The Molecular Basis of Glycogen Storage Disease Type 1a
STRUCTURE AND FUNCTION ANALYSIS OF MUTATIONS IN GLUCOSE-6-PHOSPHATASE*

Glycogen storage disease type 1a is caused by a deficiency in glucose-6-phosphatase (G6Pase), a nine-helical endoplasmic reticulum transmembrane protein required for maintenance of glucose homeostasis. To date, 75 G6Pase mutations have been identified, including 48 mutations resulting in single-amino acid substitutions. However, only 19 missense mutations have been functionally characterized. Here, we report the results of structure and function studies of the 48 missense mutations and the ΔF327 codon deletion mutation, grouped as active site, helical, and nonhelical mutations. The 5 active site mutations and 22 of the 31 helical mutations completely abolished G6Pase activity, but only 5 of the 13 nonhelical mutants were devoid of activity. Whereas the active site and nonhelical mutants supported the synthesis of G6Pase protein in a manner similar to that of the wild-type enzyme, immunoblot analysis showed that the majority (64.5%) of helical mutations destabilized G6Pase. Furthermore, we show that degradation of both wild-type and mutant G6Pase is inhibited by lactacystin, a potent proteasome inhibitor. Taken together, we have generated a data base of residual G6Pase activity retained by G6Pase mutants, established the critical roles of transmembrane helices in the stability and activity of this phosphatase, and shown that G6Pase is a substrate for proteasome-mediated degradation.

The cloning of the G6Pase gene has enabled researchers to show that GSD-1a individuals are homozygotes or compound heterozygotes for loss of function mutations in the gene (4–11). To date, 75 G6Pase mutations (including 2 reported here) have been identified in GSD-1a patients on the basis of their absence from the normal population and/or their co-segregation with the disease phenotype (reviewed in Refs. 2 and 3). Interestingly, 48 candidate mutations are missense mutations that result in single-amino acid substitutions. Characterization of these mutations will provide critical information on functionally important residues of the protein. In this study, we functionally characterize all 48 missense mutations by site-directed mutagenesis and transient expression assays. A data base of residual enzymatic activity retained by the G6Pase mutants will serve as a reference for evaluating genotype-phenotype relationships and the minimal G6Pase activity required to correct the GSD-1a phenotype.

Sequence alignment suggests that mammalian G6Pases, lipid phosphatases, acid phosphatases, and vanadium haloperoxidases share a conserved phosphatase signature motif, and in G6Pase, this occurs between residues 76 and 180 (12, 13). The crystal structure of the vanadium-containing chloroperoxidase from the plant pathogenic fungus Curvularia inaequalis has been resolved (14). The results show the active site residues in vanadium-containing chloroperoxidase are contained within the phosphatase signature motif. Based on the crystal structure and mechanism of action of vanadium-containing chloroperoxidase (13, 14), the amino acids predicted to participate in G6Pase catalysis include Lys76, Arg83, His119, Arg170, and His176. Five mutations that alter active site residues in G6Pase, K76N, R83C, R83H, H119L, and R170Q, have been identified in GSD-1a patients (4, 6, 15–17). R83C and R83H were shown to abolish phosphatase activity in transient expression assays (4, 6). In this study, we show that K76N, H119L, and R170Q also completely abolish G6Pase activity, demonstrating the importance of these residues in G6Pase catalysis.

Very little is known about the structural requirements for the correct folding and catalytic activity of G6Pase. We have shown that human G6Pase is anchored to the endoplasmic reticulum (ER) by nine transmembrane helices with the amino terminus in the lumen and the carboxyl terminus in the cytoplasm (18, 19). Therefore, the large collection of G6Pase mutations can now be studied in the context of their positions with respect to the ER and the cytoplasm. In this study, we undertake structure-function analysis of G6Pase. We show that amino acid residues that comprise the catalytic center and nonhelical regions in G6Pase play no essential role in the stability of the enzyme. On the other hand, the structural integrity of transmembrane helices is critical for the correct folding, stability, and enzymatic activity of G6Pase.

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† To whom correspondence should be addressed: Bldg. 10, Rm. 9S241, Heritable Disorders Branch, NICHD, National Institutes of Health, Bethesda, MD 20892-1830. Tel.: 301-496-1094; Fax: 301-402-6035; E-mail: chou@helix.nih.gov.
‡ The abbreviations used are: GSD-1, glycogen storage disease type 1; G6Pase, glucose-6-phosphatase; WT, wild-type; ER, endoplasmic reticulum.
Proteins with abnormal conformation are rapidly eliminated through intracellular protein degradation, which represents a quality control system in cells (20). Cytosolic proteasomes are responsible for rapid degradation of many membrane proteins, including cystic fibrosis transmembrane conductance regulator (21, 22), the major histocompatibility complex class I molecule, and the α-chains of T-cell antigen receptor (reviewed in Refs. 23–25). The proteasome pathway can be inhibited by the Streptomyces metabolite, lactacystin (26), which inhibits the proteasome specifically without inhibiting other proteases (reviewed in Ref. 27). The availability of proteasome inhibitor allows a rapid analysis in intact cells of the possible contributions of protein breakdown by the proteasomes. In this study, we show that wild-type and mutant G6Pases are predominantly degraded in the ER through the proteasome pathway.

MATERIALS AND METHODS

Mutational Analysis—The G6Pase gene in GSD-1a patients was characterized by single-strand conformational polymorphism analysis (28) on mutation detection enhancement gels (AT Biochem, Malvern, PA) containing 5% glycerol. Exon-containing fragments were amplified by PCR using primers containing intrinsic, 5′-, and 3′-untranslated sequences of the human G6Pase gene as described previously (6). The mutation-containing fragments identified by single-strand conformational polymorphism analysis were subcloned and characterized by DNA sequencing.

Construction of G6Pase Mutants and Expression in COS-1 Cells—Human G6Pase-DraIII (29) cDNA was used as a template for mutant construction by PCR. The eight-amino acid FLAG marker peptide, DYKDDDK (Scientific Imaging Systems, Eastman Kodak, CT) was used to tag the amino or carboxyl terminus of G6Pase as described previously (18). The two outside PCR primers for G6Pase mutants that contain mutations upstream of the DraIII site are nucleotides 77–96 (G1; sense) and nucleotides 625–620 of G6Pase-DraIII (12; antisense) (29) and the inside primer is for G6Pase mutants that contain mutations downstream of the DraIII site are nucleotides 611–634 (11; sense) and nucleotides 1150–1133 of G6Pase-DraIII (2; antisense). The two outside primers for G6Pase R170Q, G184F, G184V, G188S, and G222R mutants were amplified by PCR. The amplified fragment was ligated into either the pSVLHbG6Pase-DraIII-3′ fragment, the pSVLHbG6Pase-DraIII-5′ fragment, or the pSVF vector.

The mutant primers are: M5R (nucleotides 83–103), ATG→AGG at position 5; T16A (nucleotides 116–136), ACA→GCA at position 16; Q2OR (nucleotides 128–148), CAG→CGG at position 20; Q54P (nucleotides 230–250), CAG→CGG at position 54; W63R (nucleotides 257–277), TGG→CGG at position 63; G68R (nucleotides 272–292), GGA→CGA at position 68; K76N (nucleotides 296–316), AAG→AAT at position 76; W77R (nucleotides 319–311), CTG→TGG at position 77; G184E (nucleotides 553–565), TCT→CAG at position 184; G184V (nucleotides 620–640), GGA→GTA at position 184; G188S (nucleotides 632–652), GCC→GAC at position 188; G188R (nucleotides 632–652), GCC→GCG at position 188; L265P (nucleotides 701–721), TCC→CCC at position 265; A241T (nucleotides 791–811), GCC→ACC at position 241; T255I (nucleotides 833–853), ACC→ATC at position 255; P257L (nucleotides 839–859), CCC→CTC at position 257; N264K (nucleotides 860–880), AAC→AAA at position 264; L265P (nucleotides 863–883), CTG→CGG at position 265; G266V (nucleotides 886–886), GGC→GTC at position 266; G270R (nucleotides 878–898), GGC→GCG at position 270; F327L (nucleotides 1034–1054), TTC→TCT at position 327; A322; V338F (nucleotides 1082–1102), GTC→TTC at position 338; and I341N (nucleotides 1091–1111), ATC→AAC at position 341. The antisense primer for each mutant has the corresponding complementary sequence. Bold letters indicate nucleotide changes. We have also constructed carboxyl-terminal FLAG-tagged human G6Pase mutants, R83C, R83H, E110K, E110Q, V166G, G188S, G222R, W236R, G270V, R295C, ΔF327, and L345R, as described previously (4–6). The nucleotide sequence in all constructs was verified by DNA sequencing. D38V-FLAG and P178S-3′-FLAG mutants have been described previously (19).

COS-1 cells were grown at 37 °C in HEPES-buffered Dulbecco’s modified minimal essential medium supplemented with 4% fetal bovine serum. Cells in 25-cm² flasks were transfected with 10 μg of wild-type (WT) or mutant construct in a pSVL vector by the DEAE-dextran/chloroquine method as described previously (30). To correct for transfection efficiency, 2 μg of pCMVβ (BD Biosciences, CLONTECH) was cotransfected with WT or mutant G6Pase cDNA construct. After incubation at 37 °C for 2 days, the transfected cultures were harvested for phosphohydrolase and β-galactosidase assays, Western blot analysis, or RNA isolation.

Phosphohydrolase and β-Galactosidase Assays—Phosphohydrolase activity was determined essentially as described previously (4). Reaction mixtures (100 μl) contained 50 mM cadoxylate 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 phosphatase released using a standard curve constructed by a stock of inorganic phosphate solution. Nonspecific phosphatase activity was estimated by preincubating cell homogenates at pH 5 for 10 min at 37 °C, a condition that inactivates the thermolabile G6Pase (31).

A model of activity was measured by the release of O-nitropheno-

For Western blot analysis of FLAG-tagged G6Pase, proteins in transfected COS-1 lysates were separated by electrophoresis through a 13% polyacrylamide-SDS-gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore). The membranes were first incubated with a monoclonal antibody against the FLAG epitope (Scientific Imaging Systems) and then incubated with goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The immunocomplex was detected with the horseradish peroxidase-linked chemiluminescence system containing the SuperSignal West Pico Chemiluminescent substrate obtained from Pierce.

In vitro transcription-translation of G6Pase cDNA constructs in a pGEM-11Zf+ vector was performed using the troponin T coupled reticulocyte lysate system obtained from Promega Biotech (Madison, WI). 35S-Labeled protein was used as the labeled precursor. The in vitro synthesized proteins were analyzed by 12% polyacrylamide-SDS gel electrophoresis and fluoroo-autoradiography.

RESULTS

Mutations Identified in the G6Pase Gene of GSD-1a Patients—Single-strand conformational polymorphism and DNA sequencing analyses were used to identify mutations in the G6Pase gene of four GSD-1a patients. Six different mutations were identified, including T255I, S566Ea, W70X, Q347X, and ΔF327 (Table I). Two novel mutations identified in this study are T255I and S566Ea.

G6Pase Mutations That Cause GSD-1a—Characterization of missense mutations that result in single-amino acid substitutions will provide valuable information on functionally important residues in G6Pase. The 48 missense G6Pase mutations identified in GSD-1a patients are distributed throughout the primary sequence (Fig. 1). Nineteen missense mutations arise from chimeric D38V (8), W77R (9), R83C (4), R83H (6), E110K (3), E110Q (8), A124T (9), V166G (7), P178S (6), G184E (9), G188S (6), G188R (10), L211P (9), G222R (5), W236R (6), P257L (11), G270V (6), R295C (4), and L345R (6), were shown to abolish or greatly reduced G6Pase activity by site-directed mutagenesis and transient expression assays. In this study, we constructed

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35 mutants carrying G6Pase missense mutations, including 29 mutations that have not been characterized and 6 of the previously characterized mutations, W77R, A124T, G184E, L211P, G188R, and P257L. Glucose-6-phosphate hydrolytic activities of these mutants were examined after transient expression of WT or mutant G6Pase cDNA into COS-1 cells. We also included in this study the single codon deletion mutation, F327, shown to be devoid of enzymatic activity (5). To facilitate structure-function analysis, we have grouped these mutations into three categories (active site, helical, and nonhelical mutations) based on their predicted catalytic, transmembrane helical, luminal, and cytoplasmic locations in G6Pase (Fig. 1).

The amino acids predicted to be critical to glucose-6-phosphate binding and hydrolysis include Lys76, Arg83, His119, Arg170, and His176 (12–14). Five active site mutations, K76N (16), R83C (4), R83H (6), H119L (17), and R170Q (15), have been identified in the G6Pase gene of GSD-1a patients. In earlier studies (4, 6), we have shown that R83C and R83H mutants were devoid of G6Pase activity (Table II). In this study, we demonstrate that K76N, H119L, and R170Q mutations also completely abolished phosphohydrolase activity (Table II), demonstrating the importance of these residues in G6Pase catalysis.

Twelve of the 13 nonhelical G6Pase mutations are situated inside the ER lumen, and 1 (Q54P) is located in cytoplasmic loop 1 (Fig. 1). Earlier studies have shown that the E110K (2) mutation totally inactivated G6Pase, but the E110Q (8), W236R (6), and P257L (11) mutations only markedly reduced phosphatase activity. This was confirmed in the present study demonstrating that eight nonhelical mutants, M5R, T16A, E110Q, T111I, W236R, A241T, T255I, and P257L, retained residual phosphohydrolase activity (Table III). Four mutants, T16A, E110Q, T111I, and A241T, possessed >10% WT G6Pase activity. The five nonhelical mutations that completely abolished G6Pase activity include Q20R, Q54P, T108I, E110K, and P113L (Table III). Q20R and Q54P are located within the amino terminus and cytoplasmic loop 1, respectively, and E110K, T108I, and P113L are all situated within luminal loop 1 (Fig. 1).

Thirty missense mutations (excluding the active site mutation K76N in helix 2) and the ΔF327 mutation are scattered throughout the G6Pase gene in GSD-1a patients. These include 12 mutations that have been characterized and 23 mutations that have not been characterized.
throughout the nine transmembrane helices (Fig. 1). Earlier mutational studies have shown that 13 helical mutations, including D38V, G122D, A124T, W156L, V166A, P178S, L211P, G270V, R295C, G68R, L345R, completely abolished G6Pase activity, and one helical mutation, G222R, retained residual activity (reviewed in Refs. 2 and 3). In this study, we extended this analysis and showed that 22 helical mutations completely inactivated the enzyme (Table IV). The nine helical mutants that retain residual G6Pase activity include D38V, G122D, A124T, W156L, V166A, P178S, L211P, G222R, and F322L. It is interesting to note that only two mutants, G122D (helix 3) and F322L (helix 9), retained >10% WT G6Pase activity. Also, G122D and A124T are the only mutations identified in helix 3.

The Active Site and Nonhelical Residues Play No Essential Role in the Stability of G6Pase—Structure-function analyses of G6Pase would be greatly facilitated by specific antibodies to G6Pase. However, polyclonal antibodies to human G6Pase are not specific. As an alternative strategy to monitor biosynthesis of mutated G6Pase, we have constructed all 48 missense and the ΔF327 G6Pase mutants with the eight-amino acid FLAG marker peptide (DYKDDDDK) at their carboxyl termini. The FLAG tag does not interfere with the expression, stability, or activity of WT G6Pase and has been used successfully to tag human G6Pase (18, 19) for topological studies. G6Pase biosynthesis in COS-1 cells was examined by Western blot analysis using a monoclonal antibody against the FLAG epitope. In WT construct-transfected COS-1 cells, polypeptides of 41 and 37 kDa, representing glycosylated and nonglycosylated G6Pase, were synthesized (Fig. 2A). In the presence of a glycosylation inhibitor, tunicamycin (32), only the 37-kDa nonglycosylated G6Pase was detected, confirming their identities. We have previously shown that both forms of G6Pase are enzymatically active and that the nonglycosylated G6Pase retains ~40% activity (19). Immunoblot analyses showed that the active site mutants, K76N, R83C, R83H, H119L, and R170Q (Fig. 2), as well as the 13 nonhelical mutants (Fig. 3) supported the synthesis of similar amounts of G6Pase proteins as the WT construct. The results suggest that amino acid residues that comprise the catalytic center and nonhelical regions of the enzyme do not play an essential role in the correct folding and stability of G6Pase. Whereas the 41-kDa glycoprotein was the major form in WT-transfected cells, the 37-kDa nonglycosylated G6Pase became the major form in M5R-, Q20R-, Q54P-, T108I-, and W236R-transfected cells, suggesting the accumulation of incompletely processed protein in G6Pase mutant-transfected cells.

### Table III

| Mutation Location | Non-FLAG construct 3' FLAG construct |
|-------------------|-------------------------------------|
| Mock              |                                     |
| WT                |                                     |
| M5R               |                                     |
| T16A              |                                     |
| Q20R              |                                     |
| Q54P              |                                     |
| T108I             |                                     |
| E110K             |                                     |
| E110Q             |                                     |
| T111L             |                                     |
| P113L             |                                     |
| W236R             |                                     |
| A241T             |                                     |
| T255L             |                                     |
| T255L             |                                     |
| WT                |                                     |
| Mock              |                                     |
| WT                |                                     |
| M5R               |                                     |
| T16A              |                                     |
| Q20R              |                                     |
| Q54P              |                                     |
| T108I             |                                     |
| E110K             |                                     |
| E110Q             |                                     |
| T111L             |                                     |
| P113L             |                                     |
| W236R             |                                     |
| A241T             |                                     |
| T255L             |                                     |
| T255L             |                                     |

### Table IV

| Mutation Location | Non-FLAG construct 3' FLAG construct |
|-------------------|-------------------------------------|
| Mock              |                                     |
| WT                |                                     |
| M5R               |                                     |
| T16A              |                                     |
| Q20R              |                                     |
| Q54P              |                                     |
| T108I             |                                     |
| E110K             |                                     |
| E110Q             |                                     |
| T111L             |                                     |
| P113L             |                                     |
| W236R             |                                     |
| A241T             |                                     |
| T255L             |                                     |
| T255L             |                                     |

TABLE III: Phosphohydrolase activity of G6Pase nonhelical mutant constructs

TABLE IV: Phosphohydrolase activity of G6Pase helical mutant constructs

**FIG. 2. Western blot analysis of G6Pase in COS-1 cells transfected with WT or an active site mutant cDNA construct containing a 3' FLAG tag. A. effects of tunicamycin. COS-1 cells transfected with the G6Pase-WT construct were incubated in the absence or presence of tunicamycin (1 μg/ml) for 24 h before harvesting for Western blot analysis. B. Western blot analysis of active site mutants. Mock-transfected cells were used as controls. The G6Pase proteins on the Western membranes were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg of proteins.**

**FIG. 3. Western blot analysis of G6Pase in COS-1 cells transfected with WT or a nonhelical mutant cDNA construct containing a 3' FLAG tag. Mock-transfected cells were used as controls. The G6Pase proteins on the Western membranes were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg of proteins.**
after electrophoresis, the proteins were visualized by fluoro-
to the Stability of G6Pase
abolished G6Pase activity.
and P113L are the only nonhelical mutations that completely
Mutants (Table III). Again, Q20R, Q54P, T108I, E110K,
were obtained with the tagged and nontagged G6Pase nonhe-
ical mutants, like WT G6Pase, directed the synthesis of similar
lanes contained 20 μg of proteins. For in vitro transcription-
translation analysis, in vitro synthesis of G6Pase directed by 3’
FLAG-tagged WT or a mutant G6Pase construct in a pGEM-11Zf(+)
vector was performed using the troponin T-coupled reticulocyte lysate
system. L-[35S]Methionine was used as the labeled precursor, and
after electrophoresis, the proteins were visualized by fluoro-
autoradiography.

The FLAG-tagged active site mutants, like the respective
parental constructs, were devoid of G6Pase activity (Table II).
Similarly, comparable amounts of phosphohydrolase activities
were obtained with the tagged and nontagged G6Pase nonhe-
lical mutants (Table III). Again, Q20R, Q54P, T108I, E110K,
and P113L are the only nonhelical mutations that completely
abolished G6Pase activity.
The Structural Integrity of Transmembrane Helices Is Vital
to the Stability of G6Pase—The majority (64.5%) of helical
mutants, including D38V (H1), W63F/G68R (H2), V166A (H4),
G185D/G188S/G188R (H5), L211P/G222R (H6), N264K/L265P/
G266V/G270V/G270R (H7), R295C/S298P (H8), and ΔF327/
V338F/I341N/L345R (H9), supported the synthesis of reduced
levels of G6Pase proteins in COS-1 cells as compared with the WT
construct (Fig. 4). Moreover, the 41-kDa G6Pase was prefer-
entially reduced, indicating that mutations that altered the structural
integrity of transmembrane helices destabilize G6Pase. Interestingly, the steady-state level of the G184E mu-
tant, which is devoid of G6Pase activity, was higher than that
of WT G6Pase, suggesting that the G184E mutation increased
the stability of this phosphatase.
Northern blot analysis confirmed that similar levels of G6Pase transcripts were expressed in WT or mutant G6Pase-
transfected COS-1 cells (data not shown). Our results, there-
fore, demonstrate that the decrease in G6Pase biosynthesis was not due to a decrease in efficiency of expression of the
transfected cDNA construct. Moreover, the helical mutant con-
structs, like WT G6Pase, directed the synthesis of similar
amounts of G6Pase proteins in a cell-free transcription-translation
system (Fig. 4). The results indicate that transmem-
brane helices in G6Pase play a vital role in the correct folding
of the enzyme and that the abnormal mutant proteins are
rapidly degraded in the cell.
Comparative amounts of G6Pase activities were obtained
with FLAG-tagged or nontagged G6Pase helical mutants (Ta-
ble IV). Again, 22 of the 31 helical mutants were devoid of
enzymatic activity, and 9 mutants (D38V, G122D, A124T,
W156L, V166A, P178S, L211P, G222R, and F322L) retained
residual G6Pase activity.
The Proteasome Inhibitor Lactacystin Induces the Accumu-
lation of WT and Mutant G6Pase—The effect of a proteasome
inhibitor, lactacystin (26), on steady-state levels of G6Pase was
assessed by immunoblot analysis of COS-1 cells transfected
with WT or mutant G6Pase construct. In WT-transfected cells,
the steady-state levels of both 41-kDa and 37-kDa G6Pases
were markedly increased in the presence of lactacystin (Fig. 5),
indicating that G6Pase is predominantly degraded in cells
through the proteasome pathway. Lactacystin inhibits degra-
dation of both glycosylated and nonglycosylated G6Pases,
suggesting that this metabolite does not inhibit the processing and
matured levels of G6Pase. In the presence of lactacystin, a fast-
migrating band of 23 kDa was also accumulated (Fig. 5). The
nature of this polypeptide is unknown. It is also unclear whether additional intermediates are accumulated in the presen-
tce of lactacystin because the antibody recognizes the FLAG
tag at the carboxyl terminus of the enzyme.
In the absence of lactacystin, the steady-state levels of mu-
tant proteins in D38V-, G68R-, G188R-, L265P-, R295C-, and
L345R-transfected cells were lower than that in WT G6Pase-
transfected cells (Fig. 5). However, in the presence of lactacys-
tin, a marked increase in the accumulation of both the 41- and
37-kDa mutant G6Pases comparable to that of WT G6Pase was
observed (Fig. 5), indicating that proteasomes also participate
in the degradation of G6Pase mutants. In the absence of lac-
tacystin, the G184E construct supported the synthesis of in-
creased levels of G6Pase protein rather than that of WT con-
struct. In the presence of lactacystin, comparable amounts of
G184E and WT G6Pase were accumulated (Fig. 5), again sug-
uggesting that the G184E mutation increased the stability of G6Pase.

DISCUSSION
Human G6Pase is anchored to the ER by nine transmem-
brane helices with the amino terminus and catalytic center
facing inward in the lumen and the carboxyl terminus facing
outward in the cytoplasm (18, 19). In this study, we examined
phosphohydrolase activities of G6Pase mutants carrying 48
missense mutations and ΔF327 codon deletion mutation
grouped into three categories based on their predicted cata-
lytic, helical, and nonhelical locations in this phosphatase. We
have also undertaken structure-function analysis of human
G6Pase. We show that mutations that altered the active center
in G6Pase completely inactivated the enzyme but had no del-
etrious effects on the folding and stability of the protein. Eight
of the 13 nonhelical mutants retained residual G6Pase activity,
and most, if not all, supported the synthesis of WT levels of
G6Pase protein in COS-1 cells, suggesting that nonhelical mu-
tants also play no essential role in the stability of G6Pase. On the other hand, of the 31 helical mutations characterized, 22 (71%) completely abolished G6Pase activity, and 20 (64%) destabilized this phosphatase. Taken together, the results indicate that the structural integrity of transmembrane helices is vital to the stability and enzymatic activity of G6Pase. We have also provided evidence indicating that G6Pase is degraded predominantly through the proteasome pathway.

Based on the crystal structure of vanadium-containing chloroperoxidase (13, 14), the amino acids predicted to participate in G6Pase catalysis include Lys76, Arg83, His119, Arg170, and His176. As expected, the five active site mutations, K76N, R83C, R83H, H119L, and R170Q, completely inactivated the enzyme, confirming the importance of these residues in G6Pase catalysis. Whereas 22 of the 31 (71%) helical mutations completely abolished G6Pase activity, only 5 of the 13 (38%) non-helical mutants were devoid of enzymatic activity, suggesting that an active G6Pase depends upon the structural integrity of its transmembrane helices. Luminal loop 1 may also play a crucial role in catalytic activity of the enzyme because T108I, E110K, and P113L mutations, which totally inactivated G6Pase, are located within this loop. The nine helical mutants that retain residual G6Pase activity include D385V, G122D, A124T, W156L, V166A, P178S, L211P, G222R, and F322L. Earlier studies have shown that D385V (8), P178S (6), and A124T (9) mutants were devoid of G6Pase activity. The observed difference may result from the low levels of G6Pase activity retained by these mutants. Currently, we are adapting a recombinant adenosin vector-mediated expression system, which has been widely used for high-level protein expression in mammalian cells (33), to increase the sensitivity of the expression assays.

It is interesting to note that a Japanese patient homozygous for the P257L mutation, a nonhelical mutation that only partially inactivates G6Pase, had a very mild phenotype (11). The patient experienced no hypoglycemic episodes and required no dietary therapy. The data base of residual phosphohydrolase activity retained by the 49 G6Pase codon mutants should facilitate the development of novel therapeutic approaches for this disorder.

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Proteins with abnormal conformations that arise by muta-
tions or intracellular denaturation are rapidly degraded, which represents a quality control system in the cell (20, 34). Most intracellular protein degradation is catalyzed by lysosomal proteases or the ubiquitin-proteasome system (reviewed in Ref. 24). Most membrane proteins, including cystic fibrosis transmembrane conductance regulator (21, 22), the major histocompatibility complex class I molecule, and α-chains of T-cell antigen receptor, are degraded in cells by the proteasome system (reviewed in Refs. 23–25). The 26S proteasome, an ATP-dependent proteolytic complex, contains the central 20S proteasome, in which proteins are degraded, and two 19S complexes, which provide substrate specificity and regulation (reviewed in Refs. 23–25). The active site nucleophile of the proteasome is the hydroxyl group of a threonine at the amino terminus of the β subunit of proteasome. Lactacystin, a specific proteasome inhibitor, blocks proteasome function by becoming covalently linked to the hydroxyl group of threonine (26, 27). In this study, we show that the steady-state levels of WT and mutant G6Pase were markedly increased by lactacystin, indicating that degradation of G6Pase is mediated predominantly by the proteasome pathway. The marked increase in the levels of WT G6Pase by lactacystin suggests that folding of G6Pase is relatively inefficient, a phenomenon also observed for the cystic fibrosis transmembrane conductance regulator (35, 36).

In summary, we have generated a data base of residual G6Pase activity retained by 49 codon mutations to facilitate genotype-phenotype delineation, elucidated a number of structural requirements for the stability and enzymatic activity of G6Pase, and demonstrated that proteasomes mediate degradation of this phosphatase.
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