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Structure and mutation analysis of the glycogen storage disease type 1b gene

Paola Marcolongo[a,b], Virginia Barone[b], Giuseppina Priori[b], Barbara Pirola[c], Sabrina Giglio[c], Giacomo Biasucci[d], Enrico Zammarchi[e], Giancarlo Parenti[f], Ann Burchell[g], Angelo Benedetti[a], Vincenzo Sorrentino[b,h,*]

[a]*Institute of General Pathology, University of Siena, Siena, Italy*
[b]*Department of Biology and Technology (DIBIT), San Raffaele Scientific Institute, Milan, Italy*
[c]*Istituto di Biologia Generale e Genetica Medica, Università di Pavia, Pavia, Italy*
[d]*Ospedale S. Paolo, Università di Milano, Milan, Italy*
[e]*Ospedale Pediatrico Meyer, Università di Firenze, Florence, Italy*
[f]*Department of Pediatrics, Università Federico II, Naples, Italy*
[g]*Department of Obstetrics and Gynaecology, Ninewells Hospital and Medical School, University of Dundee, Dundee, Scotland, UK*
[h]*Department of Biomedical Sciences, University of Siena, Siena, Italy*

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Abstract Glycogen storage disease (GSD) 1b is the deficiency of endoplasmic reticulum glucose-6-phosphate (G6P) transport. We here report the structure of the gene encoding a protein likely to be responsible for G6P transport, and its mapping to human chromosome 11q23.3. The gene is composed of nine exons spanning a genomic region of approximately 4 kb. Primers based on the genomic sequence were used in single strand conformation polymorphism (SSCP) analysis and mutations were found in six out of seven GSD 1b patients analysed.

Key words: Glucose-6-phosphate transport; Endoplasmic reticulum; Glycogen storage disease type 1b

1. Introduction

Glycogen storage disease type 1 (GSD 1), also known as 'von Gierke disease', is an autosomal recessive disorder due to a deficiency of glucose-6-phosphatase activity (G6Pase; EC 3.1.3.9) [1,2]. This enzyme catalyses the terminal reaction of glycogenolysis and gluconeogenesis and plays a key role in the maintenance of blood glucose homeostasis [3,4]. G6Pase is mainly represented in liver and kidney where the catalytic subunit is localised in the lumen of the endoplasmic reticulum (ER). According to the 'substrate-transport' model [1,5–7], at least three transport systems are necessary to allow its activity. It has been proposed that the enzyme utilises one transport system (G6PT1) to translocate glucose-6-phosphate (G6P) from the cytosol to the lumen of the ER and two other transport systems to transport the reaction products phosphate (Pi) and glucose (G6PT2 and G6PT3 respectively) to the cytosol [8]. The liver G6PT1 transport protein(s) have not yet been identified. However G6P uptake into liver microsomal vesicles has been clearly demonstrated [9]. All the components of the G6Pase system can be absent or altered in the GSD 1 leading to different subtypes of GSD 1 [1]. GSD type 1a is due to a deficiency of the catalytic subunit (G6PC), whereas 1b, 1c and 1d forms are the consequence of a deficit in the transport of G6P, Pi and glucose respectively. All GSD subtypes show the same clinical profile with the exception of GSD 1b that usually shows, in addition to hypoglycemia and hepatomegaly, neutropenia and impaired neutrophil function and is, therefore, the most severe form [1]. Alterations of the gene coding for G6PC are responsible for type 1a GSD and several mutations abolishing or greatly reducing enzyme activity have been described [10–12]. No mutations in the G6PC gene have been found in patients with GSD 1b who are deficient in liver microsomal G6P transport [13], thus confirming that an additional protein(s) is necessary for ER G6P transport [10].

Recently, a human liver EST cDNA sequence has been identified on the basis of its homology with the coding sequence of bacterial transporters for phosphate esters [14]. The EST was most like UhpC (G6P receptor of *Escherichia coli*), a membrane protein involved in the regulation of UhpT (hexose-6-phosphate transporter of *E. coli*) [15]. The corresponding full length cDNA sequence was found to be mutated in two GSD 1b patients and therefore it is a logic candidate for encoding the putative G6P translocase, or a protein involved in the regulation of the translocase itself [14]. A splicing variant of this cDNA, containing an additional 66 bp, has been identified in brain RNA [16].

Here we describe the characterisation of the putative G6P translocase gene and its involvement in GSD 1b. The genomic region containing the gene has been cloned and all exons and intron-exon boundaries have been sequenced. The gene contains nine exons and has been localised by in situ hybridisation technique to chromosome 11q23.3. Single strand conformational polymorphism (SSCP) analysis revealed that this locus is mutated in six out of seven GSD 1b patients.

2. Materials and methods

2.1. Patients

All the seven GSD 1b patients analysed (five from Italy, one from Perù and one from Australia) showed, in addition to the typical clinical symptoms of GSD 1, neutropenia and impaired neutrophil function, and five of them were initially diagnosed by kinetic analysis of G6Pase system activity in liver biopsy samples (see Table 3). Genomic DNA was isolated from peripheral blood of patients (and their available relatives).

2.2. Methods

Exon-intron structure of the G6P translocase gene was obtained by

*Corresponding author. DIBIT, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano, Italy. Fax: +39 (2) 26434767.
E-mail: sorrentino.vincenzo@hsr.it

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Table 1
Primers and PCR conditions used for the amplification of the nine exons

Exon	PCR product size (bp)	Annealing temperature (°C)	$MgCl_2$ (mM)	Forward primer (5′→3′)	Reverse primer (5′→3′)
1	232	60	1.5	AGGCTGTGCGTCTTGGCTGGTAGGG	TTCTGTGTCCCCAGGTCCACCA
2	340	52	1.5	CCTTCTTTCATTGCTCCTGTGTTT	CTCTATGACAATCCAAACAGGCTC
3	340	66	1.5	CTGCCCCATCTGACCCCACCCTCA	AGTGGTCGGTCTGGGTGGGGGCTC
4	249	66	1.5	GGGGAGAGCAGTCAGGCAGAGCCT	CTGCTCCTTATGCCCACCCTTGTC
5	150	62	1.5	TCCCTCTTCCCACCACAACTCCCT	CCCTTCTCCTTCCTGTCCCTTCTG
6	191	62	1.2	TGTTCTGAGGACGTGACATTGCCG	CCTTGTGCCCTGCCGTGAGCC
7	168	60	1.5	TCTGGGCCTGGTTTTCTTTTCTTC	GTGAGACAGACCAGGAGAAAAACC
8	212	60	1.5	CTCTGAATGCCACTCCACTCTCCC	ACAGGTGGGGGTGAGGGAGAGACT
9	250	60	1.5	GCTTAGGTTCTTCCCTTTCCCCTG	AGAGCGTGCAGGGGGAAGGCCACC**G**

a PCR based method. Human genomic DNA was PCR amplified with Taq polymerase using the following couples of primers designed on the basis of the putative G6P translocase cDNA sequence [14]: G6PT129F (5′-AGGCTGTGCGTCTTGGCTGGTAGGG-3′) and G6PT530R (5′-ACCACTTCCGCAGGACCTTCCCACA-3′); G6PT530F (5′-TGTGGGAAGGTCCTGCGGAAGTGGT-3′) and G6PT834R (5′-GAGAGCACCCACAGGTAAGGGGACA-3′); G6PT834F (5′-TGTCCCCTTACCTGTGGGTGCTCTC-3′) and G6PT1161R (5′-CCAAATACAGCTCCCAATACCAGGA-3′); G6PT1161F (5′-TCCTGGTATTGGGAGCTGTATTTGG-3′) and G6PT1490R (5′-AGAGCGTGCAGGGGGAAGGCCACCG-3′). Briefly, PCR reactions were set up in a total volume of 100 μl containing 300–500 ng of genomic DNA, 50 pmol of each oligonucleotide primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl and 2.5 U Taq polymerase. Thirty-five cycles of amplification were performed in each case at 94°C for 1 min, at the annealing temperature (60°C) for 1 min and at 72°C for 1 min. A final elongation step of 72°C for 4 min was also carried out.

The PCR products were cloned in the p-GEM-T Easy plasmid (Promega) and subsequently sequenced by the dideoxy chain termination method using the Sequenase 2.0 kit (USB).

FISH was performed as previously described [18] using as hybridisation probe the full length G6P translocase cDNA obtained by RT-PCR. The probe was labelled by nick translation with biotin-16-dUTP (Boehringer Mannheim) according to the manufacturer's protocol. Hybridisation was performed on lymphocyte metaphases. Chromosomes were counterstained with propidium iodide 1 μg/ml and banded with diaminophenylnindole (DAPI). Only those chromosomes with signals present on both chromatids at the same band position were taken into consideration. Among 48 well-spread metaphases, 25 showed signals at 11q23.3. No other clusters of signals were found.

For the SSCP analysis [19] PCR primers were designed on the intronic sequences flanking each exon in order to be able to detect mutations both in the coding region and in the splicing consensus sequence.

Primer sequence and PCR conditions used for SSCP analysis are reported in Table 1 together with the expected size of the amplified fragments.

Different gel compositions were applied for each exon in order to increase the probability of detecting SSCP variant bands [20,21]. In particular 4–8 μl of PCR product were loaded on non-denaturing polyacrylamide gel containing 6% acrylamide prepared with a 99:1 ratio between acrylamide and bis-acrylamide and alternatively with or without 5% glycerol in 0.5×TBE. Gels were run in a cold room for 1–4 h at 30 W. DNA bands on the gel were visualised by silver staining. After SSCP analysis, each sample showing a conformational variant was sequenced using the Sequenase 2.0 kit (USB) directly from the purified PCR product. Once a mutation had been detected in any of these patients, all the available relatives were analysed.

G6Pase assays (with a range of G6P and pyrophosphate concentrations) and glycogen content analysis were carried out on microsomes isolated from the biopsy sample from patient C32 (see Table 3) as described in [22,23]. The biopsy samples from patients 3, 7, 10, and 14 (see Table 3) were split into two. One portion was immediately homogenised in 1% wet weight 0.25 M sucrose, the other was frozen with dry ice and then thawed and homogenised in distilled water 1% wet weight. Both were centrifuged at 500×*g* for 5 min. Supernatants were used for assays. Both were diluted 1 to 1 in 0.1 M G6P, 2 mM EDTA pH 6.5 for assay of phosphate released [24]. In addition 5 μl of 0.1 M acetate pH 5.0 was added to 50 μl of both supernatants which were then incubated to inactive specific G6Pase activity [25]. These samples were then diluted in 0.1 M G6P, 2 mM EDTA as above and assayed at pH 6.5 to obtain a value for non-specific phosphatase activity. All the enzyme activity values in Table 3 have been corrected for non-specific phosphatase activity.

3. Results

The human genomic region covering the putative G6P translocase gene was PCR amplified with four couples of primers designed on sequence of the putative G6P translocase cDNA. Four fragments spanning the total length of this gene were cloned and sequenced. The genomic structure of the G6P translocase is shown in Table 2. The gene spans a genomic region of approximately 4 kb and consists of nine exons. Intron size range from 113 bp to ∼560 bp. All intron-exon boundaries were sequenced and conformed to the GT/AG rule [26]. By FISH (see Section 2 for details) we have physically mapped the putative human G6P translocase gene to chromosome 11q23.3.

Table 2
Genomic structure of the human glucose-6-phosphate translocase gene

	Exon size (bp)	Intron size (bp)	5′ Splice site	3′ Splice site
1	317	≅560	TGG**gt**gagccctg	ttccctgc**ag**GGT
2	233	>275	AAG**gt**ggtgagtg	tgccccac**ag**TGG
3	244	>358	AGG**gt**gagccccc	ccacctcc**ag**GCT
4	159	>185	TAG**gt**aagatgag	cctactgc**ag**GTA
5	86	>265	AAG**gt**gagcgggc	gtatccat**ag**GCG
6	114	≅210	AAG**gt**aattaaga	gctcctct**ag**GAT
7	66	≅182	GAG**gt**gccttaaa	cccttggc**ag**CTC
8	139	113	ATG**gt**aagtgtta	cccctgac**ag**TGG
9	729			

Exon 1 and exon 9 contain 169 and 572 nucleotides of 5′ and 3′ untranslated region respectively. Exon sequences are in upper case letters and intron sequences are in lower case letters.

Table 3
Mutations identified in seven GSD 1b patients

Patient	Glucose-6-phosphatase activity		Liver glycogen	Mutation	Effect on the amino acid sequence	Alleles involved
	Untreated microsomes	Disrupted microsomes				
3	0.15	5.3	13%	1211–1212delCT (exon 8)	RFS after Ala-347. Stop codon arises after the first 400 aa	maternal and paternal
7	0.37	2.6	6.4%	1211–1212delCT (exon 8)	RFS after Ala-347. Stop codon arises after the first 400 aa	maternal
10	0.4	4.7	8.6%	normal: AAGgtggtgagt	splicing error between exons 2 and 3	maternal and paternal
				mutant: AAGgtgagt (5′ splice site of intron 2)		
14	0	1.64	9.5%	None found		
15	ND	ND	ND	350delG (exon 2)	RFS after Tyr-60. Stop codon arises after the first 94 aa	parents not available
18	ND	ND	ND	1094delG (exon 6)	RFS after Met-308. Stop codon arises after the first 311 aa	paternal
				G1068A (exon 6)	Arg300His	maternal
C32	$V_{max} = 0$	$V_{max} = 0.4$ $K_m = 0.2$	2025	1211–1212delCT (exon 8)	RFS after Ala-347. Stop codon arises after the first 400 aa	paternal

RFS, reading frame shift. The position of mutations is given according to the numbering of the previously published cDNA sequence [14]. Glucose-6-phosphatase activity values for patients 3, 7, 10 and 14 are expressed in U/mg wet weight liver using 50 mM glucose-6-phosphate as substrate; for patient C32 they are expressed as V_{max} μmoles/min/mg microsomal protein and K_m mM; the data for patient C32 have been corrected for microsomal intactness. Glycogen values are expressed as % wet weight for patients 3, 7, 10 and 14; for patient C32 they are expressed as μg/mg microsomal protein with the normal range being < 800 μg/mg microsomal protein. ND, not determined.

Starting from the genomic sequence obtained, we designed primers in the intronic sequences flanking each exon in order to be able to amplify the coding region and the splicing consensus sequence of these exons. Using these sets of primers it was possible to obtain a unique SSCP pattern for each exon analysed. SSCP band shifts were observed in three exons in the GSD 1b patients analysed. The altered exons in which an SSCP variant was observed were amplified again from genomic DNA and directly sequenced.

Table 3 summarises the G6Pase activity measured in the liver biopsy samples from five out of the seven GSD 1b patients here investigated. In all cases where the G6Pase activity was measured, it was normal under experimental conditions suitable to disrupt ER membrane integrity (i.e. when the transport proteins are not rate limiting) demonstrating that the G6PC was functioning normally and ruling out GSD type 1a. In contrast, in all untreated preparations the activity with G6P as substrate was either absent or very low (mean latency at V_{max} was 92%) indicating a G6P transport protein defect. In patient C32, G6PC activity was also measured with pyrophosphate as substrate; the individual values were all within the normal range in both disrupted microsomes, $V_{max} = 0.4$ μmoles/min/mg microsomal protein, $K_m = 0.8$ mM (control human liver values $n = 25$, $V_{max} = 0.2 \pm 0.02$ μmoles/min/mg microsomal protein, $K_m = 0.7 \pm 0.1$ mM) and intact microsomes, $V_{max} = 0.1$ μmoles/min/mg microsomal protein, $K_m = 4$ mM (control human liver values $n = 25$, $V_{max} = 0.1 \pm 0.01$ μmoles/min/mg microsomal protein, $K_m = 2.5 \pm 0.3$ mM) although the latency with pyrophosphate as substrate was somewhat higher than normal (75%).

SSCP analysis of the coding sequence and exon-intron boundaries of the human G6P translocase gene in these GSD 1b patients resulted in the detection of five novel mutations. Table 3 summarises the mutations identified which include one missense mutation, one possible splicing alteration and three frameshifts. In particular, we found a G→A transition at nucleotide 1068 causing a non-conservative amino acid substitution in patient 18 and in his carrier mother. A three base deletion in the donor splicing site of intron 2 has been found in patient 10 and in her carrier parents. This mutation is likely to determine a splicing error between exons 2 and 3, even though no direct proof has been obtained since mRNA from this patient was not available. The remaining mutations involved the deletion of one or two nucleotides causing a frameshift in the coding sequence. The deletion of the guanosine in position 350, found in the homozygous state in patient 15 (parents are not available), resulted in a stop codon after the first 94 amino acids. Another deletion (1094delG) was detected, together with the G1068A mutation, in patient 18 and in his carrier father. Two nucleotides (1211–1212delCT) were deleted in patient 3 and in his carrier parents. The same mutation was found in one allele of patient 7 and patient C32. This mutated allele was inherited in patient 7 from the mother and in patient C32 from the father. Although all the patients were screened by SSCP for all the exons, no additional mutations were found in the patients carrying the above described mutations.

4. Discussion

GSD 1b is an autosomal recessive disorder caused by a defect in the transport of G6P across the liver ER membrane. The existence of a G6P translocase has been demonstrated biochemically in liver microsomal vesicles [9]. Consistently, G6P transport has been shown to be deficient in one GSD 1b case [27]. More recently, the cDNA of a putative human G6P translocase has been identified and found mutated in two GSD 1b patients [14].

In this paper, we describe the structure and the chromosomal localisation of the gene coding for the translocase responsible for G6P transport across the ER membrane. The gene spans a region of approximately 4 kb and consists of nine exons. Exon 7 is a small exon of 66 nucleotides that has been very recently found to be present in brain mRNAs

whereas the mRNA present in liver is a splicing variant missing exon 7 [14,16]. The gene has been physically mapped to human chromosome 11q23.3. This is in agreement with recent results that have linked the GSD 1b locus to genetic markers in the same region [17]. More recently the GSD 1c locus has also been mapped to chromosome 11 in position q23–24.2 [28], suggesting that either the same gene is responsible for the two subtypes or that another closely linked gene is involved in GSD 1c.

On the basis of the gene structure, we set up SSCP conditions to analyse DNA from seven unrelated GSD 1b patients. The clinical symptoms of these patients were consistent with GSD 1b [1]. The activity of the G6Pase system in liver biopsy samples (when measured) was also consistent with classical GSD 1b [1,6,7]. In these patients we detected five novel mutations affecting the coding capability of this gene. These results, together with those previously reported by van Schaftingen and colleagues [14] strongly suggest that the gene does encode the G6P transport protein(s) necessary to allow G6Pase activity. In four patients we found mutations affecting both alleles. Analysis of parents (when available) was in agreement with the expected recessive inheritance trait [1]. The missense mutation identified (Arg300His) causes the substitution of an amino acid strictly conserved among the different species of bacteria [14,16] analysed. All the three deletions identified cause the synthesis of truncated proteins (Table 3) missing the two lysines at the carboxy terminus necessary as a retention signal in the ER membrane [29]. The described mutations in this paper are likely loss of function mutations. This is consistent with the activity data in the fresh (and intact) liver biopsy samples, where activity was either completely absent or extremely low. SSCP analysis of the other three patients revealed mutations affecting only one allele in two of them, but no mutations in the remaining one. Since genetic heterogeneity has not been reported in GSD 1b, it is likely that this could be accounted by the efficiency of the SSCP method in detecting conformational variants [21], although we cannot exclude that there could be mutations in the promoter or other untranslated regions of the gene.

In conclusion, the data reported demonstrate the association between the gene we have characterised and the GSD 1b disease. Furthermore they also represent a useful tool for genetic diagnostic procedure and for searching for (new) mutations in GSD 1b patients.

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