THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 20, Issue of May 19, pp. 11882–11886, 1995
Printed in U.S.A.

Structure-Function Analysis of Human Glucose-6-phosphatase, the Enzyme Deficient in Glycogen Storage Disease Type 1a*

(Received for publication, December 20, 1994, and in revised form, March 22, 1995)

Ke-Jian Lei, Chi-Jiunn Pan, Ji-Lan Liu, Leslie L. Shelly, and Janice Yang Chou‡

From the Human Genetics Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Glucose-6-phosphatase (G6Pase) is the enzyme deficient in glycogen storage disease type 1a, an autosomal recessive disorder. We have previously identified six mutations in the G6Pase gene of glycogen storage disease type 1a patients and demonstrated that these mutations abolished or greatly reduced enzymatic activity of G6Pase, a hydrophobic protein of 357 amino acids. Of these, four mutations (R83C, R295C, G222R, and Q347X) are missense and one (Q347X) generates a truncated G6Pase of 346 residues. To further understand the roles and structural requirements of amino acids 83, 222, 295, and those at the carboxyl terminus in G6Pase catalysis, we characterized mutant G6Pases generated by near-saturation mutagenesis of the aforementioned amino acids. Substitution of Arg-83 with amino acids of diverse structures including Lys, a conservative change, yielded mutant G6Pase with no enzymatic activity. On the other hand, substitution of Arg-295 with Lys (R295K) retained high activity, and R295N, R295S, and R295Q exhibited moderate activity. All other substitutions of Arg-295 had no G6Pase activity, suggesting that the role of Arg-295 is to stabilize the protein either by salt bridge or hydrogen-bond formation. Substitution of Gly-222, however, remained functional unless a basic (Arg or Lys), acidic (Asp), or large polar (Gln) residue was introduced, consistent with the hydrophobic requirement of codon 222, which is predicted to be in the fourth membrane-spanning domain. It is possible that Arg-83 is involved in stabilizing the enzyme (His)-phosphate intermediate formed during G6Pase catalysis. There exist 9 conserved His residues in human G6Pase. His-9, His-119, His-252, and His-353 reside on the same side of the endoplasmic reticulum membrane as Arg-83. Whereas H119A mutant G6Pase had no enzymatic activity, H9A, H252A, and H353A mutant G6Pases retained significant activity. Substitution of His-119 with amino acids of diverse structures also yielded mutant G6Pase with no activity, suggesting that His-119 is the phosphate acceptor in G6Pase catalysis. We also present data demonstrating that the carboxyl-terminal 8 residues in human G6Pase are not essential for G6Pase catalysis.

Glucose-6-phosphatase (G6Pase,[1] E.C. 3.1.3.9) catalyzes the terminal step in gluconeogenesis and glycogenolysis and is the key enzyme in glucose homeostasis (1, 2). Deficiency of G6Pase causes glycogen storage disease (GSD) type 1a, an autosomal recessive disorder with clinical manifestations of severe hypoglycemia, hepatomegaly, lactic acidemia, hyperlipidemia, hyperuricemia, and growth retardation (3, 4). To understand the molecular basis of GSD type 1a, we characterized cDNAs and genes encoding human and murine G6Pase (5, 6). Sequence analysis of the deduced G6Pase indicates that both enzymes are hydrophobic proteins containing six putative membrane-spanning segments (5, 6). This is consistent with the observations that mammalian G6Pase is tightly associated with the endoplasmic reticulum (ER) and nuclear membranes (1, 2).

In order to gain insight into the mechanism of G6Pase catalysis and the pathogenesis of the type 1a disorder, we analyzed the G6Pase gene of 12 unrelated GSD type 1a patients and identified six mutations, including one insertion (459insTA), one codon deletion (ΔF327), and four missense (5, 7, 8). Site-directed mutagenesis followed by transient expression assays demonstrated that each mutation abolished or greatly reduced G6Pase activity (5, 7, 8). The four missense mutations consist of a C to T transition at nucleotide 326 (Arg to Cys at codon 83, R83C) (5), a G to C transversion at nucleotide 743 (Gly to Arg at codon 222, G222R) (8), a C to T transition at nucleotide 962 (Arg to Cys at codon 295, R295C) (6), and a C to T transition at nucleotide 1118 (Gln to stop at codon 347, Q347X) (7). Of the 24 G6Pase alleles characterized, 9 alleles (37.5%) contain the R83C mutation, indicating that this mutation is probably the most prevalent mutation in GSD type 1a (5, 7, 8). In the present study, we examined the structural requirements of codons 83, 222, and 295 in G6Pase catalysis by constructing mutant G6Pases with nearly all possible substitutions and examined phosphohydrolase activity after transient expression of wild-type (WT) or mutant constructs in COS-1 cells. We also constructed a series of truncated G6Pase mutants to determine which of the 11 terminal amino acids was essential for activity.

Studies have shown that an enzyme-phosphate intermediate is formed during G6Pase catalysis and the phosphate acceptor in G6Pase is a His residue (9, 10). Sequence analysis and alignment reveal the presence of 9 conserved His residues in human (5), mouse (6), and rat (11) G6Pase. His-9, His-119, His 252, and His-353 are predicted to be on the same side of the ER membrane as Arg-83, Arg-295, and Gln-347 (5, 6, 8). In this study, we examined the role of these 4 His residues in G6Pase catalysis by altering each of the His residues by site-directed mutagenesis and analyzing G6Pase activity after transient expression of WT and mutant G6Pase cDNA in COS-1 cells.

MATERIALS AND METHODS

Construction of G6Pase Mutants—The phG6Pase-1 cDNA (G6Pase-WT) containing nucleotides 77–1156 of the entire coding region of the human G6Pase cDNA (5) was used as a template for mutant construction by polymerase chain reaction. The outside primers used in codon 83 are O1 (5′-AGGATGGAGGAAGGAATGAA-3′, nucleotides 77–96, sense) and O2 (5′-TTACAACGACTTCTTGTGCGGCTG-3′, nucleotides 1153–1130, antisense), which contain an additional *Xho*I or *Xba*I linker, respectively. Nucleotides 326–328 of codon 83 mutant primers (nucleotides 319–337) are R83E, GAA; R83K, AAG; R83L, CTA; R83Q, CAG;

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Bldg. 10, Rm. 9S242, NIH, Bethesda, MD 20892. Tel.: 301-496-1094; Fax: 301-402-0234.

[1] The abbreviations used are: G6Pase, glucose-6-phosphatase; GSD, glycogen storage disease; ER, endoplasmic reticulum; WT, wild-type.

and R83S, TCG. The amplified fragments were digested with *Xho*I and *Xba*I and ligated into a pSVL vector (Pharmacia Biotech Inc.).

The phG6Pase-*Dra*III construct, which retains the primary amino acid sequence of WT G6Pase but contains an additional *Dra*III site at nucleotides 614–622, was constructed by site-directed mutagenesis using the G6Pase-WT as a template. The outside primers are O1 and O2, and the two inside mutant primers are I1 (5′-CCTCACCAAGTGGTTGCTGGAGTC-3′, nucleotides 611–634, sense) and I2 (5′-AACCACTTGGTGAGGAAAATGAGC-3′, nucleotides 625–602, antisense). The amplified fragments were digested with *Xho*I and *Xba*I and ligated into a pSVL vector. The pSVLhG6Pase-*Dra*III construct exhibits WT G6Pase enzymatic activity as demonstrated by transient expression assays in COS-1 cells (see Table I). The pSVLhG6Pase-*Dra*III-5′ fragment (containing nucleotides 77–619 of hG6Pase) or the pSVLhG6Pase-*Dra*III-3′ fragment (containing nucleotides 621-1156 of hG6Pase) was obtained by digestion of pSVLhG6Pase-*Dra*III with *Xba*I/*Dra*III or *Xho*I/*Dra*III, respectively, and purified on a low melting agarose gel.

We also employed a modification of cassette mutagenesis (12) to generate multiple mutations of codons 83, 222, and 295. The two outside polymerase chain reaction primers for codon 83 (nucleotides 326–328) mutants (R83M, R83N, R83T) are O1 and I2. The sense strand of the degenerate inside primers (nucleotides 316–336) is 5′-CTTTGGACAG(A/C/G)(A/C/G/T)(G/T)CCATACTG-3′. The amplified fragments were digested with *Dra*III and *Xho*I and ligated into the pSVLhG6Pase-*Dra*III-3′ fragment. Mutants were identified by DNA sequencing.

The two outside polymerase chain reaction primers for codon 222 (nucleotides 743–745) and codon 295 (nucleotides 962–964) mutants are I1 and O2. The sense strand of the degenerate inside primers (nucleotides 731–754) for mutants G222K, G222L, G222M, G222N, G222Q, G222R, G222T, and G222V is 5′-AGCTTCGCCATC(A/C/G)(A/C/G/T)(C/G)TTTTATCTG-3′ and for mutants G222D, G222S, and G222Y is 5′-AGCTTCGCCATC(C/G/T)(A/C)TTTTTATCTG-3′. The sense strand of the degenerate inside primers (nucleotides 953–976) for mutants R295H, R295I, R295K, R295M, R295N, R295P, R295Q, and R295S is 5′-CTCCCATTC(A/C/G)(A/C/G/T)(C/G)CTCAGCTCTATT-3′ and for mutants R295E and R295Y is 5′-CTCCCATTC(G/T)(A/G)(A/T)CTCAGCTCTATT-3′. The amplified fragments were digested with *Dra*III and *Xba*I and ligated into the pSVLhG6Pase-*Dra*III-5′ fragment.

The 5′-primer for H9A mutant is 5′-ATGGAGGAAGGAATGAATGTTCTCGCTGACTTTGGGATC-3′ (nucleotides 80–118, sense) and the 3′-primer is O2. The 5′-primer for H353A mutant is O1, and the 3′-primer is 5′-TTACAACGACTTCTTGGCCG-3′ (nucleotides 1153–1134, antisense). The outside primers used in codons 119 and 252 are O1 and O2. The sense strand of the inside primers for mutant H119A is 5′-CCTCTGGCGCTGCCATGGGCACAG-3′ (nucleotides 426–449), and mutant H252A is 5′-GAATGGGTCGCCATTGACACC-3′ (nucleotides 824–844). The amplified fragments were digested with *Xho*I and *Xba*I and ligated into a pSVL vector.

The two outside polymerase chain reaction primers for codon 119 (nucleotides 434–436) mutants (H119I, H119K, H119M, H119N, H119R, and H119T) are O1 and I2. The sense strand of the degenerate inside primers is 5′-CCTCTGGC(A/C)(A/G/T/C)(G/T)GCCATGGGCACAG-3′ (nucleotides 426–449). The amplified fragments were digested with *Dra*III and *Xho*I and ligated into the pSVLhG6Pase-*Dra*III-3′ fragment.

The 5′-primer for carboxyl-terminal G6Pase mutants is O1, and the 3′-antisense primers are H353*X* (5′-TTACGGCTGGCCCAGGACCTG-3′, nucleotides 1118–1138), Q351*X* (5′-TTAGCCCAG GACCTGGGCGAG-3′, nucleotides 1112–1132), G350*X* (5′-TTACAGGACCTGGGCGAGGCA-3′, nucleotides 1109–1129), and L349*X* (5′-TTAGACCTGGGCGAGGCAGTA-3′, nucleotides 1106–1129). All constructs were confirmed by DNA sequencing.

Expression in COS-1 Cells and Northern Blot Hybridization Analysis—COS-1 cells were grown at 37 °C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with streptomycin, penicillin, and 4% fetal bovine serum. The WT or mutant G6Pase cDNA in a pSVL vector was transfected into COS-1 cells by the DEAE-dextran/chloroquine method (13). Mock transfections of COS-1 cells with the pSVL vector alone were used as controls. After incubation at 37 °C for 3 days, the transfected cultures were harvested for G6Pase assays or lysed for RNA isolation.

RNA was isolated by the guanidinium thiocyanate/CsCl method (14), separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to Nytran membranes. The filters were hybridized at 42 °C in the presence of the phG6Pase-1 probe as described previously (5).

Phosphohydrolase Assay—Cells were disrupted by sonication or three consecutive freeze-thaw cycles. Microsomal membranes were isolated by the method of Burchell *et al.* (15) from freshly prepared homogenates of WT or mutant G6Pase-transfected COS-1 cells. Disrupted microsomal membranes were prepared by incubating intact membranes in 0.2% deoxycholate for 20 min at 0 °C. The latency or intactness of microsomal preparations was assessed by assaying mannose 6-phosphate hydrolysis in intact *versus* detergent-disrupted microsomes (16).

Phosphohydrolase activity was determined essentially as described by Burchell *et al.* (15). Reaction mixtures (100 μl) contained 50 mM cacodylate buffer, pH 6.5, 10 mM glucose 6-phosphate, 2 mM EDTA, and appropriate amounts of cell homogenates and were incubated at 30 °C for 10 min. Sample absorbance was determined at 820 nm and is related to the amount of phosphate released using a standard curve constructed by a stock of inorganic phosphate solution. Essentially the same enzymatic activity was obtained in control or deoxycholate (0.2%)-treated homogenates.

TABLE I

Analysis of phosphohydrolase activity of G6Pase WT, DraIII, and codon 83 mutant constructs in COS-1 cells

Phosphohydrolase activity in whole homogenates was assayed in reactions containing 10 mM glucose 6-phosphate using two independent isolates of each construct in two separate transfections. The activity is expressed as nmol/min/mg of protein, and data are presented as the mean ± S.D.

Constructs	Phosphohydrolase activity	Constructs	Phosphohydrolase activity
Mock	12.1 ± 1.3		
G6Pase-WT	117.0 ± 12.2	G6Pase-*Dra*III	119.7 ± 7.2
R83C	12.7 ± 3.2	R83N	12.3 ± 3.8
R83E	12.6 ± 1.5	R83Q	12.6 ± 3.3
R83K	12.0 ± 2.1	R83S	13.2 ± 1.7
R83L	13.4 ± 1.8	R83T	14.1 ± 1.9
R83M	11.8 ± 1.4		

RESULTS

The Role of Arg-83 in G6Pase Catalysis—Analysis of the G6Pase genes of 12 GSD type 1a patients uncovered six mutations including four missense, R83C, G222R, R295C, and Q347*X* (5, 7, 8). R83C, R295C, and Q347*X* mutant G6Pases are enzymatically inactive (5, 7), whereas the G222R mutant has a very low phosphohydrolase activity (8). To examine the structural requirements for these amino acids as well as the length of carboxyl terminus essential for G6Pase catalysis, we constructed a series of codon 83, 222, 295, and carboxyl-terminal deletion mutants. Phosphohydrolase activity was examined in whole homogenates after transient transfection of WT or mutant G6Pase into COS-1 cells.

In earlier studies (5, 7, 8), we sequenced each G6Pase mutant construct generated by site-directed mutagenesis. It is time-consuming and impractical to analyze the large number of mutants generated by near-saturation mutagenesis. In the present study, we created a *Dra*III site at nucleotides 614–622 of the human G6Pase cDNA. The G6Pase-*Dra*III construct retains the primary amino acid sequence of WT G6Pase and exhibits WT G6Pase enzymatic activity (Table I). Therefore, mutagenesis and sequence analysis of the resulting mutant constructs can be performed using the smaller G6Pase-*Dra*III-5′ or G6Pase-*Dra*III-3′ fragment.

To study the role of Arg-83 in G6Pase catalysis, this amino acid was substituted with amino acids of diverse structures including Glu (R83E), Lys (R83K), Leu (R83L), Met (R83M), Asn (R83N), Gln (R83Q), Ser (R83S), and Thr (R83T) (Table I). Like the enzymatically inactive R83C mutant, phosphohydrolase activity was not detectable in COS-1 cells transfected with any of the G6Pase codon 83 mutants, including a conservative substitution of Lys for Arg.

Structural Requirements of Codon 295 in G6Pase Catalysis—Mutation of another arginine, Arg-295, in the G6Pase gene also causes the GSD type 1a disorder (5). To characterize the structure-function relationship of this amino acid, we altered Arg-

TABLE II
Analysis of phosphohydrolase activity of G6Pase WT and codon 295 mutant constructs in COS-1 cells

Constructs	Phosphohydrolase activity	Constructs	Phosphohydrolase activity
Mock	13.4 ± 2.4		
WT	163.5 ± 1.2 (100%)		
R295C	13.3 ± 1.8	R295H	19.3 ± 0.9 (3.9%)
R295I	14.7 ± 2.5	R295N	30.1 ± 2.3 (11.1%)
R295P	12.3 ± 2.2	R295S	30.2 ± 3.2 (11.2%)
R295Y	14.2 ± 0.8	R295Q	34.5 ± 9.0 (14%)
R295M	16.2 ± 1.4 (2%)	R295K	113.8 ± 9.0 (66.9%)
R295E	16.4 ± 1.2 (2%)		

Phosphohydrolase activity in whole homogenates was assayed in reactions containing 10 mM glucose 6-phosphate using two independent isolates of each construct in three separate transfections. The activity is expressed as nmol/min/mg of protein, and data are presented as the mean ± S.D. Numbers in parentheses represent percent of WT enzyme activity.

295 to either Glu (R295E), His (R295H), Ile (R295I), Lys (R295K), Met (R295M), Asn (R295N), Pro (R295P), Gln (R295Q), Ser (R295S), or Tyr (R295Y) and analyzed phosphohydrolase activity of the mutant G6Pases (Table II). In addition to the inactive naturally occurring R295C mutant, substitution of Arg-295 with either Ile, Pro, or Tyr abolished G6Pase catalytic activity. However, as opposed to mutations of Arg-83, R295M, R295E, or R295H mutant G6Pase exhibited low phosphohydrolase activity; R295N, R295S, or R295Q mutants exhibited moderate activity, and the R295K mutant retained high G6Pase activity (Table II).

Structural Requirements of Codon 222 in G6Pase Catalysis—Secondary structural analysis of human G6Pase predicts that codon 222 would be located in the fourth membrane-spanning domain (Fig. 1). To determine the structural requirement of codon 222 in G6Pase catalysis, we constructed codon 222 mutants by semi-saturation mutagenesis. In addition to the natural G222R mutant, the Gly-222 was also substituted with Asp (G222D), Lys (G222K), Leu (G222L), Met (G222M), Asn (G222N), Gln (G222Q), Ser (G222S), Thr (G222T), Val (G222V), or Tyr (G222Y) (Table III). G222M, G222Y, R222T, or G222S retained over 70% of WT G6Pase catalytic activity, and G222V, G222L, or G222N retained at least 50% of WT G6Pase activity. However, substitution of Gly-222 with Gln (large polar), Asp (acidic), or Arg/Lys (basic) residues greatly inhibited G6Pase phosphohydrolytic activity (Table III).

His-119 May Be the Phosphate Acceptor in G6Pase—It has been shown that the phosphate acceptor in G6Pase is a His residue (9, 10). The stringent structural requirement of codon 83 suggests that Arg-83 in G6Pase may be involved in positioning the phosphate. 4 conserved His residues at codons 9, 119, 252, and 353 are predicted to reside on the same side of the ER membrane as Arg-83 (Fig. 1). Therefore, we altered each of the 4 His residues individually to Ala, an amino acid to which a phosphate group cannot be transferred. Phosphohydrolase activity was analyzed after transient expression of WT or mutant G6Pase cDNAs in COS-1 cells (Table IV). H252A and H353A mutant G6Pases retained over 60% WT activity, and H9A mutant retained 13% activity. On the other hand, the H119A mutant G6Pase had no enzymatic activity. To establish the vital role of His-119 in G6Pase catalysis, we substituted this amino acid with either Ile (H119I), Lys (H119K), Met (H119M), Asn (H119N), Arg (H119R), or Thr (H119T) (Table IV). Like the enzymatically inactive H119A mutant, none of the other codon 119 mutants had detectable phosphohydrolase activity.

The Role of Carboxyl-terminal Residues in G6Pase Catalysis—The Q347*X* mutant, lacking the 11 carboxyl-terminal amino acids, is enzymatically inactive, in contrast to the K355*X* mutant, which retains significant G6Pase activity (7). Therefore, residues 355–357 in human G6Pase are nonessential amino acids, and residues 348–354 may play important role in G6Pase catalysis. To further examine the length of the carboxyl terminus required for G6Pase activity, we examined phosphohydrolase activity of mutant G6Pases containing sequential deletion of carboxyl-terminal residues (Table V). Deletion of residues 355–350 yielded mutant G6Pases retaining at least 40% of WT enzyme activity. However, a G6Pase of 348 residues (L349*X*) exhibited only 5% of WT G6Pase activity, demonstrating that the 8 carboxyl-terminal residues beyond this codon that contain an ER protein retention signal (5, 17) are not necessary for its catalytic activity.

G6Pase is an ER protein containing the ER transmembrane protein retention signal, KK, at residues 354 and 355 (5, 6). In earlier studies, we showed that the K355*X* mutant lacking the KK motif remained in the microsomal fractions of the transfected cells. Therefore, we examined phosphohydrolase activities in microsomal and soluble fractions of COS-1 cells transfected with either WT, H353*X*, Q351*X*, G350*X*, or L349*X* mutant (Table V). G6Pase activities were associated primarily with microsomal preparations as low or undetectable levels of enzyme activity were found in the soluble fractions of WT or mutant G6Pase transfected cells. Moreover, microsomal G6Pase in WT as well as mutant transfected cells exhibited similar latencies and heat sensitivities (Table V).

Northern Blot Hybridization Analysis—Northern blot hybridization analysis of G6Pase transcripts from transfected cells showed that WT as well as the various mutant G6Pase mRNAs were expressed at similar levels (data not shown). This indicates that the reduction in enzymatic activity was due to the defect in the G6Pase protein and not due to a decrease in transfection efficiency.

DISCUSSION

The characterization of mutations in the G6Pase gene of GSD type 1a patients that abolish or greatly reduce G6Pase activity has pinpointed a number of amino acid residues important in G6Pase catalysis (5, 7, 8). In the present report, we examine the structure-function relationship of amino acids 83, 222, and 295 and the role of carboxyl-terminal residues 348–354 in the catalytic activity of G6Pase. Replacement of Arg-83 with amino acids of diverse structures including a Lys (a conservative change) yielded a G6Pase devoid of enzymatic activity, demonstrating that the Arg at codon 83 is absolutely required for G6Pase catalysis. This is reminiscent of the case in human placental alkaline phosphatase where it has been shown that substitution of the Arg at position 166 with a Lys markedly inhibits phosphatase activity (18). Based on x-ray crystallography studies of *E. coli* alkaline phosphatase (19), it was proposed that Arg-166 is involved in positioning the phosphate, which binds to a Ser residue at the active center of placental alkaline phosphatase (18). The stringent structural requirement of codon 83 suggests that Arg-83 in G6Pase may be also involved in positioning the phosphate, which binds a His at the active center of G6Pase (9, 10). 4 His residues, His-9, His-119, His-252, and His-353 are predicted to be on the same side of the ER membrane as Arg-83 (Fig. 1). In the present study, we show that His-119, like Arg-83, is absolutely required for G6Pase activity, suggesting that His-119 is the phosphate acceptor during G6Pase catalysis. Hepatic 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (20) and acid phosphatases (21) also form enzyme-phosphate intermediates during catalysis. An Arg residue has been shown to reside adjacent to the His residue that accepts the phosphate in both families of enzymes. It is possible that these adjacent Arg residues also play a role in positioning the phosphate during catalysis.

WT G6Pase also contains an Arg at codon 295 residing at the

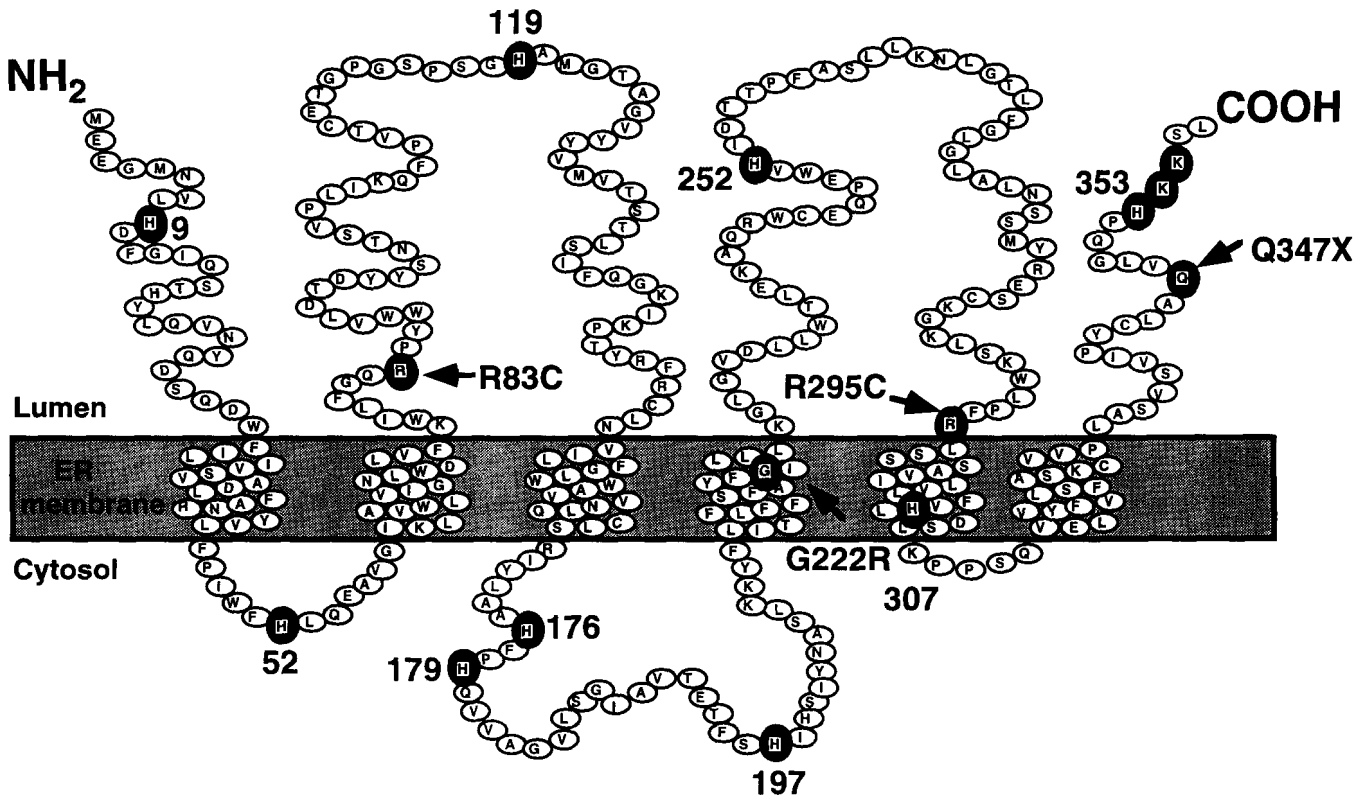

FIG. 1. **The predicted secondary structure of human G6Pase and the locations of the four missense mutations identified in GSD type 1a patients.** Transmembrane spanning domains were identified by the method of Klein *et al.* (25) using the PC/Gene Program. The four mutations are *highlighted* and denoted by *arrows*, and the His residues are *highlighted* and *numbered*.

TABLE III
Analysis of phosphohydrolase activity of G6Pase WT and codon 222 mutant constructs in COS-1 cells

Constructs	Phosphohydrolase activity	Constructs	Phosphohydrolase activity
Mock	11.7 ± 2.4		
WT	115.8 ± 4.5 (100%)		
G222R	15.9 ± 3.6 (4%)	G222N	74.1 ± 6.3 (60%)
G222K	32.0 ± 3.4 (19.5%)	G222M	87.4 ± 2.2 (72.7%)
G222Q	39.1 ± 2.6 (26.3%)	G222Y	90.3 ± 8.2 (75.5%)
G222D	39.9 ± 0.8 (27.1%)	G222T	96.5 ± 1.7 (81.5%)
G222V	67.6 ± 3.6 (53.7%)	G222S	97.6 ± 7.2 (82.5%)
G222L	70.9 ± 5.2 (56.9%)		

Phosphohydrolase activity in whole homogenates was assayed in reactions containing 10 mM glucose 6-phosphate using two independent isolates of each construct in three separate transfections. The activity is expressed as nmol/min/mg of protein, and data are presented as the mean ± S.D. Numbers in parentheses represent percent of WT enzyme activity.

TABLE IV
Analysis of phosphohydrolase activity of G6Pase WT and His mutant constructs in COS-1 cells

Constructs	Phosphohydrolase activity	Constructs	Phosphohydrolase activity
Mock	19.8 ± 2.5		
WT	173.2 ± 5.5 (100%)		
H9A	40.4 ± 2.0 (13.4%)	H119K	18.6 ± 0.7
H119A	20.9 ± 1.7	H119M	18.3 ± 0.8
H252A	126.5 ± 8.6 (69.6%)	H119N	17.7 ± 1.2
H353A	112.6 ± 8.0 (60.5%)	H119R	18.6 ± 0.7
H119I	19.6 ± 0.2	H119T	22.0 ± 2.6

Phosphohydrolase activity in whole homogenates was assayed in reactions containing 10 mM glucose 6-phosphate using two independent isolates of each construct in three separate transfections. The activity is expressed as nmol/min/mg of protein, and data are presented as the mean ± S.D. Numbers in parentheses represent percent of WT enzyme activity.

same side of the ER membrane as His-119 (Fig. 1). As opposed to Arg-83, replacement of Arg-295 with a Lys retained 67% of wild-type activity, suggesting that codon 295 needs to be basic for optimal G6Pase catalysis. However, the low phosphohydrolase activity of the R295H mutant (3.9% of wild-type activity) implies that a weakly basic residue at codon 295 is not sufficient to confer significant enzymatic activity. This is supported by the moderate but significant (11.1 and 14% of WT activity) G6Pase activity exhibited by the R295N and R295Q mutants, respectively. At the present time, we cannot rule out the possibility that Arg-295 may stabilize the negatively charged G6Pase-phosphate intermediate formed during G6Pase catalysis (9, 10). However, the fact that substituting Arg-295 with an Asn or a Gln yielded mutant G6Pases with moderate enzymatic activity suggests that Arg-295 may play a stabilizing role either by salt bridge formation or hydrogen-bonding (22, 23) with another amino acid in human G6Pase.

Using near-saturation mutagenesis, we established the structure-function requirements of codon 222 in G6Pase catalysis. Codon 222 is located in the fourth putative membrane-spanning domain in human G6Pase (Fig. 1). When the native Gly is replaced with an Arg (the mutation identified in GSD type 1a), a Lys, a Gln, or an Asp, the resulting G6Pase mutants exhibited 4, 19.5, 26.3, and 27.1% of WT enzyme activity, respectively. On the other hand, when the Gly is replaced with a Val, a Leu, an Asn, a Met, a Tyr, a Thr, or a Ser, the mutants retain 53–83% of WT G6Pase activity. Our results suggest that codon 222 cannot tolerate basic (Arg or Lys), acidic (Asp), or large polar (Gln) amino acids, which is consistent with the hydrophobic requirement of a membrane-spanning segment. Recently, Shiang *et al.* (24) showed that a Gly to Arg mutation in the transmembrane domain of the fibroblast growth factor receptor 3 causes the most common form of dwarfism. Although the molecular mechanisms underlying the dominant dwarfism

TABLE V
The role of carboxyl-terminal amino acids in G6Pase catalysis

Constructs	Phosphohydrolase activity	Latency	Thermal stability
		%	%
Mock	13.7 ± 2.0		
WT	115.8 ± 9.2 (100%)	22.5	6.0
K355*X*	80.0 ± 9.5 (65%)	ND	ND
H353*X*	74.7 ± 7.4 (60%)	22.1	9.0
Q351*X*	56.7 ± 6.7 (42.6%)	25.1	6.4
G350*X*	56.1 ± 5.0 (41.5%)	19.5	7.1
L349*X*	19.1 ± 1.2 (5.3%)		
Q347*X*	12.8 ± 2.1		

Phosphohydrolase activity in whole homogenates was assayed in reactions containing 10 mM glucose 6-phosphate using two independent isolates of each construct in two separate transfections. The activity is expressed as nmol/min/mg of protein, and data are presented as the mean ± S.D. Numbers in parentheses represent percent of WT enzyme activity. Latencies were assessed by mannose 6-phosphate phosphohydrolysis in intact (I) versus detergent-disrupted (D) microsomes, defined as $(1 - I/D) \times 100$. In WT as well as mutant-transfected cells, less than 3% of the activity remained in the supernatant fractions (soluble) after pelleting the microsomes. Thermal stability was determined by assaying glucose 6-phosphate 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 refers to the percentage of enzyme activity remaining after heat treatment. ND, not determined.

phenotype is currently unknown, it is clear that the Gly to Arg mutation has a profound effect on the function of the receptor. It is possible that the G222R mutation in the membrane-spanning domain of G6Pase inhibiting enzymatic activity is mediated through a similar mechanism.

In earlier studies, we showed that the Q347*X* mutant of 346 residues is devoid of G6Pase activity, whereas the K355*X* mutant of 354 residues is enzymatically active (7). Moreover, the K355*X* mutant lacking the ER protein retention signal (KK at residues 354 and 355) remained in the microsomal fraction of the transfected cells. This suggests that the KK motif in human G6Pase is neither essential for enzymatic activity nor for microsomal retention. In the present report, we sequentially deleted the carboxyl-terminal residues in human G6Pase and analyzed the phosphohydrolase activity and microsomal association of each mutant. Our data demonstrated that G6Pase mutants lacking 3–8 carboxyl-terminal residues retained over 40% catalytic activity, whereas deletion of 9 terminal amino acids (L349*X*) reduced activity to 5% of WT G6Pase. WT and the carboxyl-terminal truncation mutants remained associated with the microsomes of transfected cells. Therefore, the carboxyl-terminal 8 amino acids in human G6Pase are not essential for G6Pase catalytic activity or membrane retention.

Acknowledgments—We thank Drs. Margaret Chamberlin and Ida Owens for critical reading of the manuscript.

REFERENCES

1. Nordlie, R. C., and Sukalski, K. A. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., ed) 2nd Ed., pp. 349–398, Plenum Publishing Corp., New York
2. Sukalski, K. A., and Nordlie, R. C. (1989.) *Adv. Enzymol.* **62,** 93–117
3. Hers, H.-G., Van Hoof, F., and de Barsy, T. (1989) in *The Metabolic Basis of Inherited Diseases* (Scriver, C. R., Beaudet, A. L., Charles, R., Sly, W. S., and Valle, D. eds) pp. 425–452, McGraw-Hill, Inc., New York
4. Beaudet, A. L. (1991) in *Harrison's Principles of Internal Medicine* (Wilson, J. D., Braunwald, E., Isselbacher, K. J., Petersdorf, R. G. Martin, J. B., Fauci, A. S., and Root, R. K., eds) 12th Ed., pp. 1854–1860, McGraw-Hill, Inc., New York
5. Lei, K.-J., Shelly, L. L., Pan, C.-J., Sidbury, J. B., and Chou, J. Y. (1993) *Science* **262,** 580–583
6. Shelly, L. L., Lei, K.-J., Pan, C.-J., Sakata, S. F., Ruppert, S., Schutz, G., and Chou, J. Y. (1993) *J. Biol. Chem.* **268,** 21482–21485
7. Lei, K.-J., Pan, C.-J., Shelly, L. L., Liu J.-L., and Chou, J. Y. (1994) *J. Clin. Invest.* **93,** 1994–1999
8. Lei, K.-J., Shelly, L. L., Lin, B., Sidbury, J. B., Chen, Y.-T., Nordlie, R. C., and Chou, J. Y. (1994) *J. Clin. Invest.* **95,** 234–240
9. Feldman F., and Butler L. G. (1972) *Biochim. Biophys. Acta* **268,** 698–710
10. Countaway, J. L., Waddell, I. D., Burchell, A., and Arion, W. J. (1988) *J. Biol. Chem.* **263,** 2673–2678
11. Lange, A. J., Argaud, D., EI-Maghrabi, M. R., Pan, W., Maitra, S. R., and Pilkis, S. J. (1994) *Biochem. Biophys. Res. Commun.* **201,** 302–309
12. Wells, J. A., Vasser, M., Powers, D. B. (1985) *Gene (Amst.)* **34,** 315–323
13. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) *Current Protocols in Molecular Biology,* pp. 9.2.1–9.2.6, Greene Publishing and Wiley-Interscience, New York
14. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
15. Burchell, A., Hume, R., and Burchell, B. (1988) *Clin. Chim. Acta* **173,** 183–192
16. Arion, W. J., Lange, A. J., Walls, H. E., and Ballas, L. M. (1980) *J. Biol. Chem.* **255,** 10396–10406
17. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) *EMBO J.* **9,** 3153–3162
18. Hoylaerts, M. F., Manes, T., and Millan, J. L. (1992) *Biochem. J.* **286,** 23–30
19. Kim, E. E., and Wyckoff, H. W. (1991) *J. Mol. Biol.* **218,** 449–464
20. Tauler, A., Lin, K., and Pilkis, S. J. (1990) *J. Biol. Chem.* **265,** 15617–15622
21. Bazan, F., Fletterick, R., and Pilkis, S. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9642–9646
22. Roberts, V. A., Stewart, J., Benkovic S. J., Getzoff E. D. (1994) *J. Mol. Biol.* **235,** 1098–1116
23. Nandi, C. L., Singh J., Thornton, J. M. (1993) *Protein Eng.* **6,** 247–259
24. Shiang, R., Thompson, L. M., Zhu, Y.-Z., Church, D. M., Fielder, T. J., Bocian, M., Winokur, S. T., and Wasmuth, J. J. (1994) *Cell* **78,** 335–342
25. Klein, P., Kanehisa, M., DeLisi, C. (1985) *Biochim. Biophys. Acta* **815,** 468–476