatic phosphorylase b kinase deficiency with normal enzyme activity in leukocytes and erythrocytes. J Inherit Metab Dis 14:269–270

Blonden LAJ, den Dunnen JT, van Paassen HM, Wapenaar MC, Grootscholten PM, Ginjaar HB, Bakker E, et al (1989) High resolution deletion breakpoint mapping in the DMD gene by whole cosmid hybridisation. Nucleic Acid Res 17: 5611–5621

Burwinkel B, Amat L, Gray RGF, Matsuo N, Muroya K, Narisawa K, Sokol RJ, et al (1998) Variability of biochemical and clinical phenotype in X-linked liver glycogenosis with mutations in the phosphorylase kinase PHKA2 gene. Hum Genet 102:423–429

Burwinkel B, Shin YS, Bakker D, Deutsch J, Lozano MJ, Maire I, Kilimann MW (1996) Mutation hotspots in the PHKA2 gene in X-linked liver glycogenosis due to phosphorylase kinase deficiency with atypical activity in blood cells (XLG2). Hum Mol Genet 5:653–658

Cohen PTW, Burchell A, Cohen P (1976) The molecular basis of skeletal muscle phosphorylase kinase deficiency. Eur J Biochem 66:347–356

Davidson JJ, Ozcelik T, Hamacher C, Willems PJ, Francke U, Kilimann MW (1992) cDNA encoding a liver isoform of the phosphorylase kinase α subunit: its structural gene maps to the locus of X-linked liver glycogenosis. Proc Natl Acad Sci USA 89:2096–2100

Fischer R, Koller M, Flura M, Mathews S, Strehler-Page M-A, Krebs J, Penniston JT, et al (1988) Multiple divergent mRNAs code for a single human calmodulin. J Biol Chem 263:17055–17062

Gitzelmann R (1957) Glukagonprobleme bei den Glykogenspeicherkrankheiten. Helv Paediatr Acta 12:425–479

Hanks SK (1989) Messenger ribonucleic acid encoding an apparent isoform of phosphorylase kinase catalytic subunit is abundant in the adult testis. Mol Endocrinol 3:110–116

Hendrickx J, Bosshard NU, Willems P, Gitzelmann R (1998) Clinical, biochemical and molecular findings in a patient with X-linked liver glycogenosis followed for 40 years. Eur J Pediatr 157:919–923

Hendrickx J, Coucke P, Bossuyt P, Wauters J, Raeymaekers P, Marchau F, Smit GPA, et al (1993) X-linked liver glycogenosis: localization and isolation of a candidate gene. Hum Mol Genet 2:583–589

Hendrickx J, Coucke P, Dams E, Lee P, Corbeel L, Odièvre M, Fernandes JF, et al (1995) Mutations in a phosphorylase kinase gene PHKA2 are responsible for X-linked liver glycogenosis. Hum Mol Genet 4:77–83

Hendrickx J, Coucke P, Hors-Cayla MC, Smit GPA, Smeitink J, Berger R, Lee P, et al (1994) Localization of a new type of X-linked liver glycogenosis to the chromosomal region Xp22 containing the liver α-subunit of phosphorylase kinase (PHKA2). Genomics 21:620–625

Hendrickx J, Dams E, Coucke P, Lee P, Fernandes J, Willems PJ (1996) X-linked liver glycogenosis type II (XLG II) is caused by mutations in PHKA2, the gene encoding the liver α subunit of phosphorylase kinase. Hum Mol Genet 5: 649–652

Hendrickx J, Willems PJ (1996) Genetic deficiencies of the glycogen phosphorylase system. Hum Genet 97:551–556

Hirono H, Hayasaka K, Sato W, Takahashi T, Takada G (1995) Isolation of cDNA encoding the human liver phosphorylase kinase α subunit (PHKA2) and identification of a missense mutation of the PHKA2 gene in a family with liver phosphorylase kinase deficiency. Biochem Mol Biol Int 36: 505–511

Huijing F, Fernandes J (1969) X-chromosomal inheritance of liver glycogenosis with phosphorylase kinase deficiency. Am J Hum Genet 21:275–284

Keating JP, Brown BI, White NH, DiMauro S (1985) X-linked glycogen storage disease, a cause of hypotonia, hyper-

uricemia, and growth retardation. Am J Dis Child 139: 609–613

Kilimann MW, Zander NF, Kuhn CC, Crabb JW, Meyer HE, Heilmeyer LMG (1988) The α and β subunits of phosphorylase kinase are homologous: cDNA cloning and primary structure of the β subunit. Proc Natl Acad Sci USA 85: 9381–9385

Lederer B, Van Hoof R, Van Den Berghe G, Hers HG (1975) Glycogen phosphorylase and its converter enzymes in hemolysates of normal human subjects and of patients with type VI glycogen storage disease: a study of phosphorylase kinase deficiency. Biochem J 147:23–35

Lozano MJ, Benlloch T, Garcia LV, Garcia Fuentes M (1994) Hepatic phosphorylase b kinase deficiency with normal enzyme activity in erythrocytes and muscle. J Inherit Metab Dis 17:116–117

Maire I, Baussan C, Moatti N, Mathieu M, Lemonnier A (1991) Biochemical diagnosis of hepatic glycogen storage disease: 20 years of French experience. Clin Biochem 24: 169–178

Picton C, Shenolikar S, Grand R, Cohen P (1983) Calmodulin as an integral subunit of phosphorylase kinase from skeletal muscle. Methods Enzymol 102:219–277

Schimke RN, Zakheim RM, Corder RC, Hug G (1973) Glycogen storage disease type IX: benign glycogenosis of liver and hepatic phosphorylase kinase deficiency. J Pediatr 83: 1031–1034

Schneider A, Davidson JJ, Wullrich A, Kilimann MW (1993) Phosphorylase kinase deficiency in I-strain mice is associated with a frameshift mutation in the alpha subunit muscle isoform. Nat Genet 5:381–385

SenGupta B, Friedberg F, Detera-Wadleigh SD (1987) Molecular analysis of human and rat calmodulin complementary DNA clones: evidence for additional active genes in these species. J Biol Chem 262:16663–16670

Shapiro MB, Senapathy P (1987) RNA splicing of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res 15: 7155–7174

Sulston J, Du Z, Thomas K, Wilson R, Hillier L, Staden R, Halloran N, et al (1992) The *C. elegans* genome sequencing project: a beginning. Nature 356:37–41

Tuchman M, Brown BI, Burke BA, Ulstrom RA (1986) Clinical and laboratory observations in a child with hepatic phosphorylase kinase deficiency. Metabolism 35:627–633

van den Berg IET, van Beurden EACM, Malingré HEM, van Amstel HKP, Poll-The BT, Smeitink JAM, Lamers WH, et al (1995) X-linked liver phosphorylase kinase deficiency is associated with mutations in the human liver phosphorylase kinase α subunit. Am J Hum Genet 56:381–387

Wawrzynczak EJ, Perham RN (1984) Isolation and nucleotide sequence of a cDNA encoding human calmodulin. Biochem Int 9:177–185

Wehner M, Kilimann MW (1995) Human cDNA encoding the muscle isoform of the phosphorylase kinase γ subunit (PHKG1). Hum Genet 96:616–618

Willems PJ, Gerver WJM, Berger R, Fernandes J (1990) The natural history of liver glycogenosis due to phosphorylase kinase deficiency: a longitudinal study of 41 patients. Eur J Pediatr 149:268–271

Wüllrich A, Hamacher C, Schneider A, Kilimann MW (1993) The multiphosphorylation domain of the phosphorylase kinase alpha M and alpha L subunits is a hotspot of differential mRNA processing and of molecular evolution. J Biol Chem 268:23208–23214

Wüllrich-Schmoll A, Kilimann MW (1996) Structure of the human gene encoding the phosphorylase kinase β subunit (PHKB). Eur J Biochem 238:374–380

Zander NF, Meyer HE, Hoffmann-Posorske E, Crabb JW, Heilmeyer LMG, Kilimann MW (1988) cDNA cloning and complete primary structure of skeletal muscle phosphorylase kinase (α subunit). Proc Natl Acad Sci USA 85:2929–2933