ARTICLE

Glycerol kinase deficiency: residual activity explained by reduced transcription and enzyme conformation

Damayanti R Sjarif[1,9], Christina Hellerud[2,9], Johannes K Ploos van Amstel[1], Willem J Kleijer[3], Wolfgang Sperl[4], Didier Lacombe[5], Jõrn Oliver Sass[6], Frits A Beemer[1], Marinus Duran[7] and Bwee Tien Poll-The*[,8]

[1]Department of Medical Genetics, University Medical Center, Utrecht, The Netherlands; [2]Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg University, Gothenburg, Sweden; [3]Department of Clinical Genetics, Erasmus Medical Centre, Rotterdam, The Netherlands; [4]St Johanns-Spital-Landesklinik für Kinder-und Jugendheilkunde, Salzburg, Austria; [5]Department of Medical Genetics and Metabolic Diseases, CHU Pellegrin, Bordeaux Cedex, France; [6]Zentrum für Kinderheilkunde und Jugendmedizin, Universitätsklinikum Freiburg, Freiburg, Germany; [7]Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands; [8]Department of Pediatric Neurology, Academic Medical Center, Amsterdam, The Netherlands

Four unrelated patients with glyceroluria ranging from 7 to 170 mmol/l were studied. The activity of glycerol kinase (GK) in cultured fibroblasts was determined with a specific enzyme assay and with two indirect methods, that is, incorporation into macromolecules of [^{14}C] from [^{14}C]glycerol and its oxidation to [^{14}C]CO$_2$. Exon amplification and RT-PCR were used to identify mutations. In patient 1, with low activity in all three assays, we identified a c.1194A > C (E398D) missense mutation. In patient 2 with a considerable activity of the GK enzyme (22% of reference), oxidation to [^{14}C]CO$_2$ (37%) and a high incorporation of [^{14}C] into macromolecules (92%), we identified a c.182T > C (L61P) mutation that causes the enzyme to have a higher K_m for glycerol ($\sim$300 μM) than normals (2–8 μM). In patient 3, the GK activity estimated by the three different methods ranged from 16 to 22% of reference. Analysis of mRNA from the GK gene revealed three alternatively spliced transcripts. A mutation in intron 3 (g.16835G > A) resulted in an insertion of a cryptic exon between exon 2 or 3 and exon 4. Patient 4 with minor glyceroluria (7 mmol/l) and normal plasma glycerol concentration had normal activity with all three assay methods, thus excluding GK deficiency (GKD) as a cause of slight glyceroluria. To evaluate fully patients with glyceroluria, one needs to measure the GK activity and relate this and the clinical data to genetic findings. Residual enzyme activities in cultured fibroblasts can be found in GKD patients with severe clinical symptoms.
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*Correspondence: Prof Dr BT Poll-The, Department of Pediatric Neurology, Academic Medical Center, PO Box 22660, Amsterdam 1100 DD, The Netherlands. Tel: +31 20 566 75 08; Fax: +31 20 691 77 35;
E-mail: b.t.pollthe@amc.uva.nl
[9]Both authors have contributed equally to this work

Introduction
Glycerol kinase deficiency (GKD, MIM 307030) is an X-linked recessive enzyme defect characterized biochemically by hyperglycerolemia and glyceroluria. Isolated GKD can occur in patients with or without symptoms, mainly due to disturbed energy homeostasis associated with hyperketotic hypoglycemia.[1] It has recently been shown

that subjects with severe catabolic crises in childhood may become symptom free at adult age.[2] Deletion of the *GK* locus together with other loci in the Xp21 region results in the enzyme deficiency in combination with adrenal hypoplasia congenita (AHC) and/or Duchenne muscular dystrophy (DMD).[3] These patients may be mentally retarded and show dysmorphic features.[4]

In GKD patients, glycerol concentration in urine is usually >150 mmol/l (reference $\leqslant 0.2$ mmol/l) and in plasma it is $\sim 2-8$ mmol/l, whereas normally it does not exceed 0.28 mmol/l.[3] An elevated plasma glycerol concentration can erroneously result in an overestimation of plasma triglycerides known as pseudohypertriglyceridemia.[5] The GK enzyme (EC 2.7.1.30; ATP:glycerol 3-phosphotransferase) activity can be assessed to confirm the diagnosis of GKD using either a specific enzyme assay,[6] or indirect but sensitive assays that measure radio-labeled glycerol oxidation[7] or incorporation of [14C]glycerol carbon in macromolecules of intact cultured fibroblasts.[8] Until now, the activity of GK in GKD individuals has been $<1-5\%$ of control enzyme activity assayed by either assay in our laboratories,[1,6] but higher levels of GK activity have been reported from other groups.[9–11]

We here describe four patients with glyceroluria between 7 and 178 mmol/l with a wide range of clinical symptoms and different levels of GK activity. We used mutation analysis to look for aberrations in the *GK* gene that could explain a significant residual GK activity. Kinetic analysis and predictions of the enzyme structure by molecular modeling were used to study the effects of the mutations.

Patients and methods
Clinical reports
Patient 1, a 4-year-old boy, born after 37 weeks of gestation with a birth weight of 2210 g. His mother had gestational diabetes. When the patient was 2 years old, he had periods of episodic vomiting, which led to the clinical and biochemical diagnosis of GKD. At 4.5 years, he showed mild asymmetric spasticity, hyperactivity, mild mental retardation, convergent strabismus, myopia and mild facial dysmorphism. Analysis of plasma triglyceride showed pseudohypertriglyceridemia 4 mmol/l (reference 0.4–1.2 mmol/l), and analysis of urine organic acids showed massive glyceroluria (170 mmol/l).

Patient 2, a 4-year-old boy, had two episodes of somnolence after vomiting necessitating hospital admission at the age of 2 years. Ketoacidosis was a prominent finding and selective screening for inborn errors of metabolism revealed glyceroluria (58 mmol/l) and glycerolemia (4 mmol/l). Plasma cortisol concentration and serum creatine kinase activity were normal. A presumptive diagnosis of isolated GKD was carried out. In an effort to estimate the duration of a safe fasting period, a diagnostic fasting test was performed at the age of 4 years.[12] After 24 h

of fasting, blood glucose was 3.6 mmol/l (controls 2.8–3.8 mmol/l),[13] and there was a moderate metabolic acidosis (pH 7.31; base excess -8.9 mEq/l) with an impressive ketonemia and ketonuria. Plasma 3-hydroxybutyrate concentration at the end of the fasting test was 5.8 mmol/l (controls 1.7–3.2 mmol/l).[13] The psychomotor development and growth were considered to be normal.

Patient 3, a male newborn, was born at 34 weeks of gestation with a birth weight of 1950 g and severe asphyxia. At 1 h after birth, a blood gas analysis showed respiratory acidosis. Electrolyte examination showed a normal blood sodium (133–139 mmol/l) and an elevated blood potassium level (9 mmol/l) suggestive of adrenal gland imbalance. Chest radiography suggested a respiratory distress syndrome complicated by a pneumothorax. Adrenal hypoplasia was suspected because of a deficiency of the fetal adrenal zone steroid 16-OH-pregnenolone (127.5 μg/24 h, reference: 194–1293 μg/24 h) as well as the absence of the cortisol metabolite tetrahydrocortisone in the urine.[14] The activity of creatine kinase in serum was normal. Analysis of urine and plasma organic acids showed hyperglycerolemia (12 mmol/l) and glyceroluria (31 mmol/l). These findings suggested the diagnosis of complex GKD (AHC-GKD). The patient died on the fifth day of life because of circulatory collapse, before diagnostic testing for AHC could be performed.

Patient 4, a $9\frac{1}{2}$-year-old boy was born at a gestational age of 37 weeks with a birth weight of 2760 g. The pregnancy was complicated by premature rupture of the membrane. He was hospitalized for neonatal meningitis. By the age of 3 years, he was referred again because of a delayed development and hypotonia with a positive Gower's sign, and persistent unexplained microcytic anemia. The serum creatine kinase level was normal, and metabolic investigations revealed glyceroluria (7 mmol/l), whereas the glycerol levels in plasma were persistently normal.

Glycerol measurements
Glyceroluria was detected by selective screening of urinary organic acids by gas chromatography-mass spectrometry (GC-MS) of the trimethylsilyl derivatives. Glycerol was either extracted with ethyl acetate or by normal phase extraction.[15] The quantitative determination of glycerol in urine was carried out by a triglyceride test kit, which measures glycerol after hydrolysis. Plasma glycerol was measured with an enzymatic method omitting triglyceride hydrolysis.

[14C]glycerol carbon incorporation into macromolecules
GK activity was indirectly assayed in intact fibroblasts by measurement of the incorporation of [14C]glycerol carbon into trichloroacetic acid (TCA)-precipitable material essentially as described by McCabe *et al*[8] Fibroblasts were cultured in 35 mm dishes in Ham's F10 medium with

15% fetal calf serum (FCS) and antibiotics. Confluent cultures were incubated for 24 h, at 37°C in 1.5 ml F10 medium without FCS, supplemented with 1 μCi/ml of [U-^{14}C]glycerol (171 mCi/mmol, Amersham). After harvesting with trypsin, cells were disrupted in 0.5 ml ice-cold 5% TCA and keeping the tubes on ice for 10 min and subsequent centrifugation precipitated macromolecular material. Following further treatment with cold 5% TCA, the final precipitates were dissolved in 100 μl of 1 mol/l NaOH. Radioactivity in each sample was measured by scintillation counting, while protein was quantitated using the bicinchoninic acid method. The incorporation of radioactivity from [U-^{14}C]glycerol into macromolecular material was expressed as dpm [^{14}C]/μg protein.

Glycerol oxidation to CO_2

Confluent layers of fibroblasts in cell-culture flasks were trypsinized and the cells counted. Equal numbers of cells (300 000) were seeded into 25 cm^2 cell-culture flasks with minimum essential medium Eagle with Earle's salts. When the cell monolayers were confluent, the flasks were washed twice with phosphate-buffered saline and 1 μCi of [U^{14}C]glycerol (specific activity 143 mCi/mmol) in 1.2 ml Krebs Ringer phosphate buffer pH 7.4 was added to one 25 cm^2 cell-culture flask for each incubation time used. The flasks were incubated at 37°C for 2–6 h. The reaction was stopped by adding 1.2 ml 10% TCA and CO_2 was collected on a filter paper with 50 μl 1 mol/l hydroxide of Hyamine® 10-X in methanol, for an additional hour. The formed amount of [^{14}C]CO_2 was counted in a Beckman LS5000 in 2.5 ml methanol and 7.5 ml Ready Safe™ scintillation cocktail.[7]

GK activity measurements

GK activity was measured in fibroblasts after permeabilization by digitonin. The assay is based on the GK catalyzed conversion of [^{14}C]glycerol to [^{14}C]glycerol-3-phosphate, and the formed amount of [^{14}C]glycerol-3-phosphate was determined by scintillation counting.[6,7] The measurements were carried out in duplicate at two different protein concentrations between 0.5 and 3.3 g/l at a [^{14}C]glycerol concentration of 45 μmol/l. The protein determination was performed on a cell suspension dissolved in 0.5 mol/l NaOH according to the method of Lowry et al[16] Substrate saturation assay was performed as described for the enzymatic GK assay with 10 different glycerol concentrations from 1 to 110 μmol/l. Michaelis–Menten constants, K_m and V_{max}, were derived by Hanes–Woolf analysis.

Mutation analysis
Genomic – PCR

DNA was isolated from peripheral blood leukocytes and cultured fibroblasts according to established procedures. Genomic DNA of the index cases and one unrelated normal individual were used for mutation analysis. The entire protein-coding region of the GK gene was analyzed for mutations by sequencing the PCR-amplified exons. Of the 21 exons of the GK gene (Accession No. AJ252550-AJ252560, AJ252563-AJ252570), 19 were amplified using genomic primers as described previously.[1,10,17] To analyze exons 10 and 11, the Expand™ High Fidelity PCR system (Boehringer Mannheim, Germany) was used to amplify the long DNA fragments comprising exons 8–11 according to the manufacturer's instruction as described previously.[1] Sequencing was performed using the dRhodamine dye terminator cycle sequencing kit (Perkin-Elmer) and the products were analyzed on an ABI 377 sequencer (Applied Biosystems™, Foster City, CA, USA).

DNA sequence changes were confirmed by allele-specific oligonucleotide hybridization (ASOH). To hybridize duplicate dot blots of the appropriate genomic fragments, we used T4 polynucleotide kinase radio-labeled sequences;[18] ASO-Normal (TGC ATT AGA **A**GC TGT TTG T, exon 14) and (AGG AAA TTC **T**AC ATT CTG T, exon 3) and ASO-Mutation (TGC ATT AGA **C**GC TGT TTG T, patient 1) and (AGG AAA TTC **C**AC ATT CTG T, patient 2).

To establish the AHC involvement, the DAX1 gene (Dosage-sensitive sex reversal – AHC critical region on the X-chromosome, gene 1) mutation analysis was performed in patient 3 essentially as described previously.[19]

Alterations found in the DNA sequence are compared to GenBank Sequence Accession No. L13943 (The Human Gene Mutation Database, HGMD®) and numbered according to published recommendations.[20]

RT-PCR

Total RNA was purified from fibroblasts with the QIAamp® RNA Blood kit (QIAGEN GmbH, Hilden, Germany) according to their protocol. The QIAGEN® One-Step RT-PCR kit was used for the amplification of the GK Xp21-specific mRNA transcript (EMBL X78211) with primers designed to amplify this active gene copy exclusively.[21] The Xp21 GK gene was amplified as two overlapping fragments, a 667 bp fragment covering exons 1–8 and a 1190 bp fragment covering exons 8–19. Amplified fragments were separated on a 1.5% agarose gel, excised, purified and sequenced on an ABI 377 sequencer using their BigDye™ Terminator kit for the cycle-sequencing reaction. The g.16835G>A mutation (reference sequence NT_011757) found in DNA from patient 3 was confirmed by allele-specific PCR. Two PCR reactions were performed simultaneously with a sense primer detecting the normal allele (TCC TTT CTC CCT TGT TTT T**CC G**) or the mutated allele (TCC TTT CTC CCT TGT TTT T**CC A**) with an antisense primer (AGT TGT ACA GAT GGA AGT G). The sense primers were designed with an additional mismatch (marked in bold). PCR conditions were as described by the enzyme manufacturer with 200 ng DNA, 2.5 mmol/l $MgCl_2$, 100 pmol primer and an annealing temperature of 57°C.

Results

Routine analysis of urinary organic acids as their trimethyl-silyl derivates by GC-MS will not pick up the normal urine glycerol levels. All four patients had a moderately to markedly increased urinary glycerol concentration, which was readily detected by GC-MS (Table 1). Only patient 1 had a urinary glycerol concentration in the range of previously investigated GK-deficient patients, whereas it was lower in the other three patients. The plasma glycerol concentration was 10–40 times above the upper reference limit (<0.28 mmol/l) in patients 1, 2 and 3 but within the reference range in patient 4, who also had the lowest urinary glycerol concentration. A specific enzyme assay and two indirect assays demonstrated GK activity levels ranging from severe deficiency in patient 1, to normal GK activity, normal glycerol oxidation to $[^{14}C]CO_2$ and normal $[^{14}C]$glycerol carbon incorporation into macromolecules in patient 4 (Table 2). Patient 2, who showed classical manifestations of isolated GKD, had a normal incorporation into macromolecules of $[^{14}C]$ from $[^{14}C]$glycerol, but both the specific GK assay and the glycerol oxidation analysis revealed a significant GK deficiency (22 and 37% of reference value, respectively). In patient 3, the specific assay and the two indirect assays showed partial GK

deficiency (16, 17 and 22%, respectively). Kinetic analysis showed that patient 2 had an elevated K_m but most probably a normal V_{max}, while patients 1 and 3 had a normal K_m but a decreased V_{max}, the latter being in the same range as when estimated with the enzymatic assay using a glycerol concentration of 45 μmol/l (Figure 1, Table 2). Patient 4 had normal K_m and V_{max}.

The four patients were tested for GK gene abnormalities. Analysis of the GK exons by electrophoresis on ethidium bromide-stained agarose gels indicated no gross gene abnormalities in any of the patients. Subsequently, the amplified GK exons were sequenced. Patient 1 had a c.1194A>C transversion in exon 14 of the GK gene (Figure 2a), resulting in an E398D amino-acid substitution. Patient 2 showed a c.182T>C transition in exon 3 of the GK gene (Figure 2b), causing an L61P amino-acid substitution. ASOH analysis was used to screen the family members. Both patients were hemizygous and their mothers were heterozygous for the mutations (data not shown). To test whether these are common polymorphisms, we screened 100 alleles from 50 unrelated normal females. All controls had the normal sequence. Patients 3 and 4 did not show any GK gene abnormalities in the exons or in the intron regions in the immediate vicinity of

**Table 1** Clinical and biochemical presentation of four patients with glyceroluria

Patient	Clinical presentation (age of onset in years)	Plasma glycerol (mmol/l)	Urine glycerol (mmol/l)
1	Episodic vomiting, dysmorphic, mild mental retardation, hyperactivity, (2)	2.78	170
2	Altered consciousness during gastrointestinal episodes, (2)	4.1	58.2
3	Small for gestational age, respiratory distress syndrome, adrenal glands imbalance, (newborn)	12	31
4	Delayed development, hypotonia, Gower's sign (+), unexplained microcytic anemia, (3)	0.08	6.9
GKD patients[1,6]		1.3–7.9	84–315
Reference value		$\leqslant 0.28$	$\leqslant 0.2$

Table 2 Enzyme activities, kinetic and molecular genetic data

| Patient	GK assay		$[^{14}C]$glycerol oxidation to $[^{14}C]CO_2$	$[^{14}C]$glycerol carbon incorporation assay	GK kinetic data		Mutation in the GK gene
					K_m	V_{max}	
	pmol/min × mg protein	%[a]	%[a]	%[a]	μmol/l	pmol/min × mg protein	
1	3.1	3	3	8	2	3	c.1194A>C
2	16.7	22	37	92	≈300	≈80	c.182T>C
3	14.1	16	17	22	4	12	g.16835G>A
4	76.2	85	81	100	4	50[b]	No mutation
Reference range	59–124				2–8	59–124	

[a]% activity relative to fibroblast cells from a patient with normal glycerol metabolism analyzed at the same time.
[b]V_{max} value low due to protein concentration above optimum for the enzyme analysis.
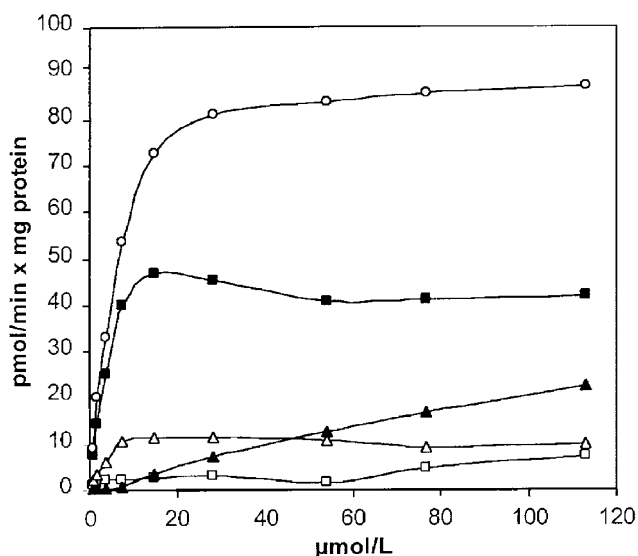
Figure 1 Substrate saturation assay. GK activity measured at different glycerol concentrations from 1 to 110 μmol/l. Patient 4 (■) and the control (○) have normal K_m and V_{max}. Patients 1 (□) and 3 (△) have normal K_m but a reduced V_{max}, whereas patient 2 (▲) has an elevated K_m but probably a normal V_{max}.
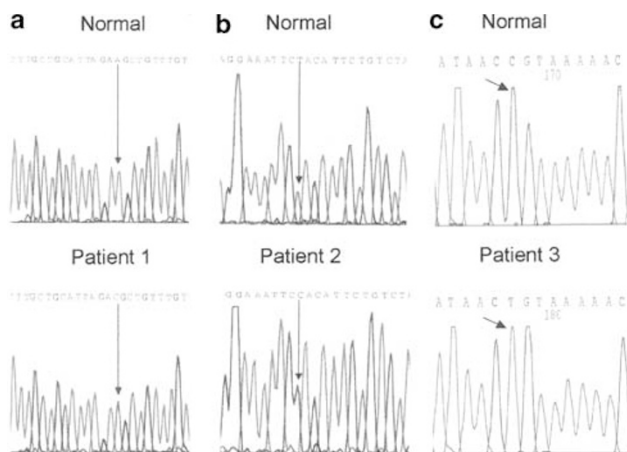
Figure 2 DNA mutations. (**a**) Sequence from exon 14 in the *GK* gene showing the c.1194A>C transversion in DNA from patient 1 and a normal control. (**b**) Sequence from exon 3 in the *GK* gene showing the c.182T>C transition in DNA from patient 2 and a normal control. (**c**) Sequence from the genomic part of intron 3 showing the g.16835G>A mutation in DNA from patient 3 and a normal control (antisense strand).

the exons. Patient 3, who was suspected to have additional AHC, did not show any alterations in the *DAX1* gene (data not shown).

Furthermore, analysis of mRNA from the Xp 21 *GK* gene was performed and amplification of the fragment contain-

ing exons 1–8 resulted in a fragment with the expected length in patients 1, 2 and 4 (Figure 3a), whereas in patient 3 the amplification resulted in three different fragments 3.1, 3.2 and, 3.3 (Figure 3b). Amplification of exons 8–19 resulted in cDNA fragments with the normal size in all four patients (data not shown). Sequencing of the fragments detected the mutations in patients 1 and 2 earlier found with the exon amplification strategy and no aberration was found in cDNA from patient 4. Sequencing of the three different cDNA fragments from patient 3 (Figure 3b), detected an unknown 212 nt long sequence that spliced to exon 4 in fragments 3.1 and 3.2. The 3.3 fragment had the normal length and the correct cDNA sequence.

An ≈2.5 kb genomic DNA fragment was amplified using a primer designed from the cryptic exon part and the GKE 3A primer.[17] After partial sequencing of the 2.5 kb fragment, a positive identification to a region of the GenBank Accession No. AC005913 was made. This 147 kb BAC clone contains the first exons in the *GK* gene and the 212 nt sequence acting as a cryptic exon is located in intron 3. Sequencing of this genomic region in DNA from patient 3 revealed a g.16835G>A mutation, in position −2 before the cryptic exon sequence (Figure 2c). The g.16835G>A mutation was not found in 100 X-chromosomes tested.

Complete sequencing of the two longer cDNA transcripts, 3.1 and 3.2, showed that the 212 nt cryptic exon was spliced in between exon 2 or 3 and exon 4 (Figure 4). The g.16835G>A mutation results in three different transcript [r. = ,r.153_259delins152 + 1_152 + 212, r.259_260ins259 + 1_259 + 212]. Translating the cDNA sequence of the two transcripts with the cryptic exon insertion predicts that they consists of premature stop codons and would probably not result in functional proteins.
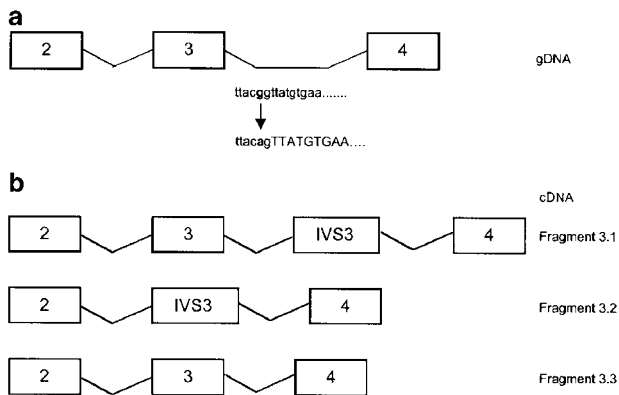
Discussion

GKD is usually suspected in the laboratory from two findings: (a) high glycerol excretion in the urine found by GC-MS in the course of investigation of urinary organic acids (patients 2, 3 and 4), (b) falsely high serum triglyceride concentration as a result of the codetermination of glycerol in the triglyceride method (patient 1).

Further studies of glycerol metabolism are needed when urine and serum glycerol are elevated. Both specific and indirect assays of GK activity are available and are adequate to detect complete GKD (enzyme activity <1–5% of reference).[1,6] In the patients reported here, partial GK deficiencies were demonstrated in three of the four cases by specific as well as indirect methods. The three methods give comparable results if the reduced GK activity results from a lower amount of transcript but with a normal enzyme conformation, as in patient 3. Different results were found in patient 2, who was shown to have a

a

1 2 3 4 5 6 7 8 9 10 marker

b

100 bp marker 3   4

Fragment
3.1 879 bp
3.2 772 bp
3.3 667 bp
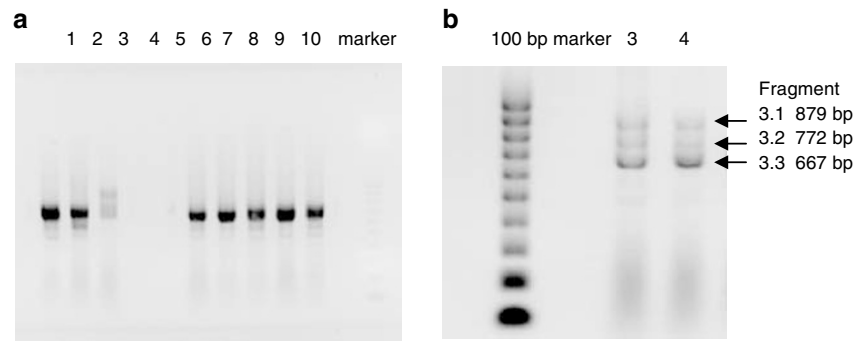
Figure 3 RT-PCR analysis of the Xp21 *GK* gene. (**a**) Agarose gel showing the amplified fragment from exons 1 to 8. In patient 2 (lane 1–2), patient 4 (lane 6) and patient 1 (lane 7–8), the amplification results in the normal 667 bp fragment as in the control sample (lanes 9–10). In mRNA from patient 3 (lane 3), the analysis results in three fragments. (**b**) Lanes 3 and 4 show the different cDNA fragments found in mRNA from patient 3. Two longer fragments 3.1 (879 bp) and 3.2 (772 bp) and the normal sized fragment 3.3.
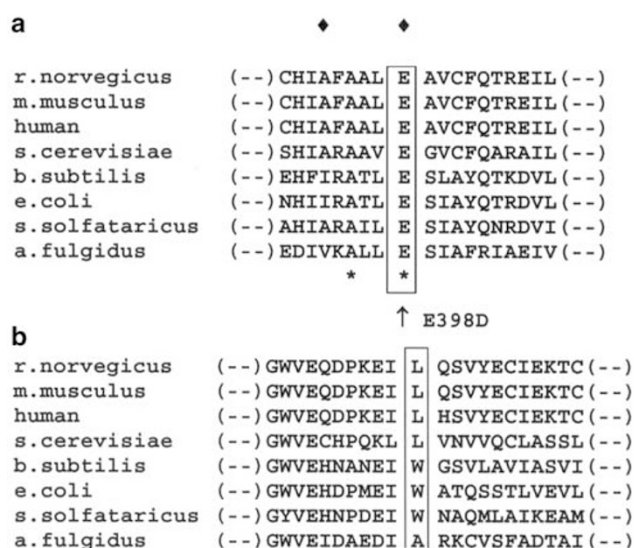
a

| 2 | 3 | 4 |

gDNA

ttacggttatgtgaa.......

↓

ttacag**TTATGTGAA**....

b

cDNA

| 2 | 3 | IVS3 | 4 | Fragment 3.1

| 2 | IVS3 | 4 | Fragment 3.2

| 2 | 3 | 4 | Fragment 3.3

Figure 4 Effect of the g.16835G>A mutation in patient 3. (**a**) Schematic representation of the genomic DNA from exons 2 to 4 with part of a cryptic exon sequence from intron 3 indicated. The g.16835G>A mutation in patient 3 (marked in bold) activates a very strong acceptor site. As a result, a 212 nt long part of intron 3 (IVS3) now act as a cryptic exon (the start is marked with capitals). (**b**) Schematic representation of the three different cDNA fragments from exons 2 to 4 found in patient 3. Fragment 3.1 (879 bp), [r.259_260ins259+1_259+212] with the cryptic exon spliced between exons 3 and 4; fragment 3.2 (772 bp), [r.153_259delins152+1_152+212] lacking exon 3 and with the cryptic exon spliced in between exons 2 and 4; and fragment 3.3 (667 bp) [r=] with a correct splicing of the three exons 2, 3 and 4.

mutation affecting the K_m for glycerol (Table 2). The specific assay showed a residual GK activity of 22% of control at the glycerol concentration of the standard assay, both indirect assays suggested higher residual activity (37% by glycerol oxidation) or even normal activity (92% by glycerol carbon incorporation). Apparently, under the

conditions of the latter indirect assay, the GK activity remains sufficiently high to maintain a normal flow towards synthesis of TCA-precipitable macromolecules. These results emphasize that the apparent level of residual enzyme activity may strongly depend on the conditions of the assay, especially in the case of variants with increased K_m or with reduced enzyme stability. Carbon incorporation into protein and glycerol oxidation are assays of glycerol metabolism that require other functioning enzymes than GK, and could therefore also be used in cases of glyceroluria and/or glycerolemia with normal GK activity in attempts to pinpoint other aberrations in glycerol metabolism than GKD.

In patient 4, a moderate glyceroluria – albeit with normal serum glycerol concentration – led to the suspicion of GKD. This diagnosis could be excluded from the measurement of GK activity. None of the indirect methods indicated an aberration in the metabolism of glycerol and thus the cause of the glyceroluria remains unknown.

DNA analysis in patients 1 and 2 showed two novel missense mutations in the *GK* gene: c.1194A>C (E398D) and c.182T>C (L61P), respectively. The glutamic acid at position 398 is conserved both in prokaryotic and eukaryotic organisms and may therefore be essential for the functioning of the GK protein (Figure 5a). Although the leucine 61 is only conserved in eukaryotes such as rat, mouse and *Saccharomyces cerevisiae* (Figure 5b), it is located in an α-helix on the outside of the GK subunit that interacts with another subunit to form the functional tetramer.[22] As proline residues are prone to interrupt helix formation, the mutation may cause a conformational change that influences the functioning of the GK protein. Also, the decreased affinity for glycerol as shown by the high K_m for glycerol in this patient supports the idea of a disturbed tetramer structure. To date, the mutations were not identified in a panel of 100 X-chromosomes of normal

a

```
                          ◆       ◆
r.norvegicus   (--)CHIAFAAL E AVCFQTREIL(--)
m.musculus     (--)CHIAFAAL E AVCFQTREIL(--)
human          (--)CHIAFAAL E AVCFQTREIL(--)
s.cerevisiae   (--)SHIARAAV E GVCFQARAIL(--)
b.subtilis     (--)EHFIRATL E SLAYQTKDVL(--)
e.coli         (--)NHIIRATL E SIAYQTRDVL(--)
s.solfataricus (--)AHIARAIL E SIAYQNRDVI(--)
a.fulgidus     (--)EDIVKALL E SIAFRIAEIV(--)
                        *   *
```

↑ E398D

b

```
r.norvegicus   (--)GWVEQDPKEI L QSVYECIEKTC(--)
m.musculus     (--)GWVEQDPKEI L QSVYECIEKTC(--)
human          (--)GWVEQDPKEI L HSVYECIEKTC(--)
s.cerevisiae   (--)GWVECHPQKL L VNVVQCLASSL(--)
b.subtilis     (--)GWVEHNANEI W GSVLAVIASVI(--)
e.coli         (--)GWVEHDPMEI W ATQSSTLVEVL(--)
s.solfataricus (--)GYVEHNPDEI W NAQMLAIKEAM(--)
a.fulgidus     (--)GWVEIDAEDI A RKCVSFADTAI(--)
```

Figure 5 Amino-acid sequence alignment. Comparison of the (**a**) glutamic acid at position 398 (E398) and (**b**) leucine at position 61 (L61) for human GK with those for *Rattus norvegicus*, *Mus musculus* and *S. cerevisiae* (eukaryotes); *B. subtilis* and *E. coli* (eubacteria); *S. solfataricus* and *A. fulgidus* (archaebacteria) (Comparative sequence analysis, http://coot.embl-heidelberg.de/Alignment/). Amino acids that are identical with those in human GK are shown with asterisks (*). A closed diamond symbol above the sequence (◆) indicates interactions with ADP in *E. coli*.[29]

controls. It is therefore probable that both missense mutations are disease-causing gene defects.

Several cases with a GK activity >5% have been reported earlier. Sargent et al[10] have described a C256R substitution in a patient with 12% GK activity. This amino-acid residue is located in a *β*-sheet close to domains involved in ATP binding,[22] and this may result in an enzyme with some residual activity. The insertion of an Alu sequence in intron 4 of the *GK* gene described by Zhang et al[11] does not effect the mRNA as judged from their RT-PCR analysis, but the residual GK activity was 32% of the mean normal control. The mutation in the intron sequence found in our patient 3 results in three different mRNA, one correctly spliced and two transcripts where the open-reading frame is disturbed, with the residual GK activity of 15–20% explained by the small amount of correctly spliced transcript. As discussed before,[21] RT-PCR analysis of the *GK* gene is tricky as there is a high homology between this gene and pseudogenes in the GK gene family. The differently spliced transcripts in our patient 3 were not detected with an RT-PCR using primers described earlier.[23] Dipple et al[9] have studied six individuals with missense mutations in the *GK* gene and report GK activities between 5 and 13% of control. They looked into the location of these amino acids in the three-dimensional structure of GK from *Escherichia coli*. In two of the patients, with ∼12% GK activity, the mutations are located in regions involved in ATP binding. An R405Q substitution is located in the same α-helix as the E398D substitution found in our patient 1.

When exon amplification and sequencing does not detect the mutation one can turn to RT-PCR analysis, as shown in patient 3. A mutation in intron 3 (g.16835G>A) created a cryptic splice acceptor site, resulting in alternative splicing of the transcript with a small amount of correctly spliced *GK* mRNA that can explain the high residual GK activity (20%) in patient 3. Similar findings were reported in another patient by Sargent et al[10] with reduced levels of GK activity.

Splice junction analysis has been used to evaluate the effect of mutations in the intron boundaries.[7,21,24] The programs calculate the individual contribution of the nucleotide to the acceptor or donor site. In the cryptic exon sequence including its surroundings, at least four possible acceptor sites exists in the wild type, close to the start of the cryptic exon, but these are probably not functional sites. Three of these have values 6.6, 5.2 and 6.2 bits at locations −1 to −3 relative to the start of the cryptic exon (data not shown). With these very similar values at adjacent locations, they probably compete strongly and are thus nonfunctional in the wild type. The g.16835G>A mutation results in a very strong and functional cryptic splice acceptor site of value 14.7 bits at location −1 relative to this fragment and thus creates a new 212 nt long cryptic exon. The cryptic acceptor site created by the mutation is more than 200 times stronger than the wild-type acceptor sites. The splice sites used for exons 2 and 3 are of moderate strength. The acceptors have values 8.5 and 8.0 bits; the donors, 6.7 and 5.0 bits, respectively. This donor for exon 3 is rather weak. Cases have been reported where upstream exons are skipped when their splice site values are much lower than those of adjacent downstream ones.[25] Thus, the extremely strong cryptic acceptor site due to the g.16835G>A mutation could cause both insertion of a cryptic exon and partial skipping of the preceding exon 3, with its weak donor site, as seen in the mRNA analysis.

Three patients presented with clinical symptoms, suggesting isolated or complex (patient 3) GKD. In these patients decreased GK activity and mutations in the *GK* gene confirmed the diagnosis of GKD. Patients 1 and 2 are typical for the isolated form of GKD from both clinical and laboratory point of view. Although patient 2 had an appreciable GK activity under optimum *in vitro* conditions, the *in vivo* flux through the glycerol pathway was apparently too low to prevent the development of symptoms. Patient 1 also has mild mental retardation. Only a few patients with isolated GKD and a mental alteration have been reported before.[1,6,9,23] In 38 cases of isolated GKD from 28 families, mental retardation and

developmental delay have been reported in five cases.[1] We have knowledge of a further 15 cases who are without mental problems (unpublished observation). Our patient 1 also had facial dysmorphism, myopia, strabismus, etc, indicating a developmental defect in addition to GKD. A severely retarded GKD patient reported by Blomquist et al[6] also had osteoporoses, multiple fractures and dental problems, which could not be explained by GKD. The clinical data concerning most reported cases are scarce, for example, in degree of mental retardation and developmental delay and follow-up period. In our opinion, the question of mental retardation as a phenotypic trait in isolated GKD[9] must be left open. There is, however, the possibility that severe catabolic episode at an early age may cause brain damage. In contrast to the situation in isolated GKD, mental retardation is very common in patients with deletion affecting DAX1, GK and DMD genes possibly by involving the IL1RAPL1 gene. This gene is located distal to the DAX1 gene and part of this gene is deleted in three brothers with a mild Becker-type muscular dystrophy, GKD, AHC and a mild mental retardation.[26]

No aberration was found in the DAX1 gene in patient 3. Although this patient presented nonspecific clinical features, the clearcut hyperglycerolemia, significant glyceroluria and a deficiency of fetal adrenal zone steroid metabolites led to the suspicion of complex GKD. RNA analysis proved that he had a mutation in the GK gene and thus the isolated form of GKD and not the complex form, as it is defined to be caused by deletion of different loci in the p21 region of the X chromosome. As the g.16835G>A mutation has recently been described in another patient with isolated GKD, normal creatine kinase activity and adrenal function,[10] it is unlikely that this mutation could explain the complex clinical picture in our patient. The possibility of another gene defect occurring together with the insertion in the GK gene cannot be excluded. Romero et al[27] reported a c.947C>T mutation in the GK gene in a patient with a deletion of exons 13–29 in the DMD gene.

Patient 4 did not have GKD and no abnormalities in the metabolism of glycerol could be detected with the two indirect assays used. This patient showed a delayed development with glyceroluria without hyperglycerolemia similar to five previously reported children with developmental abnormalities.[28] However, none of the recognized causes of increased glyceroluria, such as hyperthyroidism, von Gierke's disease, fructose-1,6-biphosphatase deficiency, chronic renal failure and strenuous exercise,[28] was present in our patient 4. Also, contamination of urine specimens was ruled out by the consistent presence of increased levels in multiple samples of this patient. The cause of his persistent glyceroluria remains unknown.

We conclude that investigation of patients with glyceroluria should start with the determination of plasma glycerol concentration. Defective glycerol metabolism as a consequence of GKD can be measured in fibroblasts, either with a specific GK assay or indirectly. Indirect GK activity assays by measurement of glycerol carbon incorporation into macromolecules or glycerol oxidation may miss some rare variants with partial GKD. Normal GK activity in indirect test may therefore need further specific enzyme analysis. Genetic analysis may help to explain residual activity and will facilitate the identification of carriers in families.

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