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ORIGINAL INVESTIGATION

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Genomic structure of the human glucose 6-phosphate translocase gene and novel mutations in the gene of a Japanese patient with glycogen storage disease type Ib

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Abstract Glycogen storage disease (GSD) type Ib is an autosomal recessive disorder caused by a deficiency in microsomal glucose 6-phosphate (G6P) translocase. A gene mutated in GSD type Ib patients has recently been isolated. We have determined the entire sequence of the human G6P translocase gene by PCR-directed sequencing. The gene spans approximately 5 kb of genomic DNA and contains eight exons. Analysis of DNA from a Japanese patient with GSD type Ib revealed new compound heterozygous mutations; a T to C transition at cDNA position 521 resulting in W118R, and an A to C transversion at the –2 splicing acceptor site of intron 1. Reverse transcription (RT)-PCR from leukocyte RNA of the patient revealed the abnormally spliced transcript. These results further support the suggestion that the gene is causative for GSD Ib and should be useful in the molecular diagnosis of such patients.

Introduction

Glycogen storage disease (GSD) type I results from defects in the glucose 6-phosphate (G6P) enzyme system. Glucose 6-phosphatase is affected in GSD type Ia and the microsomal membrane transport system of G6P in GSD types Ib, Ic, and Id (Chen and Burchell 1995). GSD type Ib (GSD Ib) results from a defect of the G6P transporter, i.e., G6P translocase (Narisawa et al. 1978; Lange et al. 1980). The clinical symptoms of GSD Ib are essentially the same as those of GSD Ia with the addition of chronic neutropenia and defective leukocyte function (Beaudet et al. 1980). Little is known, however, about the precise molecular function of G6P translocase in the liver as well as in the leukocyte. Recently, the cDNA sequence encoding a human G6P translocase has been identified and two types of point mutations were reported in the gene of two GSD Ib patients (Gerin et al. 1997).

In the present study, the entire sequence and genomic structure of the human G6P translocase gene have been determined by PCR-directed sequencing. In addition, from the direct DNA sequencing of the gene of a Japanese GSD Ib patient, we have identified new mutations in the gene.

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Materials and methods

Patient

This case, born to non-consanguineous Japanese parents, has previously been reported (Hara et al. 1986; Ihara et al. 1998). The diagnosis of GSD Ib was made by typical clinical and laboratory findings and liver enzyme assay at 4 months of age. Low glucose 6-phosphatase activity was determined in a fresh homogenate of the liver specimen, with a sixfold increase in its activity with deoxycholate treatment. Informed consent for DNA and RNA analyses was obtained from the patient, his parents, and his brother.

Sequencing of the G6P translocase gene

Genomic DNA was isolated from peripheral blood cells using a QIAAmp Blood kit (Qiagen, Tokyo, Japan). The complete G6P translocase coding region was amplified by a TaKaRa PCR thermal cycler MP (TaKaRashuzo, Otsu, Japan) with primers P1: 5'-AAGCAGGAACTGTGGTCAGA and P2: 5'-TGAAAGGTTCAGGGCTAGCC. A PCR fragment was amplified from genomic DNA of a control and the patient by long-distance PCR using TaKaRa LA Taq (TaKaRashuzo). Thermal conditions were an initial heating at 94°C for 3 min, 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min, followed by an additional extension at 72°C for 10 min. The PCR product of about 5 kb long was sequenced completely by an ABI dRhodamine Terminator Cycle Sequencing FS Ready Reaction kit with an ABI PRISM 310 automated DNA sequencer (Perkin-Elmer, Foster City, Calif., USA). The sequence primers were designed from the EMBL data library, accession number Y15409.

RNA extraction and cDNA analysis

Total RNA of the patient and controls was isolated from white blood cells using an RNeasy Blood kit (Qiagen). First-strand cDNA was

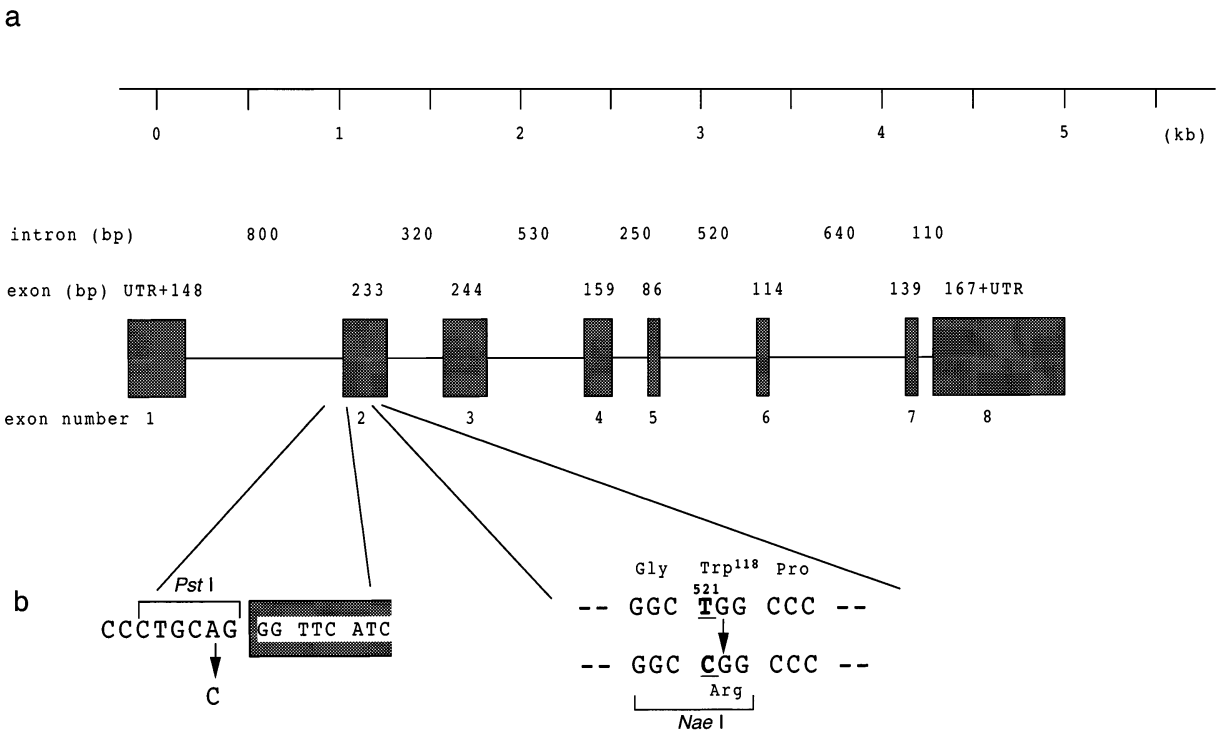

Fig. 1a,b Schematic structure of the human glucose 6-phosphate (G6P) translocase gene and nucleotide substitutions of a Japanese patient. **a** Scale in kilobases (*kb*). The exons are depicted as *shaded boxes* and the introns as a *horizontal line*. The numbering of exons is shown below the exon boxes. The sizes of exons in base pairs are shown above them. Approximate sizes of introns are shown above the exon sizes. **b** Nucleotide substitutions at –2 INS1 and in exon 2 are shown below the gene structure

generated from the total RNA derived from 1.5 ml whole blood using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Tokyo, Japan) with random hexamers. PCR amplification of the G6P translocase cDNA was performed by denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, for 40 cycles with a final 7-min extension at 72°C. PCR primers were P3: 5'-CAGATCTGGGAACTGTGAGA and P4: 5'-AGCCACTGATAACTTCCAGC. The target cDNA was further amplified by nested primer sets, P1 and P2, and PCR thermal conditions were as follows: initial heat at 94°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, for 30 cycles with a final 10-min extension at 72°C. The reaction products were analyzed in a 2.5% agarose gel. For the sequence analyses of two different fragments, each band was excised from the agarose gel, extracted, and purified using a QIAquick Gel Extraction kit (Qiagen). The purified fragments were sequenced by automated DNA sequencer.

PCR–restriction enzyme analysis

One point mutation at position 521 created a *Nae*I site, and the other at the position –2 IVS1 destroyed a *Pst*I restriction site. For screening these substitutions on the gene, digestion of PCR products with restriction enzymes was carried out. DNA from exon 2 to exon 3 of the patient, his family members and normal controls was amplified using primers P5: 5'-TACTGACACTGTGAGCACTG and P6: 5'-TCTGCCTGCTAAATGAGTGC. The PCR products were purified by a QIAquick PCR Purification kit (Qiagen). Then, purified fragments were digested with *Nae*I or *Pst*I restriction enzymes at 37°C for 1 h, followed by separation in a 2.5% agarose gel.

Results

We amplified and sequenced the G6P translocase gene containing the entire protein-coding exons and their internal introns from a normal control DNA. The human G6P translocase gene consists of eight exons and seven introns spanning approximately 5 kb (Fig. 1a). The sequences of the exon/intron boundaries are shown in Table 1.

The whole region was amplified and sequenced from the DNA of a Japanese GSD Ib patient. We found two point mutations. One was a T to C transition at cDNA position 521 (amino acid 118) in exon 2. This substitution caused an amino acid change from tryptophan to arginine. The other was an A to C transversion at the intron 1 splicing–acceptor site (Fig. 1b). The point mutation at position 521 created a *Nae*I site, and the other at the position –2 IVS1 destroyed a *Pst*I restriction site (Fig. 1b). The nucleotide substitutions were confirmed by restriction enzyme digestion of PCR fragments (Fig. 2).

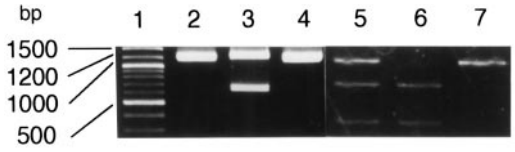

Fig. 2 Electrophoretic analyses of PCR products followed by *Nae*I or *Pst*I digestion. *Lane 1* DNA size markers (100-bp ladder), *lane 2* patient without *Nae*I digestion, *lane 3* patient with *Nae*I digestion, *lane 4* control with *Nae*I digestion, *lane 5* patient with *Pst*I digestion, *lane 6* control with *Pst*I digestion, *lane 7* patient without *Pst*I digestion. PCR product (1380 bp) is cleaved to 694-bp and 686-bp fragments by *Nae*I in the presence of the T to C transition at cDNA position 521, and is not cleaved to 901-bp and 479-bp fragments by *Pst*I in the presence of the A to C transversion at the –2 splicing acceptor site of intron 1

Table 1 Exon–intron boundaries and size of exons in the human glucose 6-phosphate translocase gene. Intron sequence and parts of the 5′- and 3′-end untranslated sequences (*UTR*) are shown by lower-case letters and exon sequences by upper-case letters, with the amino acid numbering based on the predicted cDNA sequence (EMBL accession number Y15409). The translation stop codon is indicated by an asterisk

Exon number	Exon boundaries			Exon size (bp)
1	5′ utr	ATG GCA GCC	·····	GAT TTG G gtgagccctgaaaca	UTR+148
		Met Ala Ala		Asp Leu	
2	taatgttccctgcag	GG TTC ATC	·····	CTG CGG AAG gtgagtgcttccaaa	233
		Gly Phe Ile		Leu Arg Lys	
3	ccctctgccccacag	TGG TTT GAG	·····	AAG AAG G gtgagccccccaccca	244
		Trp Phe Glu		Lys Lys	
4	ttttcccacctccag	GC TCC TTG	·····	CTT GTA G gtaagatgagcatgg	159
		Gly Ser Leu		Leu Val	
5	aactccctactgcag	GT AGC TCC	·····	ATG GCA AAG gtgagcgggcagaag	86
		Gly Ser Ser		Met Ala Lys	
6	ggactgtatccatag	GCG GGA CTG	·····	TCC CCC AAG gtaattaagaaaggc	114
		Ala Gly Leu		Ser Pro Lys	
7	ctctgcccttggcag	CTC TGG ATC	·····	GCC AAT G gtaagtgttaacttc	139
		Leu Trp Ile		Ala Asn	
8	ctttccccctgacag	TG GGC GGC	·····	GCT GAG TGA 3′ utr	167+UTR
		Val Gly Gly		Ala Glu *	

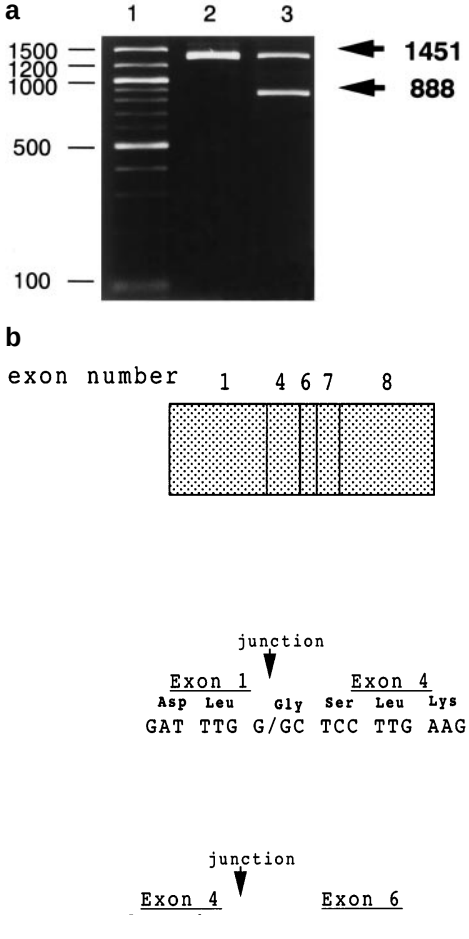

Fig. 3a,b Detection of G6P translocase mRNA in leukocytes by RT-nested PCR. **a** Agarose gel electrophoresis of PCR products. *Lane 1* DNA size markers (100-bp ladder), *lane 2* control, *lane 3* patient. **b** *Top* Schematic presentation of the aberrant G6P translocase transcript. *Middle* The exon 1–exon 4 junction. *Bottom* The exon 4–exon 6 junction. Amino acid sequences derived from the cDNA report are shown above the nucleotide sequence and the predicted amino acids of the abnormal transcript are shown below

This nucleotide change would lead to abnormal splicing and transcriptional aberration. As shown in Fig. 3, a short transcript was detected by RT-nested PCR for the G6P translocase gene. Direct sequencing of the short fragment revealed that an aberrant splicing resulted in skipping of exons 2, 3, and 5.

For the genetic analysis of the affected pedigree, DNA from his family members and the patient were analyzed by PCR followed by restriction enzyme digestion. Appearance of a new *Nae*I site was noted in the patient and his father. Abolition of a *Pst*I site was found in the patient, his mother, and brother. These mutations in family members were confirmed by direct DNA sequencing of the PCR fragments used for the restriction enzyme digestion analysis (data not shown). Neither appearance of a new *Nae*I site nor abolition of a *Pst*I site at these positions were found in 70 alleles of normal controls (data not shown).

Discussion

We have reoprted here the entire sequence of the human G6P translocase gene and its genomic structure. The gene spans about 5 kb and consists of at least eight exons as a protein coding region. As we amplified the gene by using 5’ and 3’ primers which were located about 50 bp upstream or downstream of the untranslated regions, there remained a

possibility that other untranslated exons might exist. As the gene for GSD Ib has recently been mapped to 11q23 (Annabi et al. 1998), the genomic data of the G6P translocase gene will make it possible to perform further localization of the gene.

The gene product is estimated to function as a transporter for a monophosphate ester by structural comparison to various types of bacterial monophosphate ester transporter proteins (Gerin et al. 1997). The bacterial transporter proteins as well as a human G6P translocase have 12 transmembrane helices. The genomic structure showed that helix 1 is in exon 1, helices 2–4 in exon 2, helices 5 and 6 in exon 3, helix 7 in exon 4, helix 8 in exon 5, helix 9 in exon 6, helix 10 and the upper region of helix 11 in exon 7, and the lower region of helix 11 and helix 12 in exon 8, indicating close correlation between transmembrane helices and exons.

Based on the genome sequence data, we analyzed DNA of a patient with GSD Ib. We found two types of nucleotide substitutions. The T to C substitution at cDNA nucleotide position 521 was a missense mutation from tryptophan to arginine at amino acid position 118. This tryptophan locates in the fourth transmembrane helix. This amino acid change might alter the protein structure at the transmembrane region.

The second mutation at the –2 splicing acceptor site of intron 1 was estimated to cause abnormal splicing. In fact, an aberrant splicing resulted in skipping of exons 2, 3, and 5, proven by RT-nested PCR and direct sequencing; exons 1 and 4 joined in-frame, but exons 4 and 6 were connected out-of-frame (Fig. 3). As a result, the gene product was estimated to consist of 49 amino acids from exon 1, 53 from exon 4, and 34, which showed aberrant amino acids by frameshift, from exon 6. It is likely that the abnormal protein has little function as a transporter of G6P because it lacked 10 of the 12 predicted transmembrane helices as well as two lysine residues from its carboxyl terminus that are essential for the protein to be in the endoplasmic reticulum (Jackson et al. 1990). Therefore, this abnormal protein could function neither as a translocase enzyme nor be located as a transmembrane protein in the endoplasmic reticulum. Family study revealed that his mother and father were carriers of each mutation. PCR followed by restriction enzyme digestion of DNA from normal controls showed that neither of the substitutions were present on 70 alleles. These results strongly suggested that the substitutions were causative mutations of GSD Ib. To determine whether these mutations were common to Japanese GSD Ib patients, mutation screening of more patients should be undertaken. Further molecular analyses will be of great help in understanding the pathogenesis of GSD Ib disease.

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