Cloning and Characterization of cDNAs Encoding a Candidate Glycogen Storage Disease Type 1b Protein in Rodents*

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Glycogen storage disease type 1 (GSD-1), also known as von Gierke disease, is a group of autosomal recessive disorders with a combined incidence of approximately 1 in 100,000 live births (1, 2). Patients present with hypoglycemia, hepatomegaly, kidney enlargement, growth retardation, lactic acidemia, hyperlipidemia, and hyperuricemia. The GSD-1 abnormality is caused by a deficiency in the activity of the microsomal enzyme glucose-6-phosphatase (G6Pase). GSD-1a presents with the same clinical symptoms of GSD-1a that reflect a defect in the metabolism of G6P (1, 2). However, unlike other GSD-1 subgroups, these patients suffer additional infectious complications due to heritable neutropenia and functional deficiencies of neutrophils and monocytes, which add to the severity of the disease (10, 11). Treatment of GSD-1b patients consists of a combination of dietary therapy (1, 2) to correct the symptoms of G6Pase deficiency and a human granulocyte-macrophage colony stimulating factor therapy (12, 13) to restore neutrophil/monocyte functions and to reduce the frequency of infection. Earlier studies indicate that GSD-1b is distinct from GSD-1a (8, 10), and accordingly, no mutations have been found in the G6Pase gene of GSD-1b patients (6).

Recently, two complementary approaches have been used to identify the GSD-1b locus. Using linkage analysis, we have mapped the GSD-1b locus to human chromosome 11q23 (14). Using homology to bacterial transporters for phosphate esters as a criterion, Gerin et al. (15) identified a candidate human cDNA that encodes a transmembrane protein resembling the bacterial G6P receptor, UhpC (16), as well as bacterial phosphate ester transporters. The encoded GSD-1b protein is predicted to contain an endoplasmic reticulum (ER) transmembrane protein retention motif at its carboxyl terminus, consistent with its proposed relationship to the G6Pase enzyme.

In this study, we report the cloning and characterization of cDNAs encoding the putative GSD-1b proteins in the mouse and rat. Moreover, we demonstrate that the human cDNA maps to chromosome 11q23, the site for the GSD-1b locus. We further characterize the patterns of tissue-specific and developmental expression of this gene in the mouse. Our results provide strong evidence that this protein is the putative GSD-1b gene product.

MATERIALS AND METHODS

Characterization of Chromosomal 11q23 P1-derived Artificial Chromosome Clones—Clones of P1-derived artificial chromosomes (PACs) spanning chromosome 11q23 were kindly provided by Dr. Glen A. Evans (University of Texas Southwestern Medical Center, Dallas, TX). DNAs from PAC clones were isolated according to the procedures provided by Genome Systems, Inc. (St Louis, MO), digested with restriction enzyme NotI, analyzed by pulsed field gel electrophoresis using the CHEF Mapper XA Pulsed Field Gel Electrophoresis System (Bio-Rad), and blotted to Nytran membranes (Schleicher & Schuell, ). The filters were hybridized with probes derived from the candidate human GSD-1b cDNA (15) following the procedures of Ausubel et al. (17).

Cloning of Human, Murine, and Rat GSD-1b cDNAs—The coding regions of human (hGSD-1b), murine (mGSD-1b), and rat (rGSD-1b) cDNA clones were isolated by reverse transcriptase-polymerase chain reaction (PCR) amplification of the respective human, murine, or rat liver poly(A) RNA using two oligonucleotide primers derived from nucleotides 166 to 192 (sense) and 1439 to 1459 (antisense) of the human GSD-1b cDNA (15). The 5′- and 3′-untranslated regions of murine and rat cDNAs were cloned and sequenced using 5′ and 3′ rapid amplification of cDNA ends (RACE) systems (Life Technologies, Inc.).
Candidate Murine and Rat GSD-1b cDNAs

FIG. 1. Mapping of the candidate hGSD-1b cDNA to human chromosome 11q23. DNAs isolated from PAC clones mapping to the GSD-1b locus on human chromosome 11q23 were digested with NotI, separated by pulsed field gel electrophoresis, and hybridized with probes containing nucleotides 1490–1514, 1–235 (5'), or 1726–2013 (3') in the candidate human GSD-1b cDNA. kb, kilobases pairs.

The PCR or RACE products were cloned in a pGEM7Z vector (Promega, Madison, WI) or a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The GSD-1b cDNA sequences were verified by sequencing three or more PCR and RACE products using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer).

**Primer Extension**—Murine or rat liver poly(A)+ RNA (5 pg) was incubated overnight at 42 °C with 10^6 cpm of a 5'-γ-32P-labeled oligonucleotide primer in hybridization buffer (10 mM Tris-HCl, pH 8, 150 mM KCl, and 1 mM EDTA). The sample was extended with avian myeloblastosis virus reverse transcriptase (20 units, Boehringer Mannheim) for 60 min at 42 °C in 40 μl of a solution containing 50 mM Tris-HCl, pH 8, 50 mM KCl, 2.5 mM MgCl2, and 0.5 mM each of dNTP. The extended fragments were analyzed on 8% polyacrylamide-urea sequencing gels. The murine antisense primer contained nucleotides 119–143 of mGSD-1b cDNA, and the rat antisense primers contained nucleotides 152–175 of rGSD-1b cDNA.

**Northern Blot Hybridization Analysis**—Total RNA was isolated by guanidinium thiocyanate-CsCl method (18), and poly(A)+ RNA was obtained by oligo(dT)-cellulose chromatography. RNA was fractionated by electrophoresis through 1.2% agarose gels containing 2.2M formaldehyde, and transferred to a Nytran membrane by electrolabelling. The filters were hybridized at 62 °C in a buffer containing 5× SSC, 50% formamide, 50 mM sodium phosphate, pH 6.5, 5× Denhardt, 1% SDS, 200 μg/ml sonicated salmon sperm DNA, and a uniformly labeled riboprobe. Two sets of liver or kidney RNA preparations were used, and similar results were obtained. The data presented are from a representative experiment.

**Isolation of Human Neutrophils and Monocytes**—Neutrophils/monocytes were isolated from fresh human blood following the procedures of Coligan et al. (19). Briefly, neutrophils/monocytes were enriched by sequential sedimentation in dextran-saline and pelleted by density centrifugation in endotoxin-free Ficoll-sodium diatrizoate (Ficoll-Paque Plus, Amersham Pharmacia Biotech). The co-pelleted red blood cells were then removed by hypotonic lysis.

**RESULTS**

The Candidate GSD-1b cDNA Maps to Human Chromosome 11q23—Using linkage analysis, we have recently mapped the GSD-1b locus to human chromosome 11q23 (14). Meanwhile, Gerin et al. (15) have identified a candidate human GSD-1b cDNA that encodes a transmembrane protein by sequence homology to bacterial phosphate ester transporters. Mutations in this gene have been identified in two GSD-1b patients, suggesting that this candidate human GSD-1b cDNA (Fig. 1). Clone 748h13, which did not hybridize to this probe, was used as a control.

Probes containing either 5' or 3' sequences of the human GSD-1b cDNA were then hybridized to DNAs from clones 1004c12, 460c21, and 748h13. Results in Fig. 1 show that the human GSD-1b sequence is contained within chromosome 11q23 PAC clones, 1004c12 and 460c21, demonstrating that this human cDNA encodes the GSD-1b protein.

Cloning and Sequencing of Murine and Rat GSD-1b cDNAs—Reverse transcriptase-PCR and RACE were utilized to isolate candidate murine (GenBank accession number AF080469) and rat (GenBank accession number AF080468) GSD-1b cDNA clones. Both encode a transmembrane protein of 429 amino acids and contain ER protein retention signals, KKEG (murine) and KKA (rat), at their respective carboxyl termini (Fig. 2). The transcription initiation sites of murine and rat GSD-1b were determined by primer extension (Fig. 3). Using an oligonucleotide probe corresponding to nucleotides 119–143 in murine GSD-1b, two extended fragments of 151 and 147 bases were observed with murine liver RNA (Fig. 3), mapping the start sites 8 and 4 bases upstream of the end of the cDNA clone. Similarly, using an oligonucleotide probe corresponding to nucleotides 152–175 in rat GSD-1b, three extended fragments of 203, 190, and 179 bases were observed with rat liver RNA (Fig. 3), mapping the start sites 28, 15, and 4 bases upstream of the end of the cDNA clone.

The encoded murine and rat proteins share 98% amino acid sequence homology as well as 95 and 93% sequence homology, respectively, to the human protein (Fig. 2). The hydropathy profiles analyzed by the algorithm of Hoffman and Stoffel (20) predict that murine, rat, and human GSD-1b proteins contain ten transmembrane helices (Fig. 2).

**The Developmental Profile of the GSD-1b Transcript**—In the murine liver, GSD-1b mRNA was clearly detectable at 16 days of gestation and increased to adult levels shortly after at 18 days of gestation (Fig. 4A). On the other hand, studies have shown that both hepatic G6Pase mRNA (Fig. 4A) and activity were detectable at 18 days of gestation, with a marked increase at parturition, leveling off to adult levels by postnatal day 2 (21, 22). In the murine kidney, GSD-1b mRNA was already expressed at a significant level by 19 days of gestation, increasing to adult levels by age 7 days (Fig. 4B). This profile again differed from that of G6Pase. It has been shown that kidney G6Pase mRNA expression (Fig. 4B) and activity can be detected around birth. Both increase with age and peak around weaning (22, 23). Therefore, GSD-1b and G6Pase mRNAs had dissimilar developmental profiles in both liver and kidney of the mouse.

**The GSD-1b Transcript Is More Ubiquitously Expressed than the G6Pase mRNA**—It has been shown that G6Pase mRNA is expressed in the liver, kidney, and intestine but is not detectable in testis, brain, muscle, lung, heart, spleen, pancreas, and adipose tissues (24, 25). Northern blot hybridization analysis showed that the GSD-1b transcript was strongly expressed in adult mouse liver, kidney, large intestine, small intestine, and skeletal muscle and to a lesser extent in brain and heart (Fig. 5). Detectable but low expression was observed in placenta, spleen, stomach, testis, and uterus (Fig. 5). As expected, the G6Pase transcript was detectable only in the adult liver, kidney, and small intestine (Fig. 5). The size of the GSD-1b transcript in brain and heart appeared larger than those in other tissues. A 1.3-kilobase transcript containing the coding region of the GSD-1b mRNA was obtained by reverse transcriptase-PCR of brain or liver RNA (data not shown), suggesting that the size of the two transcripts differ due to the length of their 5'- or 3'-untranslated regions.

In addition to functional G6Pase deficiency, GSD-1b patients also suffer from neutropenia and neutrophil dysfunction (10, 11). We therefore examined expression of GSD-1b and G6Pase genes in human neutrophils/monocytes by Northern blot anal-
The GSD-1b mRNA is expressed in human neutrophils/monocytes and in all cell lines examined, including HepG2 hepatoma cells, promonocyte lines, U937 and THP-1, COS-1, JEG-3 choriocarcinoma, and HeLa S3 cells (Fig. 6). On the other hand, the G6Pase transcript is expressed at high levels only in HepG2 cells (Fig. 6).

**DISCUSSION**

In this study, we demonstrate that the putative GSD-1b cDNA isolated by Gerin et al. (15) maps to the GSD-1b locus on human chromosome 11q23 (14), making it a strong candidate gene for the GSD-1b disorder. We have also isolated and characterized candidate murine and rat GSD-1b cDNA clones and shown that both encode ER transmembrane proteins of 429 amino acid residues. Analysis of the hydropathy profiles by the algorithm of Hoffman and Stoffel (20) predicts that mammalian GSD-1b products are anchored in the ER membrane by ten transmembrane helices. The GSD-1b protein is structurally similar to both a bacterial sugar phosphate transporter, UhpT, and a bacterial G6P receptor/sensor, UhpC (16). Interestingly, this algorithm predicts that UhpT and UhpC contain nine and ten transmembrane helices, respectively. The transmembrane topology of the GSD-1b protein has yet to be determined.

The developmental patterns of GSD-1b and G6Pase tran-
scripts differed in both the murine liver and kidney. The GSD-1b transcript appeared at a much earlier stage than G6Pase and increased to adult levels rapidly at 18 days of gestation. It has been proposed that GSD-1b is caused by defects in a putative microsomal G6P translocase (8, 9) that transports G6P from the cytoplasm into the ER lumen where the G6Pase active site is located (26, 27). Transport studies using hepatic microsomes from GSD-1b patients support this hypothesis (28). Using G6Pase-deficient mice, we have shown that knockout of the G6Pase gene destroys both G6Pase activity and G6P transport, indicating that G6Pase activity is required for efficient G6P transport (29). We have further shown that hepatic microsomal G6P transport activity in the mouse was only detectable after birth and increased gradually to peak around 4 weeks of age (22). Therefore, the absence or very low levels of G6Pase activity in the fetal liver explain only the lack of hepatic G6P transport during embryonic development. If the presence of the GSD-1b transcript is a reflection of the G6Pase product, the discordance between GSD-1b/G6Pase mRNA and G6P transport activity suggests the involvement of additional factor(s) in modulating the microsomal G6P transport function. It has been shown that glucokinase, which catalyzes the phosphorylation of glucose to G6P, plays a role in mediating the increase in G6Pase mRNA by glucose (30). This suggests that glucose regulation of the G6Pase gene expression is actually mediated in part by G6P, a substrate or a ligand for the GSD-1b product. Therefore, the G6P transport activity of the GSD-1b protein could be controlled by G6P levels in the cytoplasm, which is regulated to some extent by glucokinase in the liver. Interestingly, hepatic glucokinase mRNA and activity first appear in the liver only at the 15th postnatal day (31). The role of glucokinase in microsomal G6P transport is currently under investigation.

How does a defect in the GSD-1b protein lead to heritable neutropenia and functional deficiencies of neutrophils and monocytes? Studies have shown that neutrophils and monocytes from GSD-1b patients exhibit impaired chemotaxis, mobility, and migration as well as diminished respiratory burst, hexose monophosphate shunt, and phagocytic activities (10, 11, 32). It has also been documented that neutrophils and monocytes from GSD-1b patients are unable to sequester Ca2+, and that they show a decreased ability to respond to Ca2+-mediated agonists (33). Changes in intracellular concentrations of Ca2+ modulate many physiological functions through signal transduction mechanisms (reviewed in Ref. 34). G6P stimulates glycolysis and the hexose monophosphate shunt, which provide energy for chemotaxis and phagocytosis (11, 32). Enzymes catalyzing the reaction of the hexose monophosphate shunt are present both in the cytoplasm and in the ER (35). Moreover, G6P enhances ATP-dependent microsomal Ca2+ sequestration in a variety of tissues including liver, kidney, brain, and heart (36–38), which results in the intraluminal accumulation of G6P (38). Unlike G6Pase, which is expressed in high levels only in the liver, kidney, and intestine (3), we show in this study that the GSD-1b transcript is expressed in numerous tissues, including human neutrophils/monocytes. This suggests that the GSD-1b protein could be a bi-functional protein. In gluconeogenic tissues that express high levels of the G6Pase gene and produce high concentrations of G6P, the G6Pase protein would behave as a G6P translocase. Whereas the same protein would act as a G6P receptor/sensor in tissues that express low or nondetectable levels of the G6Pase gene. In this respect, an altered GSD-1b protein would impair glycolysis, the hexose monophosphate shunt, and the G6P-dependent sequestration of Ca2+, resulting in the observed functional deficiencies in neutrophils and monocytes in GSD-1b patients.

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