

complete deficiency of MAOA activity and abnormal aggressive behavior in affected males. This observation raises a number of important questions. First, the frequency of MAOA deficiency in the population has to be determined. Second, given the wide range of variation of MAOA activity in the normal population (18), one could ask whether aggressive behavior is confined to complete MAOA deficiency. Third, animal models could help to determine the various neurochemical alterations that are induced by selective MAOA deficiency and their secondary effects on the organism. Such studies might also suggest possibilities for treatment of the metabolic disturbance caused by the MAOA deficiency state. Finally, the possibility of hypertensive crises in selective MAOA deficiency through increased sensitivity to dietary and pharmacologic amines has not yet been investigated.

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## Mutations in the Glucose-6-Phosphatase Gene That Cause Glycogen Storage Disease Type 1a

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Glycogen storage disease (GSD) type 1a is caused by the deficiency of D-glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis. Despite both a high incidence and morbidity, the molecular mechanisms underlying this deficiency have eluded characterization. In the present study, the molecular and biochemical characterization of the human G6Pase complementary DNA, its gene, and the expressed protein, which is indistinguishable from human microsomal G6Pase, are reported. Several mutations in the G6Pase gene of affected individuals that completely inactivate the enzyme have been identified. These results establish the molecular basis of this disease and open the way for future gene therapy.

**Glucose-6-phosphatase** (E.C. 3.1.3.9), the key enzyme in the homeostatic regulation of blood glucose concentrations, catalyzes the terminal step in gluconeogenesis

and glycogenolysis (1-3). Deficiency of G6Pase causes GSD type 1a (von Gierke disease), an autosomal recessive disorder with an incidence of one in 100,000 to 300,000 (1, 2). This metabolic disease typically manifests during the first year of life with severe hypoglycemia and hepatomegaly caused by the accumulation of glycogen. Individuals with GSD type 1a exhibit

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growth retardation, delayed puberty, lactic acidemia, hyperlipidemia, hyperuricemia, and in adults a high incidence of hepatic adenomas. Current treatments focus solely on controlling symptomatic hypoglycemia; patients receive frequent feedings by mouth or, in some cases, continuous nighttime feeding by a nasogastric tube. Despite recognition for decades that G6Pase deficiency is the cause of GSD type 1a, the enzyme has eluded molecular characterization as a result of its tight association with the endoplasmic reticulum (ER) and nuclear membranes. Characterization of the human G6Pase gene and the mutations causing G6Pase deficiency are essential for understanding the molecular basis of GSD type 1a and developing therapies for the disease.

Our strategy was first to isolate a murine G6Pase complementary DNA (cDNA) (4) from a normal mouse liver cDNA library by screening differentially (5) with mRNA populations representing the normal and the albino mutant mouse; the mutant mouse expresses markedly reduced G6Pase activity (6). With a pair of oligonucleotide primers derived from the murine G6Pase cDNA, we isolated a human G6Pase cDNA clone, phG6Pase-1 (nucleotides 77 to 1156), by

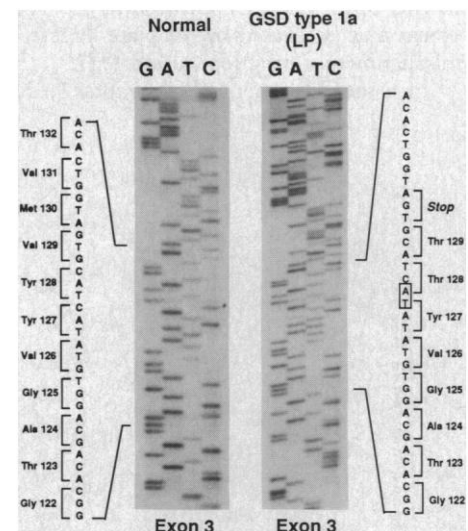
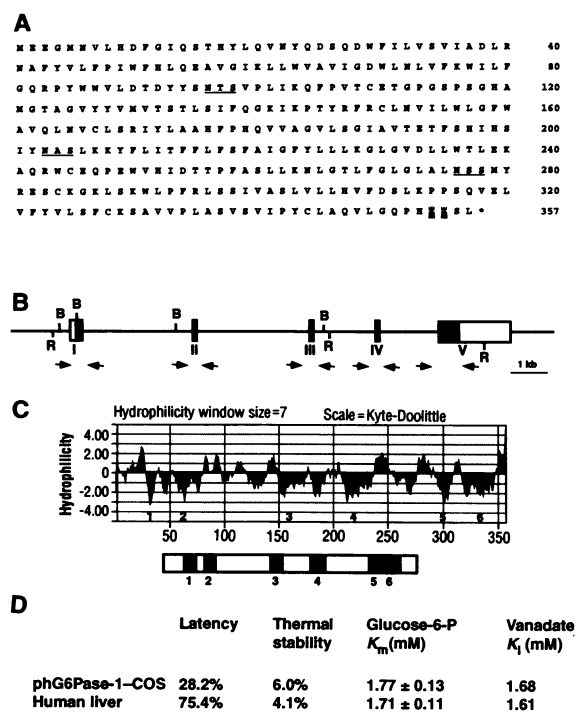
reverse transcriptase-polymerase chain reaction (RT-PCR) with human liver polyadenylated [poly(A)<sup>+</sup>] RNA as the template (7). The phG6Pase-1 cDNA was used to isolate a genomic clone containing the entire human G6Pase transcription unit (Fig. 1B) (7). The human G6Pase gene spans ~12.5 kb and consists of five exons: I (309 base pairs (bp)), II (110 bp), III (106 bp), IV (116 bp), and V (larger than 2000 bp, including a coding region of 509 bp). The 5'- and 3'-untranslated regions of the human G6Pase mRNA were identified by a combination of primer extension, sequencing of the human G6Pase genomic clone, and RT-PCR (8). ER localization of the human G6Pase is predicted by the presence of an ER protein retention signal KK (9), positioned three and four amino acids from the COOH-terminus, respectively (Fig. 1A). The hydropathy index analysis (10, 11) predicts that human G6Pase is an extremely hydrophobic protein containing six putative membrane-spanning segments (Fig. 1C).

To demonstrate the functional identity of the human G6Pase cDNA, we performed biochemical studies (12) of microsomal preparations isolated from COS-1 cells that had been transiently transfected with the

phG6Pase-1 cDNA (Fig. 1D). Hepatic G6Pase is known to exhibit latency, referring to the portion of enzymatic activity that is not expressed unless the microsomes are disrupted (3, 13). Latency values for mannose-6-P phosphohydrolase activity reported for human liver microsomes varied from 23 to 26% (14, 15) to 95% (16). Human liver microsomes isolated in the present study exhibited a latency value of 75% (Fig. 1D). Microsomes isolated from rat hepatocytes or hepatoma cells exhibit reduced latencies (17), comparable with that from phG6Pase-1-transfected COS-1 cells which displayed a latency of 28% (Fig. 1D). Cultured cells thus exhibit similar reduced latencies.

Hepatic G6Pase is completely inactivated by incubation of the microsomal preparation at pH 5.0 for 10 min at 37°C (18). Incubation of microsomes isolated from phG6Pase-1-transfected COS-1 cells or human livers at 37°C for 10 min at pH 5.0 virtually abolished glucose-6-P phosphohydrolase activity (Fig. 1D), demonstrating that the expressed enzyme is indistinguishable from the human liver microsomal G6Pase. Kinetic studies (Fig. 1D) indicated that  $K_m$  (Michaelis constant) values for glucose-6-P and  $K_i$  (inhibition constant) values for a competitive inhibitor, vanadate (19), were indistinguishable between microsomes isolated from phG6Pase-1-trans-

**Fig. 1.** (A) Predicted amino acid sequence of human G6Pase cDNA and (B) structural organization of the G6Pase transcription unit. The cDNA sequence has been deposited in GenBank (accession number V01120). (A) The translation termination codon is marked by an asterisk, the ER protein retention signal (KK) is shaded and double underlined, and potential Asn-linked glycosylation sites are underlined (29). A search for a signal peptide consensus sequence, by the method of Von Heijne (30) using the PC/Gene Program Release 6.5 (IntelliGenetics, Inc.), indicates that the G6Pase polypeptide does not contain a signal peptide. (B) The exon coding regions are indicated by filled boxes and the untranslated regions by open boxes. Arrows illustrate oligonucleotide primers used for amplification of exons. B, Bam HI; R, Eco RI. (C) Hydropathy plot and transmembrane domain structure of the deduced human G6Pase polypeptide. The hydropathy plot (10) was analyzed with the MacVector DNA/Protein Sequence Analysis Program. Negative values indicate hydrophobic residues. Transmembrane-spanning domains (11) were identified with the PC/Gene Program. Numbers 1 to 6 refer to putative membrane-spanning segments, diagrammatically illustrated as filled boxes. (D) Characteristics of microsomal G6Pase phosphohydrolase activity in phG6Pase-1-transfected COS-1 cells and human liver. Latencies were assessed by mannose-6-P phosphohydrolysis in intact (I) versus detergent-disrupted (D) microsomes, defined as  $[1 - (I/D)] \times 100$ . Two microsomal preparations from phG6Pase-1-transfected COS-1 cells or human livers were analyzed. Thermal stability was determined by assaying glucose-6-P phosphohydrolase activity in deoxycholate (0.2%)-disrupted microsomes before and after incubation for 10 min at 37°C in 50 mM cacodylate buffer (pH 5.0), and the stability values refer to enzyme activities remaining after heat treatment.



**Fig. 2.** Autoradiograms of Sanger nucleotide sequencing reactions of the G6Pase gene from normal and GSD type 1a patient LP. The G6Pase gene of patient LP contains a TA insertion (boxed) at nucleotide 459 in exon 3 generating a stop codon at nucleotides 467 to 469. The predicted mutant G6Pase is a severely truncated protein of 129 amino acids. The genomic DNA of patient LP, a Mexican American, was isolated from lymphoblasts (GM09036) obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, New Jersey). The liver biopsy of patient LP contained no detectable G6Pase activity.

fectected COS-1 cells and human livers. Taken together, our data demonstrate that this cDNA encodes human microsomal G6Pase, the enzyme deficient in GSD type 1a patients.

To identify the G6Pase gene mutations in GSD type 1a patients, we PCR amplified (20) the coding regions of each of the five exons and all intron-exon junction regions of this gene with five pairs of oligonucleotide primers containing intronic sequences (Fig. 1B). The amplified fragments were cloned and five subclones of each exon were sequenced. Sequencing data of the defective gene were compared with those of a normal G6Pase gene. Analysis of the G6Pase gene in a GSD type 1a patient (LP) showed that exons 1, 2, 4, and 5 were normal (21). However, exon 3 of the G6Pase gene had a TA insertion at nucleotide 459 that was identified in each of the five exon 3 subclones examined (Fig. 2). The 2-bp insertion alters the reading frame of the encoded protein such that a stop codon is generated at nucleotides 467 to 469. The predicted mutant G6Pase is a severely truncated protein of 129 amino acids. These results indicate that LP is homozygous for the TA insertion and predict that the mother (only parent available) would be heterozygous for the insertion at this locus. As expected, a TA insertion at nucleotide 459 was demonstrated in two of five exon 3 subclones of the gene from the mother of LP (21). Duplication of the TA repeat may be caused by template-directed misalignment during replication (22).

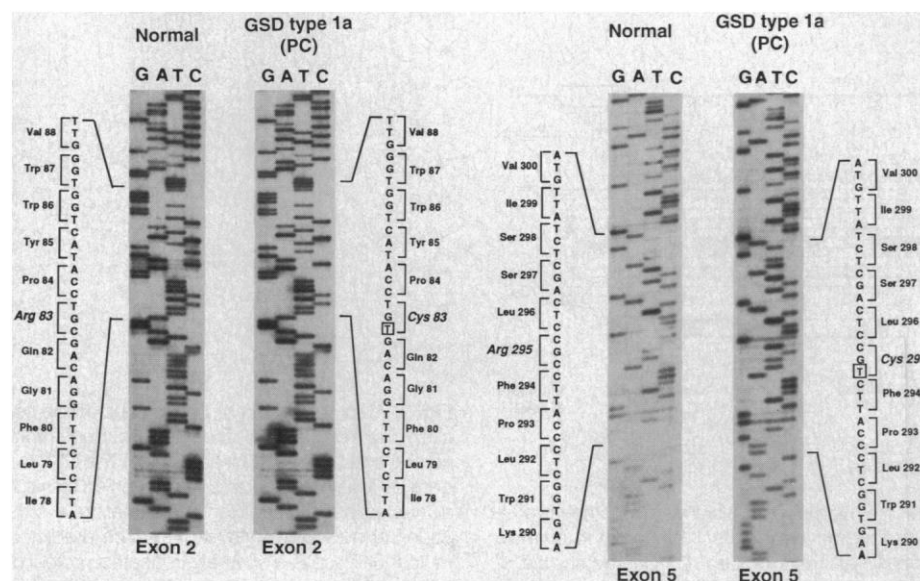
In a second patient (PC), exons 1, 3,

and 4 were normal (21); however, exons 2 and 5 each contained a C to T mutation at nucleotides 326 and 962, respectively (Fig. 3). Both mutations were found to convert an Arg codon to a Cys codon (codon 83 in exon 2 and codon 295 in exon 5). Our finding, that only two of the five subclones from either exon 2 or 5 exhibit the mutation, suggests that patient PC is a compound heterozygote with different mutations in the two G6Pase alleles. We confirmed this by sequencing exon subclones obtained from the G6Pase gene of both parents. The father had a normal exon 2 and the mother, a normal exon 5. Two of the five exon 5 subclones from the gene of the father contained a C to T mutation at nucleotide 962 converting an Arg to a Cys at codon 295. Three of the five exon 2 subclones from the gene of the mother contained a C to T mutation at nucleotide 326 converting an Arg to a Cys at codon 83 (21). Methylation of the CpG doublet, a hot spot for mutation in bacteria and eukaryotes, may be responsible for the two C to T transitions observed in patient PC (23).

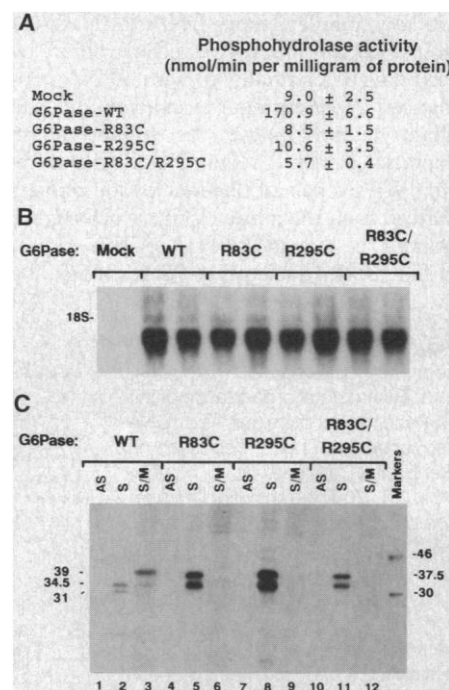
The liver biopsy of patient PC had no detectable G6Pase activity, suggesting that a mutation that substitutes either Arg<sup>83</sup> or Arg<sup>295</sup> to a Cys residue yields a mutant G6Pase with undetectable phosphohydrolase activity. To confirm our conclusions, we constructed three G6Pase mutants (24) that changed either Arg<sup>83</sup> to Cys<sup>83</sup> (G6Pase-R83C), Arg<sup>295</sup> to Cys<sup>295</sup> (G6Pase-R295C), or both Arg residues to Cys (G6Pase-R83C/R295C). Phosphohydrolase activities were

analyzed after transient expression of the wild-type or mutant G6Pase in COS-1 cells (Fig. 4A). As predicted, Arg to Cys substitution at either codon 83 or 295 abolished G6Pase phosphohydrolase activity. Northern (RNA) hybridization analysis of G6Pase transcripts from transfected COS-1 cells showed that wild-type and mutant G6Pase mRNAs were expressed at similar amounts (Fig. 4B). Moreover, translation of mutant G6Pase mRNA indicated that G6Pase-R83C, G6Pase-R295C, or G6Pase-R83C/R295C RNA supported the synthesis of polypeptides of 34.5 and 37.5 kD (Fig. 4C).

There are three potential Asn-linked glycosylation sites in the deduced protein (Fig. 1A). The G6Pase wild-type mRNA support-



**Fig. 3.** Autoradiograms of Sanger nucleotide sequencing reactions of the G6Pase gene from normal and GSD type 1a patient PC. Patient PC contains two C to T (boxed) mutations at nucleotides 326 (exon 2) and 962 (exon 5), respectively. The predicted mutant G6Pase of patient PC contains Cys residues at codon 83 and 295 instead of Arg residues found in normal G6Pase. The genomic DNA of patient PC was isolated from a blood sample. The liver biopsy of patient LP exhibited no detectable G6Pase activity.



**Fig. 4.** (A) Analysis of G6Pase phosphohydrolase activity and (B) mRNA expression after transient expression of wild-type and mutant G6Pase cDNAs in COS-1 cells. Phosphohydrolase activity was assayed in reactions containing 10 mM glucose-6-P with two independent isolates of each construct. Total RNA was separated by formaldehyde-agarose gel electrophoresis, blotted onto a Nytran membrane (Schleicher & Schuell), and hybridized to the phG6Pase-1 probe labeled by random priming. Transfection of each construct was done in duplicate. (C) SDS-polyacrylamide gel electrophoresis analysis of G6Pase polypeptides. In vitro transcription-translation of wild-type (WT) or mutant G6Pase cDNA in a pGEM-7Zf(+) vector (Promega) was done with the TnT-coupled reticulocyte lysate system (Promega) and analyzed in both sense (S, lanes 2, 5, 8, and 11) and antisense (AS, lanes 1, 4, 7, and 10) orientations. Protein processing was also examined by in vitro transcription-translation done in the presence of canine microsomal membranes (S/M, lanes 3, 6, 9, and 12).

ed the synthesis of two polypeptides of 31 and 34.5 kD that were processed to glycosylated polypeptides of 34.5 and 39 kD in the presence of canine microsomal membranes (Fig. 4C). This is in agreement with previous findings that rat microsomal G6Pase migrates as two tightly associated glycopolypeptides of 36.5 kD (25, 26). Surprisingly, all three mutant proteins, G6Pase-R83C, G6Pase-R295C, and G6Pase-R83C/R295C, exhibited a higher apparent molecular size and markedly reduced processing of the G6Pase polypeptides (Fig. 4C). Our data suggest that in human G6Pase, mutation of Arg at either codon 83 or 295 to a Cys induces conformational changes that inactivate the enzyme.

Currently, there are two proposed models of G6Pase catalysis. The conformation-substrate-transport model (27) proposes that G6Pase is a membrane channel protein with both catalytic and substrate (or product) transport functions. On the other hand, the translocase-catalytic unit model (28) suggests that G6Pase is a multicomponent complex consisting of a catalytic unit, G6Pase, situated on the luminal surface of the ER that gains access to substrates in the cytosol by means of associated translocases. The latter model has been used to explain the phenotypic divergence observed in the four subgroups of GSD type 1 patients (1a, 1b, 1c, and 1d). These GSD subgroups correspond to defects in G6Pase, the putative glucose-6-P translocase, phosphate or pyrophosphate translocase, and glucose translocase, respectively (1–3, 14, 15). This classification was based on the observations that GSD type 1b or 1c patients exhibit normal glucose-6-P or pyrophosphate hydrolytic activity in microsomes from liver samples previously frozen, but not in fresh liver biopsy specimens (14, 15). The results are consistent with disruption of microsomes by freezing that abolishes the translocation requirement. It is equally possible that activation of the enzyme after microsomal disruption could be caused by a conformational alteration of the membrane-bound G6Pase. The characterization of the G6Pase gene makes it possible to clearly distinguish between the models of G6Pase catalysis.

The identification of mutations in GSD type 1a patients has established the molecular basis of the type 1a disorder. Knowledge of the mutations may be applied to prenatal diagnosis and the design of therapies (including gene therapy).

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- The transcription initiation site of the G6Pase mRNA was determined by primer extension with human liver poly(A)<sup>+</sup> RNA as a template and an antisense oligonucleotide primer corresponding to nucleotides 124 to 142 of the human G6Pase cDNA. To demonstrate that the 3'-untranslated region of the G6Pase cDNA (nucleotides 1154 to 3095) was contained in the region 3' of the termination codon in exon 5 of the human G6Pase gene, we utilized four pairs of oligonucleotide primers to amplify individually the 3'-untranslated region of the G6Pase mRNA by RT-PCR with human liver poly(A)<sup>+</sup> RNA as a template. The four pairs of primers are 3UT1 (nucleotides 1155 to 1172, sense) and 3UT2 (nucleotides 1934 to 1950, antisense); 3UT3 (nucleotides 1886 to 1902, sense) and 3UT4 (nucleotides 2242 to 2258, antisense); 3UT5 (nucleotides 2490 to 2506, antisense); and 3UT3 and 3UT6 (2783 to 2800, antisense). Four predicted fragments of 796, 373, 621, and 915 bp were obtained, subcloned, and their identities confirmed by DNA sequencing. A consensus motif for polyadenylation (AATAAA) is located at nucleotides 3053 to 3058.
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- We used phG6Pase-1 (G6Pase-WT, nucleotides 77 to 1156 containing the entire coding region of the human G6Pase cDNA) as a template for mutant construction by site-directed mutagenesis [R. Higuchi, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA, 1990), pp. 177–183]. The two outside PCR primers are O1 (5'-AGGATGGAG-GAAGGAATGAA-3', nucleotides 77 to 96) and O2 (5'-TTACACGACTTCTGTGCGCGCTG-3', nucleotides 1153 to 1130). The two pairs of inside mutant primers for the G6Pase-R83C mutant are M1S (5'-TGGACAGTGCCATACTGGTGG-3', nucleotides 319 to 340) and M1AS (5'-CCACCAGTA-TGGACaCTGTCCA-3', nucleotides 340 to 319), and for the G6Pase-R295C mutant are M2S (5'-GCTCCCATTCtGCCTCAGCTC-3', nucleotides 952 to 972) and M2AS (5'-GAGCTGAGGCaGAATG-GGAGC-3', nucleotides 972 to 952). The mutant bases are indicated by lowercase letters. The outside primers O1 and O2 contain an additional Xho I or Xba I linker, respectively. The double mutant, G6Pase-R83C/R295C, was constructed with G6Pase-R83C as a template and primers M2S and M2AS. The amplified fragments were digested with Xho I and Xba I and ligated into a pSVL vector. All constructs were confirmed by DNA sequencing.
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