Inactivation of the Glucose 6-Phosphate Transporter Causes Glycogen Storage Disease Type 1b*

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Glycogen storage disease type 1b (GSD-1b) is proposed to be caused by a deficiency in microsomal glucose 6-phosphate (G6P) transport, causing a loss of glucose-6-phosphatase activity and glucose homeostasis. However, for decades, this disorder has defied molecular characterization. In this study, we characterize the structural organization of the G6P transporter gene and identify mutations in the gene that segregate with the GSD-1b disorder. We report the functional characterization of the recombinant G6P transporter and demonstrate that mutations uncovered in GSD-1b patients disrupt G6P transport. Our results, for the first time, define a molecular basis for functional deficiency in GSD-1b and raise the possibility that the defective G6P transporter contributes to neutropenia and neutrophil/monocyte dysfunctions characteristic of GSD-1b patients.

Glycogen storage disease type 1 (GSD-1) is a group of autosomal recessive disorders characterized by hypoglycemia, hepatomegaly, kidney enlargement, growth retardation, lactic acidemia, hyperlipidemia, and hyperuricemia (1, 2). This abnormality is caused by a deficiency in the activity of microsomal glucose-6-phosphatase (G6Pase), a key enzyme in glucose homeostasis, which converts glucose 6-phosphate (G6P) to glucose and phosphate (3). The most prevalent form of GSD-1, GSD-1a, is caused by mutations in the G6Pase gene (4–7) that map to chromosome 17 (5). A more severe form of this disorder, GSD-1b, manifests functional G6Pase deficiency (6), is caused by mutations in the gene (4–7) that map to chromosome 17 (5). A more severe form of this disorder, GSD-1b, although not associated with defects in the G6Pase gene (6), manifests functional G6Pase deficiency in vivo. However, elevated G6Pase catalytic activity can be demonstrated in vitro when hepatic microsomal membranes from these patients are disrupted (8, 9). In addition to having a functional G6Pase deficiency, GSD-1b patients also manifest infections because of a heritable neutropenia and functional deficiencies of neutrophils and monocytes (10, 11).

G6Pase is an endoplasmic reticulum (ER) membrane-spanning protein with the active site facing the lumen (12). It has been proposed that hydrolysis of G6P by intact microsomes requires the participation of several integral membrane proteins, including the G6Pase catalytic unit and a G6P transporter (13). Kinetic studies of G6P hydrolysis (9) suggest that GSD-1b is caused by a defect in microsomal G6P transport, an observation supported by studies (14) using hepatic microsomal strands from GSD-1b patients.

Two complementary approaches have been used to identify the GSD-1b gene. By linkage analysis, we mapped the GSD-1b locus to human chromosome 11q23 (15). By screening an expressed sequence tag data base using a sequence homologous to bacterial transporters of phosphate esters, Gerin et al. (16) identified a candidate human cDNA that encodes a protein predicted to contain an ER transmembrane protein retention motif. This cDNA was recently mapped to chromosome 11 (17–19). However, characterization of the transporter protein and its function, until now, has remained elusive.

We now report the structural organization of the human GSD-1b gene, G6PT, and identify mutations in this gene that segregate with the disorder in members of nine GSD-1b families. We developed a functional G6P transporter assay for the G6P transporter protein and showed that mutations recovered in patients with GSD-1b abolish or greatly reduce G6P transport activity. Our study establishes the molecular basis of the GSD-1b disorder and provides ways for the development of DNA-based diagnostic tests for this disorder.

MATERIALS AND METHODS

Characterization of the Human G6PT Gene and Mutation Analysis—The human G6PT cDNA probe (16) was used to screen a bacterial artificial chromosome (BAC) library by Research Genetics Inc. (Huntsville, AL), and clone 110-11, which contains the G6PT gene, was characterized. BAC DNAs were prepared according to the protocol provided by Research Genetics. DNA sequencing was performed using the ABI Prism 310 genetic analyzer (Perkin-Elmer).

Eight consanguineous families (families 1–8) and one non-consanguineous family (family 9) included in this study were previously used to map the GSD-1b locus to chromosome 11q23 (15). The G6PT gene in GSD-1b patients and available family members was characterized by single-strand conformation polymorphism (SSCP) analysis (20) on mutation detection enhancement gels (AT Biochem, Malvern, PA) containing 5% glycerol. Exon-containing fragments were amplified by polymerase chain reaction using primers containing intronic, 5′ or 3′ untranslated sequences of the human G6PT gene. The sense and antisense primers used are: exon 1 (5′-CTGTCAGAGGGTGCTGCTCT-3′ and 5′-CTGAGTGTCGTTCTTTCGCCCA-3′, 248 bp), exon 2 (5′-TCCTCCTCGTGCTTTTCTCC-3′, 73 bp), exon 3 (5′-TAAGACATTTCGTTCTCCGAGG-3′, 285 bp), exon 4 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp), exon 5 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp), exon 6 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp), exon 7 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp), exon 8 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp), and exon 9 (5′-ATGAGGGTCCGGTGCTACCT-3′, 222 bp). SSCP analysis of the 252G→A mutation in members of family 2 was performed by

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF097831. § These authors contributed equally to this work. || To whom correspondence should be addressed: 98241, Bldg. 10, NIH, Bethesda, MD 20892-1830. Tel: 301-496-1094; Fax: 301-402-7784; E-mail: chou@helix.nih.gov.

The abbreviations used are: GSD-1, glycogen storage disease type 1; G6Pase, glucose-6-phosphatase; G6P, glucose 6-phosphate; ER, endoplasmic reticulum; BAC, bacterial artificial chromosome; SSCP, single strand conformation polymorphism; bp, base pair; CHA, chlorogenic acid; WT, wild-type.
reverse transcriptase-polymerase chain reaction amplification of total RNA isolated from cell lines of family members using two oligonucleotide primers derived from nucleotides 139–160 (sense) and 376–395 (antisense) of the human G6PT cDNA (I*, 287 bp). The mutation containing fragments identified by SSCP was subcloned and characterized by DNA sequencing.

Construction of G6PT Mutants and Expression in COS-1 Cells—Nucleotides 166–1486 of the human G6PT cDNA in a pSVL vector (Amersham Pharmacia Biotech) were used as a template for mutant construction by polymerase chain reaction. The two outside primers are nucleotides 166–186 (sense) and 1466–1486 of the G6PT cDNA (16). Codon 28 (CGC) mutant primers contain C
\[\text{A}^C\text{C}\] at position 28; codon 149 (GGA) mutant primers contain G
\[\text{A}^G\text{A}\] at position 149, and codon 183 (TGT) mutant primers contain C
\[\text{G}^C\text{T}\] at position 183. Bold letters indicate nucleotide changes. After polymerase chain reaction, the amplified fragment was ligated into the pSVL vector. All constructs were verified by DNA sequencing.

Transfection in COS-1 cells was performed as described previously (4, 12). Each construct was present at 30 µg, and the pSVL vector DNA was included in each transfection to a final concentration of 60 µg of plasmid DNA/150-cm² flask. Mock transfections with the pSVL vector (60 µg) alone were used as controls. After incubation at 37 °C for 3 days, the transfected cultures were either harvested for microsomal G6P uptake and phosphohydrolase assays or lysed for RNA isolation. Transfection was conducted at a saturation amount of G6PT cDNA (30 µg/150-cm² flask), and increasing the G6PT cDNA by 2-fold did not alter microsomal G6P uptake activity.

G6P Uptake, Phosphohydrolase, and Northern Blot Analyses—G6P uptake measurements were performed essentially as described previously (21), except sodium cacodylate buffer, which increases G6P uptake efficiency, was used in this study. Briefly, microsomes (40 µg) were incubated in a reaction mixture (100 µl) containing 50 mM sodium cacodylate buffer, pH 6.5, 250 mM sucrose, and 0.2 mM [U-14C]G6P (50 µCi/µmol). The reaction was stopped at the appropriate time by the addition of 50 volumes of an ice-cold solution containing 50 mM Tris-HCl, pH 7.4, and 250 mM sucrose and filtered immediately through a nitrocellulose filter (BA85, Schleicher & Schuell). Microsomes permeabilized with 0.2% deoxycholate, which abolished G6P uptake, were used as negative controls. Two to three independent experiments were conducted, and at least two G6P uptake studies were performed for each microsomal preparation. Statistical analysis using the unpaired t test was performed with the GraphPad Prism Program (GraphPad Software, San Diego, CA).

Phosphohydrolase assays were performed as described previously (4). Dissrupted microsomal membranes were prepared by incubating intact microsomes in 0.2% deoxycholate for 20 min at 0 °C. Nonspecific phosphatase activity was estimated by preincubating microsomal preparations at pH 5 for 10 min at 37 °C, a condition that inactivates the

| Table I Mutations identified in the G6PT gene of GSD-1b patients |
|------------------|-----------------|-----------------|
| Family | Exon | Mutation | Effect on coding sequence |
| 1 | 8 | 1211delCT | Frameshift at 348, stop at 400 |
| 2 | 1 | 252G→A/R28H | Arg→His at 28 |
| 3 | 8 | 1211delCT | Frameshift at 348, stop at 400 |
| 4 | 1 | 252G→A/R28H | Arg→His at 28 |
| 5 | 2 | 338delTCGGCAG | Frameshift at 57, stop at 72 |
| 6 | 4 | 911C→T/G248X | Gin→stop at 248 |
| 7 | 9 | 1349G→A/W393X | Trp→stop at 393 |
| 8 | 3 | 615G→A/G149E | Gly→Glu at 149 |
| 9 | 2 | 550 + 1G→T | AGgt→AGtt at 550 + 1, 5’ splice site |
| 9 | 3 | 716T→C/C183R | Cys→Arg at 183 |
RESULTS AND DISCUSSION

To establish the molecular basis of the GSD-1b disorder, we characterized the structural organization of the human GSD-1b gene, G6PT, isolated from a BAC clone. The G6PT gene (GenBank™ accession number AF097831) spans approximately 5.3 kilobases (Fig. 1A) and consists of 9 exons: I, 317 bp; II, 233 bp; III, 244 bp; IV, 159 bp; V, 86 bp; VI, 114 bp; VII, 66 bp; VIII, 139 bp; and IX, 748 bp. Exon VII, which was identified in the brain G6PT transcript (23), is not present in the liver cDNA (16). Southern blot analyses of human genomic DNA and the BAC clone showed that exon sequences were contained within a single HindIII (~21 kilobases), SpeI (~12 kilobases), or HindIII/SpeI (~9.4 kilobases) fragment (data not shown), suggesting that human G6PT is a single copy gene.

SSCP analysis was used to detect mutations in the G6PT gene in members of the nine GSD-1b families studied for mapping the GSD-1b locus to chromosome 11q23 (15). Exons I, II, III, IV, V, VI, VII, VIII, and IX, along with their intron junctions, were amplified and analyzed. Homozygous mutations that segregate with the disorder were detected in members of consanguineous GSD-1b families, 1–8 (Fig. 1B). A total of six different mutations was identified (Table I). The two deletion mutations, 1211delCT and 338delTCGGCAG, resulted in frameshifts that introduce a premature termination codon.

SSCP analysis (Fig. 1B) also detected a missense mutation (716T→C) (Table I) in one allele of the two patients from the non-consanguineous family 9. This mutation was inherited maternally (Fig. 1B). Because GSD-1b is an autosomal recessive disorder, a second G6PT mutant allele was anticipated, and this mutation was identified after sequencing five G6PT cDNA clones from each patient. Whereas approximately half of the cDNA clones carried the 716T→C mutation, the other half carried a deletion of exon 2 sequences. Sequencing genomic subclones from both patients confirmed that deletion of the exon 2 sequence was caused by a mutation, 550+1G→T, that results in the loss of the splice acceptor for exon 2 (Table I). Sequencing of genomic subclones from both parents also showed that the 550+1G→T mutation is paternally inherited.

Kinetic studies of G6P hydrolysis (9) and transport (14) suggest that GSD-1b is caused by a deficiency in microsomal G6P transport. Using G6Pase-deficient mice, we have shown that G6Pase activity is required for G6P transport into the microsomes (21). To investigate the function of the G6PT protein and its relationship to the G6Pase enzyme, we examined microsomal G6P transport in transient expression studies. COS-1 cells express G6PT but not G6Pase RNA transcripts (19); also a very low level of G6P uptake was detected in microsomes isolated from mock-transfected cells (Fig. 2A). Microsomal G6P transport activity was significantly increased in cells transfected with the G6PT cDNA, indicating that G6P can function as a transporter (Fig. 2A). This is consistent with studies (24) showing that G6P can be taken up by ER/sarcoplasmic reticulum isolated from the brain and the heart, which express the G6PT but not the G6Pase gene (19). Microsomal G6P transport was also slightly increased in cells transfected with a G6Pase cDNA, although the increase was determined to be statistically insignificant. However, G6P uptake was markedly increased in microsomes isolated from COS-1 cells trans-
fected with both G6PT and G6Pase cDNAs (Fig. 2A), demonstrating that G6Pase activity facilitates G6P transport into the microsomal lumen by the G6PT protein. The mutual dependence of G6P transporter and G6Pase enzyme on hydrolysis of G6P explains the functional G6Pase deficiency manifested by GSD-1a patients with a defective G6Pase gene as well as by GSD-1b patients with an intact G6Pase gene.

To further elucidate G6P transport activity of the G6PT protein, we examined the effect of chlorogenic acid (CHA) on G6P uptake. CHA inhibits G6P hydrolysis in intact hepatic microsomes but not in fully disrupted microsomes (25), suggesting that it might be a specific inhibitor of the G6P transporter. If inhibition of phosphohydrolase activity by CHA in intact hepatic microsomes results from inhibition of the G6P transporter, microsomal G6P uptake should be specifically reduced by this inhibitor. Indeed, 1 mM CHA markedly reduced G6P uptake in mouse hepatic microsomes (Fig. 2B) as well as in microsomes isolated from COS-1 cells transfected with G6PT and G6Pase cDNAs (Fig. 2C) or the G6PT cDNA alone (Fig. 2D). CHA appeared to have no effect on G6P transport in microsomes of G6Pase-transfected COS-1 cells (Fig. 2E).

Three missense mutations, R28H, G149E, and C183R, were identified in the G6PT gene of patients from the nine GSD-1b families studied (Table I). To determine the importance of these amino acids in G6P transport, we constructed G6PT mutants and examined microsomal G6P transport activity in transient expression studies. G6P transport in intact microsomes from cells transfected with the G6PT-R28H (Fig. 3A), G6PT-G149E (Fig. 3B), or G6PT-C183R (Fig. 3C) cDNA alone was barely detectable. Co-transfecting COS-1 cells with G6PT-R28H (Fig. 3A), G6PT-G149E (Fig. 3B), or G6PT-C183R (Fig. 3C) and a G6Pase cDNA did not restore G6P transport. Our study, for the first time, established the molecular basis of the GSD-1b disorder.

Similar amounts of G6P hydrolytic activity were detected in disrupted microsomes of cells transfected either with a G6Pase cDNA alone or a G6Pase and a G6PT cDNA (Fig. 3D). Furthermore, co-transfection of a G6Pase cDNA with the wild-type (WT) G6PT cDNA greatly increased phosphohydrolase activity in intact microsomes, suggesting that a functional G6PT protein facilitates G6P hydrolysis. This is in agreement with studies on hepatic microsomes isolated from GSD-1b patients, which suggest that the G6PT protein is required for G6Pase catalysis in vivo.

Northern blot analysis confirmed that similar levels of G6PT and G6Pase transcripts were expressed in WT or mutant G6PT-transfected COS-1 cells (Fig. 3E). Our data therefore demonstrate that the decrease in G6P uptake was because of a defective G6PT protein and not because of a decrease in efficiency of expression of the transfected genes.

In addition to functional G6Pase deficiency, GSD-1b patients also suffer from neutropenia and functional deficiencies of neutrophils and monocytes (10, 11). Neutrophils from GSD-1b patients exhibit impaired mobility and chemotaxis as well as diminished respiratory burst, hexose monophosphate shunt, and phagocytotic activities (reviewed in Ref. 11). Moreover, neutrophils and monocytes from GSD-1b patients are unable to sequester Ca<sup>2+</sup> (26). Human neutrophils/monocytes express the G6PT but not the G6Pase gene (19), and the G6PT transcript expressed in neutrophils/monocytes is
identical to the liver transcript. This raised the question as to whether the G6PT protein plays the same role in gluconeogenic and non-gluconeogenic tissues. It has been shown that G6P enhances ATP-dependent microsomal Ca\(^{2+}\) sequestration (24, 27), and the presence of ATP and Ca\(^{2+}\) also leads to a higher level of G6P accumulation in the ER lumen (24). In neutrophils and monocytes, G6P stimulates glycolysis and hexose monophosphate shunt activity, which provide the major source of energy for chemotaxis and phagocytosis. Thus, it is possible that the G6PT protein has dual roles dependent upon the tissue or cells in which it is expressed. In gluconeogenic tissues that express high levels of the gene the primary function of the G6PT protein is to transport G6P to the ER to be hydrolyzed by G6Pase to produce glucose and phosphate. In other tissues or cells, including neutrophils and monocytes, the G6PT protein may function as a G6P receptor/sensor that regulates Ca\(^{2+}\) sequestration, glycolysis, and hexose monophosphate shunt activity. This could provide an explanation for the observations that despite possessing an intact G6Pase gene, GSD-1b patients manifest neutrophil and monocyte dysfunctions as well as a functional G6Pase deficiency.

The knowledge of the G6PT gene structure now permits the generation of a G6PT-deficient mouse model to increase our understanding of the biology and pathophysiology of GSD-1b and to facilitate the development of novel therapeutic approaches for this disorder.

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