Transmembrane Topology of Glucose-6-Phosphatase*

(Received for publication, November 18, 1997, and in revised form, December 18, 1997)

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Deficiency of microsomal glucose-6-phosphatase (G6Pase), the key enzyme in glucose homeostasis, causes glycogen storage disease type 1a, an autosomal recessive disorder. Characterization of the transmembrane topology of G6Pase should facilitate the identification of amino acid residues contributing to the active site and broaden our understanding of the effects of mutations that cause glycogen storage disease type 1a. Using N- and C-terminal tagged G6Pase, we show that in intact microsomes, the N terminus is resistant to protease digestion, whereas the C terminus is sensitive to such treatment. Our results demonstrate that G6Pase possesses an odd number of transmembrane helices, with its N and C termini facing the endoplasmic reticulum lumen and the cytoplasm, respectively. During catalysis, a phosphoryl-enzyme intermediate is formed, and the phosphoryl acceptor in G6Pase is a His residue. Sequence alignment suggests that mammalian G6Pases, lipid phosphatases, acid phosphatases, and a vanadium-containing chloroperoxidase (whose tertiary structure is known) share a conserved phosphatase motif. Active-site alignment of the vanadium-containing chloroperoxidase and G6Pases predicts that Arg-83, His-119, and His-176 in G6Pase contribute to the active site and that His-176 is the residue that covalently binds the phosphoryl moiety during catalysis. This alignment also predicts that Arg-83, His-119, and His-176 reside on the same side of the endoplasmic reticulum membrane, which is supported by the recently predicted nine-transmembrane helical model for G6Pase. We have previously shown that Arg-83 is involved in positioning the phosphate during catalysis and that His-119 is essential for G6Pase activity. Here we demonstrate that substitution of His-176 with structurally similar or dissimilar amino acids inactivates the enzyme, suggesting that His-176 could be the phosphoryl acceptor in G6Pase during catalysis.

Glucose-6-phosphatase (G6Pase⁵; EC 3.1.3.9), which catalyzes the terminal step in gluconeogenesis and glycolysis, is the key enzyme in glucose homeostasis (1). In humans, deficiency in microsomal G6Pase causes glycogen storage disease type 1a (GSD-1a), also known as von Gierke's disease (2). It is an autosomal recessive disorder with clinical manifestations of severe hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, hyperlipidemia, hyperuricemia, and lactic acidemia (2, 3). G6Pase is tightly associated with the endoplasmic reticulum (ER) membranes (1), and enzymatic activity in intact hepatic microsomes is resistant to limited proteolysis, suggesting that the active site of G6Pase is not exposed to the cytoplasm (4–7). To understand the biology and pathophysiology of GSD-1a, we have characterized the murine (8) and human (9) G6Pase cDNAs and genes and showed that mammalian G6Pases are hydrophobic proteins of 357 amino acids. Analysis of the hydrophathy profiles by the algorithm used in the PC/Genet program (10) predicted that mammalian G6Pases are anchored in the ER membrane by six putative transmembrane helices (8, 9). During catalysis, a phosphoryl moiety is transferred from Glu-6-P to a His residue in G6Pase, forming a phosphoryl-enzyme intermediate (11–13). Structure-function studies suggest that Arg-83 in G6Pase is involved in stabilizing the phosphoryl-enzyme intermediate formed during catalysis (14). These studies also show that His-119 is essential for G6Pase activity, suggesting that this His residue could be the phosphoryl acceptor (14).

Recently, the tertiary structure of a vanadium-containing chloroperoxidase from the fungus Curvularia inaequalis has been determined (15). Vanadate, which is a strong competitive inhibitor for G6Pase (16), is structurally similar to phosphate. Moreover, apochloroperoxidase can function as a phosphatase (17). Sequence analysis indicates that the amino acids contributing to the active site of the vanadium-containing chloroperoxidase are also conserved in lipid phosphatases, acid phosphatases, and mammalian G6Pases (17, 18), even though their overall amino acid identities are very low. Therefore, the vanadium-containing chloroperoxidase structure could provide clues to the structure of G6Pase. Alignment of the active-site residues of the vanadium-containing chloroperoxidase with the proposed phosphate-binding site of G6Pase (19) supports our earlier proposal (14) that Arg-83 is a candidate for positioning the phosphoryl moiety during catalysis. However, this alignment predicts that His-176 is the residue that covalently binds the phosphoryl moiety (19), not His-119 as suggested earlier (14). If His-176 is the phosphoryl acceptor, the six-transmembrane helical model of G6Pase must be re-evaluated because it places His-176 on the opposite side of the ER membrane from Arg-83 and His-119 (14). The hydrophathy profiles analyzed by a newly developed algorithm (20) predict that G6Pase contains nine transmembrane helices, which would place Arg-83, His-119, and His-176 on the same side of the ER membrane (19).

The number of transmembrane helices dictates the luminal or cytoplasmic location of the N or C terminus of G6Pase. The nine-transmembrane helical model predicts that only one terminus of G6Pase faces the cytoplasm, which will be sensitive to protease digestion. On the other hand, the six-transmembrane helical model predicts that both the N and C termini of G6Pase face either the ER lumen or the cytoplasm, and thus, both termini should have the same sensitivity to protease digestion. In this study, we performed protease protection assays using N- and C-terminal tagged G6Pase and showed that G6Pase contains an odd number of transmembrane helices, with its N

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The abbreviations used are: G6Pase, glucose-6-phosphatase; GSD-1a, glycogen storage disease type 1a; ER, endoplasmic reticulum; WT, wild-type.
terminals facing the ER lumen and its C terminus facing the cytoplasm. To examine the role of His-176 in G6Pase during catalysis, we substituted codon 176 with amino acids of different structures by site-directed mutagenesis and analyzed G6Pase activity after transient expression of wild-type (WT) and mutant G6Pase cDNAs in COS-1 cells.

MATERIALS AND METHODS

Generation of Mutant G6Pase Constructs—The pH6G6Pase-DraIII construct, which contains an additional DraIII site at nucleotides 614–622 but retains the primary amino acid sequence of WT human G6Pase and contains WT enzymatic activity (14), was used as a template for mutant construction by polymerase chain reaction. The eight-amino acid FLAG marker peptide DYKDDDDK (Kodak Scientific Imaging Systems) was used to tag the N and C termini of G6Pase. The 5′-primer for the N-terminal FLAG-tagged G6Pase (G6Pase-5′-FLAG) coding contained an ATG initiation codon followed by the 24-base pair FLAG coding sequence (5′-GACTACAAGGACGACGATGACAAG-3′) and nucleotides 80–98 of human G6Pase (9); the 3′-primer contained nucleotides 625 to 602 (1-2) of human G6Pase-DraIII (14). The amplified fragment was ligated into the pSVLhG6Pase-DraIII 3′-fragment (14). The 5′-primer for C-terminal FLAG-tagged G6Pase (G6Pase-3′-FLAG) contained nucleotides 611–634 (1-1) of human G6Pase-DraIII (14); the 3′-primer contained the last coding nucleotides (1133) of human G6Pase (9) followed by the 24-base pair FLAG coding sequence and a termination codon. The amplified fragments were ligated into the pSVLhG6Pase-DraIII 5′-fragment (14).

The 5′-primer for G6Pase-(5–357) and G6Pase-(5–357)-3′FLAG, with an N-terminal deletion of residues 1–4, contained nucleotides 92–110 of human G6Pase, and the ATG initiation codon was provided by nucleotides 92–94 of human G6Pase. The 5′-primer for G6Pase-(14–357) and G6Pase-(14–357)-3′ FLAG, with an N-terminal deletion of residues 1–13, contained an ATG initiation codon followed by nucleotides 119–139 of human G6Pase. The 3′-primer for G6Pase-(5–357) and G6Pase-(14–357) is 1-2, and after polymerase chain reaction, the amplified fragment was ligated into the pSVLhG6Pase-DraIII 3′-fragment. The 3′-primer for G6Pase-(5–357)-3′FLAG and G6Pase-(14–357)-3′FLAG contained the last coding nucleotides (1150 to 1133) of human G6Pase followed by the 24-base pair FLAG coding sequence and a termination codon. After polymerase chain reaction, the amplified fragment was ligated into the pSVL vector (Pharmacia Biotech Inc.).

The two outside polymerase chain reaction primers for codon 176 (nucleotides 605–607) mutants are nucleotides 77–96 (sense) and nucleotides 1130–1149 (antisense) of human G6Pase (9). Codon 176 (CAT) mutant primers (nucleotides 588–616) are as follows: H176A, GCC; H176T, ATT; H176K, AAG; H176M, ATG; H176N, AAT; H176S, TCT; and H176R, CGT. The amplified fragments were ligated into the pSVL vector. All constructs were verified by DNA sequencing.

Expression in COS-1 Cells and Northern Blot and Western Blot Hybridization Analyses—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 G6Pase construct in a pSVL vector was transfected into COS-1 cells by the DEAE-dextran/chloroquine method (21). 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 either harvested for G6Pase assays and Western blot analysis or lysed for RNA isolation.

RNA was isolated by the guanidinium thiocyanate/CsCl method (22), separated by electrophoresis on 1.2% agarose gels containing 2.2 mM formaldehyde, and transferred to Nytran membranes (Schleicher & Schuell). The filters were hybridized at 42 °C in the presence of the pH6G6Pase-1 probe as described previously (9).

For Western blot analysis of tagged G6Pase, microsomal proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel and blotted onto polyvinylidine fluoride membranes (Millipore Corp., Bedford, MA). The filters were incubated with a monoclonal antibody against the FLAG epitope (Kodak Scientific Imaging Systems). The immunocomplexes were then incubated with a secondary antibody conjugated to alkaline phosphatase and visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Kirkgaard & Perry Laboratories, Inc., Gaithersburg, MD).

Phosphohydrolase Assay—Microsomal preparations and phosphohydrolase assays in intact or disrupted microsomes were performed essentially as described (8). Appropriate amounts of microsomal proteins were incubated at 30 °C for 10 min in reaction mixtures (100 μl) containing 50 mM sodium cacodylate buffer, pH 6.5, 10 mM Glu-6-P, and 2 mM EDTA. 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. Disrupted microsomal membranes were prepared by incubating intact membranes in 0.2% deoxycholate for 20 min at 0 °C. Nonspecific phosphatase activity in microsomes was estimated by preincubating microsomal preparations at pH 5 for 10 min at 37 °C, a condition that inactivates the thermolabile G6Pase (23). The latency or intactness of microsomal preparations was assessed by comparing mannose-6-phosphohydrolase activities in intact and detergent-disrupted microsomes (1). Liver microsomes with latency values of 93–95% were used in this study.

Protease Protection Assays—Mouse liver microsomes or cell homogenates from G6Pase-WT, G6Pase-5′FLAG, or G6Pase-3′FLAG-transfected COS-1 cells were used for protease protection assays. Microsomes or cell homogenates were treated with trypsin (Type XII, 500 μg/mg of protein) or proteinase K (50 μg/mg of protein) for 30 min at room temperature. Phenylmethylsulfonyl fluoride (final concentration of 5 mM) was then added to inactivate proteinase K, and phenylmethylsulfonyl fluoride and trypsin inhibitor (final concentration of 6 mg/mg of protein) were added to inactivate trypsin. The reaction mixtures were diluted 100-fold to 10 ml with cold buffer A (0.25 M sucrose and 5 mM HEPES, pH 7.4) and centrifuged at 100,000 × g for 1 h at 4 °C. The microsomal pellets were resuspended in buffer A and used for either hydrophosphatase assays or Western blot analysis. Microsomes or cell homogenates treated first with 0.5% deoxycholate and then with trypsin or proteinase K were used as controls.

RESULTS

Characterization of N-terminal Deleted and/or Tagged G6Pase Mutants—G6Pase is tightly associated with the ER membrane (1). Therefore, protease protection assays using N- and C-terminal tagged G6Pase constructs should allow us to assess whether G6Pase possesses an even or odd number of transmembrane segments as well as the location of its N and C termini with respect to the ER lumen. We have previously shown that the eight C-terminal residues of human G6Pase are not required for activity (14), suggesting that a small C-terminal tag should not markedly disturb G6Pase activity. To investigate whether N-terminal perturbations affect G6Pase, we examined the effects of N-terminal deletions on enzymatic activity. While deletion of residues 1–13 (G6Pase-(14–357)) abolished G6Pase activity, deletion of residues 1–4 yielded mutant G6Pase (G6Pase-(5–357)) retaining ~60% of WT enzymatic activity (Fig. 1), suggesting that a small N-terminal tag should also not greatly disturb G6Pase activity.

| G6Pase mRNA | Phosphohydrolase Activity | mmol/min |
|-------------|--------------------------|----------|
| Mock        |                          | 12.0 ± 0.5 |
| G6Pase-WT   |                          | 164.4 ± 12.7 (100%) |
| G6Pase-(5/357) |                        | 102.8 ± 8.1 (95.9%) |
| G6Pase-(14/357) |                     | 13.4 ± 1.8 |
| G6Pase-5′FLAG |                        | 119.6 ± 9.1 (70.6%) |
| G6Pase-3′FLAG |                        | 122.6 ± 11.2 (72.6%) |
| G6Pase-(5/357)-3′FLAG |                | 106.7 ± 9.5 (62.1%) |
| G6Pase-(14/357)-3′FLAG |             | 11.2 ± 1.0 |

FIG. 1. Analysis of G6Pase mRNA expression and phosphohydrolase activity after transient expression of WT and mutant G6Pase cDNA constructs in COS-1 cells. Total RNA was separated by formaldehyde-agarose gel electrophoresis, blotted onto a Nytran membrane, and hybridized to the phG6Pase-1 probe labeled by random priming. G6Pase activity was determined in deoxycholate (0.2%)-disrupted microsomes as described under “Materials and Methods” using two independent isolates of each construct in three separate transfections. Data are presented as the mean ± SD. Numbers in parentheses represent percent of WT enzymatic activity.
To tag G6Pase, we used the eight-amino acid FLAG marker peptide DYKDDDDK, which is small and hydrophilic and should offer minimal disruption to the native protein configuration of the enzyme. As expected, the N-terminal (G6Pase-FLAG) and C-terminal (G6Pase-3FLAG) tagged G6Pase constructs retained >70% of WT enzymatic activity when analyzed after transient transfection in COS-1 cells (Fig. 1). Moreover, the G6Pase-(5–357)-3FLAG construct also retained >60% of WT activity. Northern blot analysis of G6Pase transcripts showed that WT and N-terminal deleted and tagged G6Pase constructs directed the expression of similar levels of G6Pase mRNA in transfected cells (Fig. 1). Our data show that G6Pase mRNA expression was not affected by the addition of N- or C-terminal FLAG or by the deletion of up to 14 N-terminal residues. Therefore, the decrease in enzymatic activity of the tagged or G6Pase-(5–357) constructs and the loss of activity of the G6Pase-(14–357) and G6Pase-(14–357)-3FLAG constructs were not due to a decrease in transcription efficiency.

Transmembrane Topology of G6Pase—The nine-transmembrane helical model (19, 20) predicts that the N and C termini of G6Pase would be situated at the opposite sides of the ER membrane. On the other hand, the six-transmembrane helical model (10, 14) predicts that both the N and C termini of G6Pase would be situated at the same side of the ER membrane. Therefore, the FLAG tag at the luminal N or C terminus should be sensitive to proteolysis, whereas the FLAG tag at the cytoplasmic N or C terminus should be sensitive to protease digestion. Intact microsomes, isolated from G6Pase-5FLAG- and G6Pase-3FLAG-transfected COS-1 cells, were subjected to digestion by protease K or trypsin, in the absence or presence of deoxycholate. Proteinase K is a broad spectrum protease exhibiting no pronounced cleavage specificity (24), whereas trypsin cleaves peptide bonds between Lys or Arg and an unspecific amino acid (25). The presence of proteases, G6Pase activity in intact microsomes is also resistant to limited proteolysis, we examined microsomal G6Pase activity from mock-transfected cells has been subtracted from the activity in microsomes from G6Pase-WT-, G6Pase-5FLAG-, and G6Pase-3FLAG-transfected cells.

**FIG. 2.** Sensitivity of N and C termini of human G6Pase to digestion by protease K or trypsin. Intact microsomes, isolated from G6Pase-5FLAG- and G6Pase-3FLAG-transfected COS-1 cells, were subjected to digestion by protease K (50 μg/mg of microsomal protein) or trypsin (500 μg/mg of microsomal protein) for 30 min at room temperature as described under “Materials and Methods.” Mock-transfected cells were used as controls. The presence or absence of the FLAG epitope was analyzed by Western blot hybridization and visualized by an anti-FLAG monoclonal antibody. The G6Pase-FLAG proteins are indicated by arrows.

**TABLE I**

| Phosphohydrolase activity | Intact microsomes | Detergent microsomes | Microsomes disrupted after proteolysis | Microsomes disrupted before proteolysis |
|---------------------------|------------------|---------------------|---------------------------------------|----------------------------------------|
| Mouse liver               |                  |                     |                                       |                                        |
| None                      | 100.0 ± 2.5      | 160.6 ± 7.0         |                                       |                                        |
| Proteinase K, G6Pase-WT   | 82.0 ± 3.2       | 168.2 ± 3.3         | 3.3 ± 0.5                             |                                        |
| Trypsin                   | 79.4 ± 2.6       | 136.4 ± 1.5         | 44.5 ± 0.8                            |                                        |
| Proteinase K, G6Pase-5FLAG| 57.0 ± 4.0       | 115.8 ± 1.4         | 1.1 ± 0.1                             |                                        |
| Trypsin                   | 71.0 ± 6.0       | 167.2 ± 4.9         | 17.7 ± 3.5                            |                                        |
| Proteinase K, G6Pase-3FLAG| 82.9 ± 8.5       | 143.8 ± 2.3         |                                       |                                        |
| Trypsin                   | 59.1 ± 5.1       | 122.6 ± 1.4         | 11.2 ± 2.5                            |                                        |
| Trypsin                   | 74.1 ± 3.5       | 155.2 ± 5.2         | 52.4 ± 3.0                            |                                        |
| Proteinase K, G6Pase-WT   | 69.1 ± 0.6       | 126.5 ± 7.1         |                                       |                                        |
| Trypsin                   | 41.5 ± 0.3       | 84.3 ± 3.5          | 4.5 ± 0.6                             |                                        |
| Trypsin                   | 53.5 ± 4.5       | 132.5 ± 2.0         | 23.9 ± 3.3                            |                                        |

G6Pase, we used a trypsin concentration (500 μg/mg of microsomal protein) that was at least 50-fold higher than previously used for intact liver microsomes (4–7). We therefore examined the effects of proteinase K and of a higher concentration of trypsin on hepatic microsomal G6Pase activity in the absence or presence of deoxycholate (Table I). In the absence of proteases, G6Pase activity in intact hepatic microsomes was lower than the activity in detergent-permeabilized microsomes (Table I), a characteristic of mammalian microsomal G6Pase (1). Both proteinase K and trypsin moderately inhibited G6Pase activity in intact liver microsomes. However, enzymatic activity increased when detergent was added to intact microsomes after prior inactivation of either protease (Table I). On the other hand, both proteases markedly inhibited G6Pase activity in detergent-disrupted microsomes.

To demonstrate that in vitro expressed WT or tagged G6Pases in intact microsomes are also resistant to limited proteolysis, we examined microsomal G6Pase activity from G6Pase-WT-, G6Pase-5FLAG-, and G6Pase-3FLAG-transfected COS-1 cells after digestion by proteinase K or trypsin in the absence or presence of deoxycholate (Table I). In the absence of proteases, G6Pase activity in intact microsomes iso-
and not due to a decrease in transfection efficiency. Levels (data not shown). This indicates that the reduction in G6Pase activity from transfected cells showed that WT as well as the various mutants had detectable G6Pase activity. (Table II). Consistent with our hypothesis, none of these codon substitutions with seven different substitutions at codon 176 (Ala (H176A), Ile (H176I), Lys (H176K), Met (H176M), Asn (H176N), Ser (H176S), and Arg (H176R)), and the resulting G6Pase activity was analyzed after transient expression in COS-1 cells. Phosphohydrolase activity was assayed as described under “Materials and Methods” using two independent isolates of each construct in three separate transfections. Data are presented as means ± S.D.

### DISCUSSION

In this study, we have characterized the orientation of human G6Pase in the ER and demonstrated that this enzyme contains an odd number of transmembrane helices, with the N terminus localized in the ER lumen and the C terminus in the cytoplasm. Our data best support the nine-transmembrane helical model for G6Pase (19, 20), rather than the six-transmembrane helical model (14) previously predicted (10). According to the nine-transmembrane helical topology, the residues predicted to compose the active center in G6Pase, Arg-83, His-119, and His-176, are all situated on the luminal side of the ER membrane (Fig. 3). Our study demonstrates that G6Pase possesses an odd number of transmembrane helices, supporting the new nine-transmembrane helical model (19, 20). According to this model, Arg-83, His-119, and His-176, which are predicted to contribute to the active site of G6Pase, reside on the same side of the ER membrane (Fig. 3). In an earlier study, we have shown that Arg-83 is involved in positioning the phosphoryl moiety and that His-119 is absolutely required for G6Pase activity (14). The acidic region of vanadium-containing chloroperoxidase and mammalian G6Pases suggests that Arg-83 in G6Pase is a candidate for positioning the phosphate, His-119 is a proposed acid-base group in catalysis, and His-176 is the residue that covalently binds the phosphoryl moiety (17, 19). If His-176 is indeed the phosphoryl acceptor, then this residue should be essential for G6Pase activity. Therefore, we generated mutant G6Pase constructs with seven different substitutions at codon 176 (Ala (H176A), Ile (H176I), Lys (H176K), Met (H176M), Asn (H176N), Ser (H176S), and Arg (H176R)), and the resulting G6Pase activity was analyzed after transient expression in COS-1 cells (Table II). Consistent with our hypothesis, none of these codon 176 mutants had detectable G6Pase activity.

Northern blot hybridization analysis of G6Pase transcripts from transfected cells showed that WT as well as the various codon 176 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.

### FIG. 3. The predicted nine-transmembrane helical structure of human G6Pase. The amino acids predicted to contribute to the active site of G6Pase are shaded. The locations of 16 missense mutations and one codon deletion mutation identified in the G6Pase gene of GSD-1a patients are shown in black. Arg-83 is one of the residues that contributes to the active center and is mutated in some GSD-1a patients. Transmembrane helices were identified by the algorithm of Hoffman and Stoffel (20).

| Constructs | Phosphohydrolase activity (nmol/min/mg) |
|------------|----------------------------------------|
| Mock       | 13.1 ± 0.1                             |
| G6Pase-WT  | 121.2 ± 9.8                            |
| H176A      | 11.3 ± 1.0                             |
| H176I      | 12.0 ± 1.2                             |
| H176K      | 11.5 ± 0.5                             |
| H176M      | 12.3 ± 0.6                             |
| H176N      | 13.0 ± 0.6                             |
| H176S      | 13.8 ± 2.0                             |
| H176R      | 12.2 ± 0.71                            |

## Membrane Topology of G6Pase

*Phosphohydrolase activity of codon 176 mutant constructs*

Phosphohydrolase activity was assayed as described under “Materials and Methods” using two independent isolates of each construct in three separate transfections. Data are presented as means ± S.D. The predicted nine-transmembrane helical structure of human G6Pase is shown in Fig. 3. The amino acids predicted to contribute to the active site of G6Pase are shaded. The locations of 16 missense mutations and one codon deletion mutation identified in the G6Pase gene of GSD-1a patients are shown in black. Arg-83 is one of the residues that contributes to the active center and is mutated in some GSD-1a patients. Transmembrane helices were identified by the algorithm of Hoffman and Stoffel (20).
will be the focus of future studies. Alignment of the active sites of vanadium-containing chloroperoxidase and mammalian G6Pases suggests that, in addition to Arg-83, Lys-76 and Arg-170 in human G6Pase also participate in positioning the phosphoryl moiety, and Ser-117 and Gly-118 may participate in hydrogen bonding (Fig. 3). It will be of interest to study the roles of these residues during G6Pase catalysis.

Microsomes are closed vesicles with a defined cytoplasmic side orientation (26). Therefore, protein domains exposed on the outside of the ER can be selectively digested by proteases. G6Pase activity in intact liver microsomes is resistant to limited proteolysis, suggesting that the active site is not on the cytoplasmic side of the ER membrane (4–7). In this study, we demonstrate that G6Pase activity in intact microsomes isolated from mouse liver as well as from G6Pase-WT-, G6Pase-5FLAG-, and G6Pase-3FLAG-transfected COS-1 cells is resistant to limited proteolysis. Therefore, the orientation of the in vitro expressed G6Pase in the ER is similar to that of liver microsomal G6Pase. To date, at least 29 mutations have been identified in the G6Pase gene of GSD-1a patients (9, 27–33). Seventeen amino acids in human G6Pase were altered by the known missense and codon deletion mutations. Thirteen missense mutations and the codon deletion mutation (ΔF327) uncovered in this laboratory were shown to abolish or greatly reduce G6Pase activity in transient expression assays (9, 27, 28, 33). The 17 amino acids mutated in the G6Pase gene of GSD-1a patients are illustrated in Fig. 3. According to the nine-transmembrane helical topology of G6Pase, the four loops facing the cytoplasm are relatively short, varying from 8 to 12 residues. The two large loops, which are situated between helices 2 and 3 (37 residues, loop 1L) and helices 6 and 7 (33 residues, loop 3L), are located on the luminal side of the ER. Among the 17 amino acids mutated in GSD-1a patients, 14 are situated in transmembrane helices 1–9, two are located in loop 1L, and one in loop 3L (Fig. 3). No missense or codon deletion mutations have yet been identified in the four cytoplasmic loops or N- and C-terminal domains of human G6Pase. Therefore, G6Pase activity depends on the structural integrity of the transmembrane helices, and residues in the two large luminal loops also play crucial roles. It is tempting to suggest that during catalysis, the structural requirement of the cytoplasmic loops and N- and C-terminal domains are less stringent than the other features of human G6Pase.

It is worth noting that the apparent molecular mass of the G6Pase protein in intact microsomes was not noticeably altered after digestion by either proteinase K or trypsin. This was demonstrated by Western blot analysis of the G6Pase-5FLAG protein before and after proteolysis. This is expected since trypsin is predicted to cleave off a small peptide (KSL, amino acids 355–357 of human G6Pase) of 0.3 kDa, which would not substantially reduce the apparent molecular mass of the G6Pase protein. Additionally, G6Pase activity increased to the levels of the permeabilized control microsomes when detergent was added to intact microsomes, after inactivation of either protease. Our data indicate that only a small number of C-terminal residues were removed by either proteinase K or trypsin and that the tertiary structure of G6Pase remained intact after proteolysis. It appears that the short cytoplasmic loops are not readily accessible to proteolysis, suggesting that they are closely associated with the ER membranes.

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