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## Two New Mutations in the Glucose-6-Phosphatase Gene Cause Glycogen Storage Disease in Hungarian Patients

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### Key Words

Glucose-6-phosphatase

Mutations

E110Q

D38V

G222R

R83C

Expression assays

GSD-1a

### Abstract

Glycogen storage disease type 1a (von Gierke disease, GSD-1A) is caused by the deficiency of microsomal glucose-6-phosphatase (G6Pase) activity which catalyzes the final common step of glycogenolysis and gluconeogenesis. The cloning of the G6Pase cDNA and characterization of the human G6Pase gene enabled the identification of the mutations causing GSD-1a. This, in turn, allows the development of non-invasive DNA-based diagnosis that provides reliable carrier testing and prenatal diagnosis. Here we report on two new mutations E110Q and D38V causing GSD-1a in two Hungarian patients. The analyses of these mutations by site-directed mutagenesis followed by transient expression assays demonstrated that E110Q retains 17% of G6Pase enzymatic activity while the D38V abolishes the enzymatic activity. The patient with the E110Q has G222R as his other mutation. G222R was also shown to preserve about 4% of the G6Pase enzymatic activity. Nevertheless, the patient presented with the classical severe symptomatology of the GSD-1a.

### Introduction

Glycogen storage disease type 1a (von Gierke disease, GSD1a), one of the most common types of glycogen storage diseases, is caused by the deficiency of microsomal glucose-6-phosphatase (G6Pase) activity, which catalyzes the final common step of glycogenolysis and gluconeogenesis. The enzyme is normally expressed in the liver, kidney and intestinal mucosa and its absence is associated with excessive accumulation of glycogen in these organs. GSD-1a is transmitted as an autosomal recessive trait with an estimated overall frequency of 1:100,000. The disease manifests itself during the early years of life with hepatomegaly, severe fasting hypoglycemia, bleeding dia-

thesis, lactic acidemia, hyperlipidemia and hyperuricemia. Growth retardation and delayed sexual maturation become prominent during the second decade of life. Long-term complications include gout, hepatic adenomas, osteoporosis and renal disease [1-3]. Currently, the diagnosis of GSD-1a is established by demonstrating the lack of G6Pase activity on a liver biopsy specimen. Carrier testing and prenatal diagnosis on the basis of enzyme activity are not possible. The cloning of the G6Pase cDNA and characterization of the human G6Pase gene by Lei et al. [4] enabled the characterization of the mutations causing GSD-1a [4-9]. This, in turn, allowed the development of non-invasive DNA-based diagnosis that provides reliable carrier testing and prenatal diagnosis.

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The identification of the mutations causing GSD-1a provides an insight into the structure-function relation of G6Pase catalysis by revealing the structural roles of specific amino acids. Here we report on two new mutations causing GSD-1a in two Hungarian patients and the analysis of these mutations on their effect on the G6Pase activity by site-directed mutagenesis followed by transient expression assays.

## Patients

D.O., male, was born on April 21, 1990 after 37 weeks gestation as a second child to non-consanguineous Hungarian parents. The first child is healthy. At age 5 months, he started to suffer from episodes of restlessness and vomiting during the early morning hours. On hospital admission at 7 months of age because of *Escherichia coli* and Rotavirus gastroenteritis an extreme hepatomegaly was found. Blood biochemistry revealed fasting hypoglycemia, metabolic acidosis, hyperlactic acidemia, hypertriglyceridemia and hypercholesterolemia. G6Pase activity performed on fresh liver biopsy material according to Hers [10] showed zero activity. The infant was started on dietary treatment with frequent meals, restriction of glucose and lactose, two meals with uncooked starch during the night and xanthine oxidase inhibitor to treat hyperuricemia. At present he has no psychomotor retardation and his physical growth is between the 3rd and 10th percentile.

P.D. was born on October 9, 1987 after 42 weeks of gestation to non-consanguineous Hungarian parents and has a younger healthy brother. He was admitted to hospital at 2 months of age because of diarrhea and restlessness. On admission, hepatomegaly, fasting hypoglycemia and metabolic acidosis were noted. Blood biochemistry showed hyperlipoproteinemia and hyperuricemia. A fasting glucagon test resulted in a flat blood glucose curve. Liver biopsy revealed G6Pase activity of 0.5 nmol/min/mg tissue, measured according to Hers [10] at a glucose-6-phosphate (G6P) concentration of 100 mM, after subtraction of nonspecific phosphatase activities. As control values range between 4 and 6 nmol/min/mg tissue, the patient's activity represents about 10% of normal activity. As of 1995, he was treated by frequent meals exclusive of lactose and fructose and nocturnal gastric drip which had a beneficial effect on his physical growth (8.5 cm/year) and improved his abnormal lipoprotein values.

## Methods

Extraction of DNA, polymerase chain reaction (PCR) amplification, single-stranded conformation polymorphism (SSCP) was performed as described elsewhere [8] except for the SSCP of the D38V that was observed only under the conditions of electrophoresis on 0.75 × MDE gel, in 0.6 × TBE at 4°C, 8 W for 16 h. Sequence analysis was performed on the whole PCR product purified by the GeneClean kit (BIO101, Calif., USA) by the SequiTherm Cycle Sequencing kit (Epicentre Technologies, Wisc., USA) and was confirmed on two independent PCR products on both strands.

The E110Q and mutations were introduced into the G6Pase cDNA by site-directed mutagenesis as described in Lei et al. [6], by

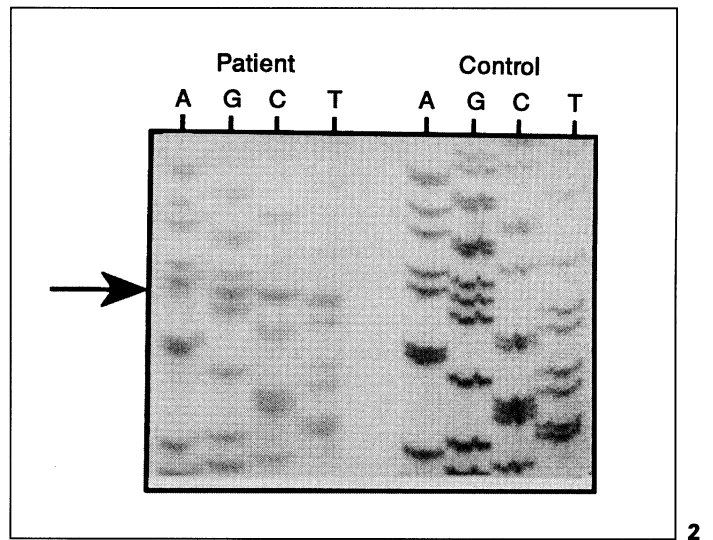
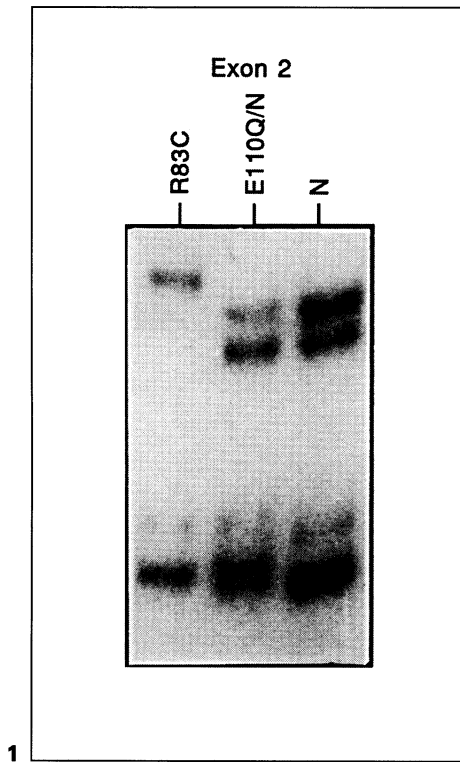
use of the phGpase-1 cDNA which retains the primary amino acid sequence (nucleotides 77–1,156) and activity of wild-type G6Pase. Constructs were transfected into COS 1 cells as described in Lei et al. [6]. Northern hybridization analysis of G6Pase transcripts from transfected cells showed that WT, D38V and E110Q mutant G6Pase were expressed at similar levels. Transfection efficiency varies from experiment to experiment as efficiency is affected by many factors, including cell passage numbers, culture conditions, media, serum, etc. However, within each experiment, the transfection efficiency of the various plasmids differs very little as monitored by Northern blot analysis for G6Pase mRNA expression. Therefore for each transfection experiment, the wild type and vector alone (mock) controls were included. The mutated and the wild-type constructs were tested for enzymatic activity after transient expression in COS-1 cells.

Phosphohydrolase activity was determined essentially as described by Burchel et al. [11] and detailed in Lei et al. [6]. reaction mixtures (100 µl) contained 50 mM cacodylate buffer pH 6.5, 2 mM EDTA, the G6P concentrations specified (0.5, 1 or 10 mM) and appropriate amounts of cell homogenates were incubated at 30°C for 10 min. Protein determination was done using the Micro BCA protein assay of Pierce (Rockford, Ill., USA). Sample absorbance was determined at 820 nm and is related to the amount of phosphate released using standard curve constructed by a stock of inorganic phosphate.

## Results

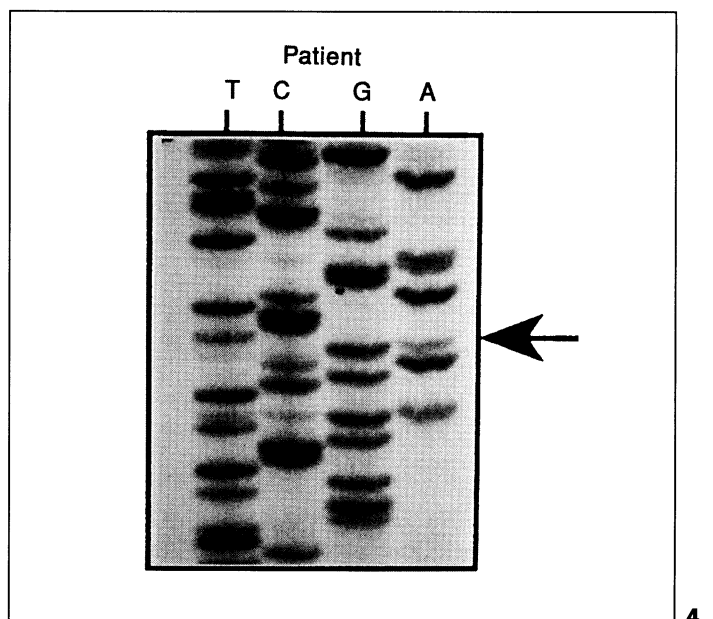
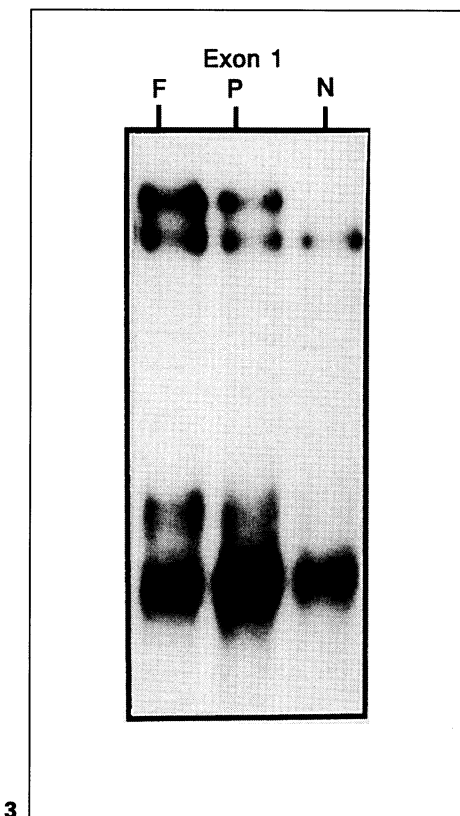
### *Identification of the Mutations in the G6Pase Gene*

The five exons of the G6Pase gene were amplified separately and subjected to SSCP analysis to verify the site of the mutations. Patient P.D. showed abnormal heterozygote SSCP patterns in exons 2 and 5. The SSCP of the normal allele and a PCR product with a deviated mobility that was different from the R83C pattern (fig. 1). Sequencing the PCR product of exon 2 of P.D. revealed a G to C transversion in position 407 in addition to the normal sequence (fig. 2). This will result in the replacement of the glutamic acid at position 110 by glutamine. The deviant pattern in the fifth exon (not shown) was caused by a G to C transversion at position 743 causing G222R (not shown), a mutation reported before [4]. Patient D.O. showed heterozygous SSCP patterns in the first and second exons. The abnormal SSCP pattern of exon 1 was inherited from his father (fig. 3). The SSCP pattern in his second exon was that of the R83C mutation also found in his mother (not shown), this SSCP finding was confirmed by sequencing (not shown). The abnormal SSCP pattern of exon 1 was found by sequencing to result from an A to T transversion at position 192 (fig. 4). This will cause replacement of the aspartic acid at codon 38 by valine.



**Fig. 1.** SSCP analysis of exon 2 of patient P.D. in comparison to exon 2 of control and of an R83 homozygous individual. The SSCP pattern of the PCR product of P.D. is the middle lane marked E110Q/N, showing both the control and deviant patterns.

**Fig. 2.** Sequence analysis of the PCR product of exon 2 of patient P.D. in comparison to a healthy control. The G→C transversion at position 407 is marked by an arrow.



**Fig. 3.** SSCP analysis of exon 1 of patient D.O. and his father in comparison to control. F = Father; P = patient; N = normal healthy control.

**Fig. 4.** Sequence analysis of the PCR product of exon 1 of patient D.O. The position 192 where the T comigrates with the A is marked by an arrow.

**Table 1.** Analysis of G6Pase activity of wild type and mutant G6pase cDNAs in COS-1 cells

Construct	Phosphohydrolase activity, nmol/min/mg protein		
	10 mM G6P	1 mM G6P	0.5 mM G6P
Mock	34.9 ± 3.5	9.6 ± 0.6	1.6 ± 0.3
WT	316.1 ± 13	122.9 ± 8.5	52.5 ± 1.6
E110Q	86.3 ± 6 (18.2%)	30.1 ± 0.4 (18%)	9.9 ± 1.8 (16.3%)
D38V	36.2 ± 1		

Phosphohydrolase activity in whole homogenate was assayed in reactions containing the noted concentrations of G6P. The data are presented as mean ± SEM. Two different isolates of each construct and three independent transfection experiments were conducted. Data are given for one representative transfection experiment. The mock construct represents cells transfected with the expression vector. Wild-type (WT) is the wild type cDNA sequence. D38V and E110Q represent the wild-type construct into which the respective mutations were introduced. The percent activity of the E100Q construct relative to the wild-type construct is given in parentheses. Northern blot analysis of G1Pase transcripts from transfected cells showed that WT, D38V and E110Q mutant G6Pase mRNA were expressed at similar levels (data not shown).

#### *Effects of the Mutations on the G6Pase Phosphohydrolase Activity*

In order to confirm that these codon changes cause GSD-1a they were introduced into the G6Pase cDNA by site-directed mutagenesis and the enzymatic activity of the mutated construct was compared to that of the wild-type construct after transient expression in COS-1 cells (table 1). The change of the aspartic acid at codon 38 by valine completely abolishes the enzymatic activity while the replacement of the glutamic acid by glutamine leaves 17% of the enzymatic activity. To verify whether the replacement of glutamic acid by glutamine affects  $K_m$  or  $V_{max}$ , G6Pase activity of the mutant was also tested at 1 and 0.5 mM (similar to the physiologic value) G6P, the same ratio of activity of E110Q construct to the wild type construct was preserved (table 1).

#### **Discussion**

GSD-1a is a severe metabolic disease involving carbohydrate, purine and lipid metabolism. The severity of the disease, the need of performing liver biopsies for diagnosis and the inability to establish prenatal diagnosis make the identification of the mutations clinically impor-

tant. The identification of the mutations causing GSD-1a enables the families to benefit from DNA-based analysis for the detection of heterozygotes and prenatal diagnosis that can be made from blood, chorionic villus cells or amniocytes. The distinct SSCP patterns of both E110Q and V116G mutations enable easy detection of affected individuals and heterozygotes. Indeed, prenatal diagnosis using SSCP analysis was successfully employed in several GSD-1a families [10]. Successfully identifying most mutations responsible for GSD-1A in a population would mean that those patients in whom GSD-1a was suspected could be screened for the mutations prevalent in the population to avoid the liver biopsy. Hungarians are shown here to have two of the common mutations reported in other populations (R83C and G222R) as well as different mutations, demonstrating the need to characterize the mutations for each population in order to be able to benefit from DNA-based diagnosis.

Patient P.D. has two mutations that retain about 4–17% of enzymatic activity; nevertheless, he presented with the classical severe symptoms of GSD-1A. Thus, apparently a higher activity of the enzyme is necessary for maintaining adequate rates of glycogenolysis and gluconeogenesis. The presentation of GSD-1a is modified by many factors in addition to G6Pase activity, including the frequency of feeds, intercurrent infections as well as hepatic glucose production by alternative pathways, thus in contrast with many other inborn errors, the small residual enzyme activity may not make a big difference to the clinical problem.

#### *Structure Function Requirements of the Human G6Pase*

The replacement of the negatively charged glutamic acid at codon 110 by the uncharged polar glutamine leaves 17% of enzymatic activity, which appears to affect the  $V_{max}$ . Codon 110 is situated in the lumen side between the arginine at codon 83 and the histidine at 119. The arginine at position 83 was suggested to be involved in the positioning of the phosphate which binds the histidine at position 119 that acts as the acceptor [11]. The E110Q mutation demonstrates the requirement of keeping the electrical charges in this domain as the size of glutamic acid would be similar to that of glutamine.

The aspartic acid at codon 38 is localized in the first putative transmembrane domain. Our report is the first to identify a mutation in this domain. The replacement of the negative charge of aspartic acid with the nonpolar valine is not compatible with enzymatic activity (table 1), thus suggesting an important role for the negative charge

in the conformation of the transmembrane domain, that could depend on the neutralization of the positive charge of the arginine at codon 40, the single basic amino acid in this transmembrane domain. In addition, the aspartic acid may function in the trafficking and docking of the enzyme in the membrane. The integrity of the transmembrane domains for enzyme catalysis appears to be important for the G6Pase enzymatic activity since three mutations affecting the third, fourth and sixth transmembrane domains were already reported to abolish completely or greatly reduce the G6Pase enzymatic activity (V166G [8], Lei et al., unpublished; G222R [4] and  $\Delta$ F327 [5], respectively). The importance of the transmembrane domains could be explained by their function in the tight association of G6Pase with the G6P transport. It was recently

demonstrated that in G6Pase knockout mice both G6Pase activity and G6P transport are destroyed [12], thus providing direct evidence for the tight coupling between G6Pase activity and G6P transport which had previously been inferred from kinetic studies of G6Pase catalysis [13]. In addition, the transmembrane domains probably have a role in the strong association of the G6Pase with the endoplasmic reticulum membrane [14].

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