Asparagine-linked Oligosaccharides Are Localized to a Luminal Hydrophilic Loop in Human Glucose-6-Phosphatase

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Deficiency of glucose-6-phosphatase (G6Pase), an endoplasmic reticulum transmembrane glycoprotein, causes glycogen storage disease type 1a. We have recently shown that human G6Pase contains an odd number of transmembrane segments, supporting a nine-transmembrane helical model for this enzyme. Sequence analysis predicts the presence of three potential asparagine (N)-linked glycosylation sites, N96TS, N203AS, and N276SS, conserved among mammalian G6Pases. According to this model, Asn96, located in a 37-residue luminal loop, is a potential acceptor for oligosaccharides, whereas Asn203 and Asn276, located in a 12-residue cytoplasmic loop and helix 7, respectively, would not be utilized for this purpose. We therefore characterized mutant G6Pases lacking one, two, or all three potential N-linked glycosylation sites. Western blot and in vitro translation studies showed that G6Pase is glycosylated only at Asn96, further validating the nine-transmembrane topology model. Substituting Asn96 with an Ala (N96A) moderately reduced enzymatic activity and had no effect on G6Pase synthesis or degradation, suggesting that oligosaccharide chains do not play a major role in protecting the enzyme from proteolytic degradation. In contrast, mutation of Asn276 to an Ala (N276A) destabilized the enzyme and markedly reduced enzymatic activity. We present additional evidence suggesting that the integrity of transmembrane helices is essential for G6Pase stability and catalytic activity.

Glucose-6-phosphatase (G6Pase, EC 3.1.3.9), which catalyzes the terminal step in gluconeogenesis and glycogenolysis, 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 disease (2). It is an autosomal recessive disorder with clinical manifestations of hypoglycemia, growth retardation, hepatomegaly, kidney enlargement, lactic acidemia, hyperlipidemia, and hyperuricemia (2, 3). G6Pase is a transmembrane protein tightly associated with the endoplasmic reticulum (ER) (1). Using N- or C-terminal-tagged G6Pase constructs, we have shown that human G6Pase contains an odd number of transmembrane helices with the N terminus localized in the ER lumen and the C terminus in the cytoplasm (4). Our data support a nine-transmembrane helical model previously proposed for the enzyme based on hydropathy analysis (5).

G6Pase is a glycoprotein (6–8) and three potential asparagine (N)-linked glycosylation sites at N96TS, N203AS, and N276SS are predicted from sequence analysis (7–10). All three sites are conserved in human (7), mouse (8), rat (9), and canine (10) G6Pases. Thus, G6Pase belongs to a class of multispans membrane glycoproteins that are N-glycosylated. Although the presence of a consensus glycosylation site within the amino acid sequence of a membrane protein is suggestive of a glycoprotein, not all consensus sites are necessarily utilized. A survey of mammalian multispans membrane proteins has suggested that, for a potential N-linked glycosylation site to be utilized, it must be situated on the luminal side of the ER, and the size of the hydrophilic loop must be at least 33 amino acids in length (11, 12). Analysis of protein glycosylation thus provides useful topological information for membrane proteins.

The nine-transmembrane topology model predicts that human G6Pase would contain four short (8–12 residues) cytoplasmic loops, and two short (7 and 8 residues) and two large (33 and 37 residues) luminal loops (4). According to this model, N96TS would be situated in a 37-residue luminal loop, thus it is the only glycosylation site in human G6Pase that satisfies the criteria as an acceptor for oligosaccharides. In the present study, we constructed mutant G6Pases lacking one, two, or all three potential N-linked glycosylation sites and examined G6Pase synthesis directed by wild-type (WT) or mutant G6Pase in transient expression, Western blot, and in vitro translation studies. Our data demonstrate that G6Pase is glycosylated only at Asn96, further validating the nine-transmembrane helical model. We also show that oligosaccharide side chains do not play a major role in protecting the enzyme from proteolytic degradation. Furthermore, the structural integrity of transmembrane segments in human G6Pase is essential for enzyme activity and stability.

MATERIALS AND METHODS
Construction of G6Pase Mutants—Human G6Pase (7) or G6Pase-DraIII (13) cDNAs were used as templates for mutant construction by polymerase chain reaction. The eight-amino acid FLAG marker peptide, DYKDDDDK (Scientific Imaging Systems, Eastman Kodak Co.) was used to tag the C terminus of G6Pase as described previously (4). The two outside polymerase chain reaction primers for the N96A mutant are nucleotides 77–96 (G1, sense) of human G6Pase (7) and nucleotides 625–602 of G6Pase-DraIII (12, antisense (13), for the N203A mutant are nucleotides 611–634 (I1, sense) of G6Pase-DraIII and nucleotides 1150–1133 of human G6Pase (G2, antisense), and for the N276A mutant are I1 and G2. Codon 96 (AAC) mutant primers (nucleotides 356–376) contain GCC (Ala) at position 96, codon 203 (AAT) mutant primers (nucleotides 677–697) contain GCT (Ala) at position 203, and codon 276 (AAC) mutant primers (nucleotides 896–916) contain GCC (Ala) at position 276. After the polymerase chain reaction, the amplified fragment was ligated either into the pSVLhG6Pase-DraIII-5 fragment (N203A and N276A) or the pSVLhG6Pase-DraIII-5 fragment (N96A) as a template. The primers for N203A-3’ FLAG and N276A-3’ FLAG mutants are I1 and nucleotides (1150–1133) of human G6Pase, followed by the 24-base pair FLAG coding sequence and a termination codon (G2-3’ FLAG, antisense) using N203A or N276A, respectively, as a template. After the
polymerase chain reaction, the amplified fragment was ligated into the pSVLhG6Pase-DraIII-5' fragment. N96A-3'FLAG, D38V-3'FLAG, and P178S-3'FLAG were constructed by substituting the pSVLhG6Pase-DraIII-3' fragment in N96A, D38V (14), or P178S (15) construct with the G6Pase-WT-3'FLAG-DraIII-3' fragment (4). All constructs were verified by DNA sequencing.

Expression in COS-1 Cells and Northern 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 (16). Mock transfections of COS-1 cells with the pSVL vector alone were used as controls. After incubation at 37 °C for 2–3 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 (17), separated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde, and transferred to Nytran membranes (Schleicher & Schuell). The filters were hybridized at 42 °C in the presence of the phG6Pase-1 (7) or β-actin probe as described previously (4).

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

Phosphohydrolase Assay—Phosphohydrolase activity was determined essentially as described by Burchell et al. (18). Reaction mixtures (100 μl) contained 50 mM cacodylate buffer, pH 6.5, 10 mM glucose-6-P, 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. 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 (19).

In Vitro Transcription and Translation—In vitro transcription-translation of a WT or mutant cDNA construct, in a pGEM-11Zf(+) vector (Promega Biotech), was performed using the TNT coupled reticulocyte lysate system obtained from Promega Biotech. [35S]L-Methionine was used as the labeled precursor. The in vitro synthesized proteins were analyzed by SDS-polyacrylamide gel electrophoresis, 10% gel, and fluorography.

RESULTS

Characterization of G6Pase Mutants Lacking One or More Glycosylation Sites—We have recently shown that human G6Pase contains an odd number of transmembrane domains with the N terminus localized in the ER lumen and the C terminus in the cytoplasm (4). Our data support a nine-transmembrane topology for G6Pase (5), which places the three potential N-linked glycosylation sites in a 37-residue luminal loop (N96TS), a 12-residue cytoplasmic loop (N203AS), and helix 7 (N276SS) (Fig. 1). According to this model, only Asn96, which satisfies the criteria as an acceptor for oligosaccharides (11, 12), would be utilized. To further evaluate the nine-transmembrane model and to determine the extent of N-linked glycosylation in human G6Pase, we constructed a series of mutants that disrupted one (N96A, N203A, and N276A), two (N96A/N203A, N96A/N276A, and N203A/N276A), or all three (N96A/N203A/N276A) consensus glycosylation sites in human G6Pase. Phosphohydrolase activity was examined in whole homogenates after transient transfection of WT or mutant G6Pase cDNA constructs into COS-1 cells.

Phosphohydrolase activities of N96A and N203A mutants,
TABLE I

Phosphohydrolase activity of human G6Pase glycosylation mutants

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 the mean ± SD.

| Constructs | Phosphohydrolase activity | Constructs | Phosphohydrolase activity |
|------------|---------------------------|------------|---------------------------|
| Mock       | 12.3 ± 0.5                | Mock       | 12.3 ± 0.5                |
| WT         | 113.6 ± 10.9 (100)*        | WT-3-FLAG  | 86.5 ± 9.1 (100)          |
| N96A       | 50.2 ± 3.2 (37.4)         | N96A-3-FLAG| 44.8 ± 0.8 (43.8)         |
| N203A      | 49.3 ± 0.2 (36.5)         | N203A-3-FLAG| 47.1 ± 4.3 (46.9)         |
| N276A      | 23.2 ± 1.1 (9.9)          | N276A-3-FLAG| 23.0 ± 1.2 (14.4)         |
| N96A/N203A | 35.2 ± 0.2 (22.6)         | N96A/N203A-3-FLAG| 31.5 ± 3.8 (25.9)         |
| N96A/N276A | 19.4 ± 0.7 (2.4)          | N96A/N276A-3-FLAG| 12.9 ± 1.9 (0.8)          |
| N203A/N276A| 20.1 ± 2.0 (9.7)          | N203A/N276A-3-FLAG| 20.2 ± 2.2 (10.6)         |
| N96A/N203A/N276A| 14.6 ± 0.1 (2.3) | N96A/N203A/N276A-3-FLAG| 12.7 ± 1.4 (0.7)         |

* Numbers in parentheses are percent.

Fig. 2. A, Western blot analysis of G6Pase synthesis after transient expression of 3'FLAG-tagged WT and mutant G6Pase cDNA constructs in COS-1 cells. COS-1 cells were transfected with WT or G6Pase mutant cDNA constructs that disrupt one, two, or all three potential N-linked glycosylation sites. After incubation at 37 °C for 48 h in the absence or presence of tunicamycin (1 μg/ml), the transfected cultures were harvested for Western blot analysis and probed with a monoclonal antibody against the FLAG epitope. Mock transfected cells were used as controls. Each lane contained 20 μg of protein. B, analysis of G6Pase mRNA expression after transient expression of 3'FLAG-tagged WT and mutant G6Pase cDNA constructs in COS-1 cells. Total RNA (10 μg/lane) was separated by formaldehyde-agarose gel electrophoresis, blotted onto a Nitran membrane, and hybridized to the pFLAGG6Pase-1 (7) or β-actin probe labeled by random priming.

which disrupt glycosylation sites 1 and 2, respectively, were approximately 37% of WT enzymatic activity (Table I). The N276A mutant, which disrupts site 3, retained 10% of WT activity (Table I). Double glycosylation mutants, N96A/N203A, N96A/N276A, and N96A/N276A, retained 22.6, 9.7, and 2.4% of WT activity, respectively, and the triple mutant, N96A/N203A/N276A, retained only 2.3% of WT activity. Our data suggest that amino acid at position 276 plays a more critical role in G6Pase catalysis than amino acids at position 96 and 203.

To examine G6Pase synthesis in transfected COS-1 cells, we tagged the G6Pase constructs using the eight-amino acid FLAG marker peptide, DYKDDDDK, which has been successfully used to study the topology of human G6Pase (4). Results in Table I show that phosphohydrolase activity of the tagged G6Pase was comparable to the respective parental construct.

Human G6Pase Is Glycosylated at Asn96—To characterize the glycosylation site(s) utilized in human G6Pase, COS-1 cells were transfected with a FLAG-tagged WT or mutant construct in the absence or presence of a protein glycosylation inhibitor, tunicamycin (28). G6Pase synthesis in transfected cells was analyzed by Western blot using an anti-FLAG monoclonal antibody (Fig. 2A). The WT-FLAG construct supported the synthesis of a 36-kDa polypeptide in the absence of tunicamycin and a 32-kDa polypeptide in the presence of tunicamycin, suggesting that the apparent molecular mass of the nonglycosylated G6Pase migrated as a 32-kDa protein. The synthesis of a low level of the 32-kDa product in the absence of tunicamycin suggests that glycosylation in COS-1 cells may be incomplete. Both the N203A-3'FLAG and N276A-3'FLAG constructs supported the synthesis of polypeptides of 36 and 32 kDa in the absence and presence of tunicamycin, respectively (Fig. 2A), indicating that Asn203 and Asn276 in human G6Pase were not utilized as acceptors for oligosaccharides. On the other hand, the N96A-3'FLAG construct supported the synthesis of a 32-kDa polypeptide, both in the absence and presence of tunicamycin (Fig. 2A). Only 3'FLAG constructs, N96A/N203A, N96A/N276A, and N96A/N203A/N276A, that disrupted the consensus site at position 96, abolished glycosylation in G6Pase. Our data further validate the nine transmembrane consensus site at position 96, abolished glycosylation in G6Pase.

Roles of Transmembrane Helices in G6Pase—Western blot analysis (Fig. 2A) showed that WT-3'FLAG, N96A-3'FLAG and N203A-3'FLAG constructs supported the synthesis of similar levels of G6Pase proteins that were considerably higher than the levels directed by the N276A-3'FLAG mutant. Likewise, markedly reduced levels of G6Pase synthesis were observed...
WT as well as mutant G6Pase indicate the nonglycosylated and glycosylated G6Pase.

Fig. 3. Analysis of G6Pase by in vitro transcription-translation of WT or mutant cDNA constructs using L-[35S]methionine as the labeled precursor. The amount loaded on the polyacrylamide-SDS gel of the in vitro synthesized proteins in the presence of canine microsomal membranes to that in the absence of membranes is 4:1. After electrophoresis, the proteins were visualized by fluorography. Arrows indicate the nonglycosylated and glycosylated G6Pase.

with double or triple glycosylation mutants harboring the N203A mutation (Fig. 2A). Northern blot hybridization analysis of G6Pase transcripts from transfected cells showed that WT as well as mutant G6Pase mRNAs were expressed at similar levels (Fig. 2B). This suggests that the reduction in G6Pase protein levels was not due to a decrease in transfection efficiency, but due to either a decrease in enzyme synthesis or an increase in enzyme degradation. In vitro translation experiments (Fig. 3) showed that N276A and WT mRNA were able to direct the synthesis of similar amounts of G6Pase protein, suggesting that the N276A mutant enzyme is more susceptible to degradation than the WT enzyme.

To investigate whether the structural integrity of transmembrane helices is important to G6Pase stability, we examined the effect of other transmembrane helical mutations on G6Pase synthesis. Asp38, located in helix 1, and Pro178, located in helix 5, were chosen because mutations (D38V and P178S) associated with these two residues have been identified in the G6Pase gene of GSD-1a patients and were shown to abolish G6Pase activity (14, 15). COS-1 cells were transfected with D38V-3FLAG or P178S-3FLAG constructs and G6Pase synthesis examined by Western blot analysis. COS-1 cells transfected with WT-3FLAG, N96A-3FLAG, N203A-3FLAG, or N276A-3FLAG construct were used as controls. D38V-3FLAG and P178S-3FLAG, like the WT construct, supported the synthesis of two polypeptides of 36 (major) and 32 kDa (minor), but at significantly lower levels than that of the WT construct (Fig. 4). Our data suggest that mutations that alter the structural integrity of transmembrane helices in G6Pase destabilize the enzyme. The total absence of enzymatic activity of the D38V and P178S mutants (14, 15) raises the possibility that the low levels of mutant protein synthesized may not fold properly to perform catalytic functions.

DISCUSSION

A survey of mammalian multispan membrane proteins showed that N-linked oligosaccharides are localized to single extracytosolic segments that are at least 33 residues in length (11, 12). Thus, N-linked glycosylation is a useful topological marker for membrane proteins. In earlier studies, we showed that human G6Pase contains an odd number of transmembrane helices with the N terminus localized in the ER lumen and the C terminus in the cytoplasm (4). Our data support a nine-transmembrane helical model proposed for G6Pase based on hydrophathy analysis (5) which predicts that the three consensus glycosylation sites in human G6Pase would be located in a 37-residue luminal loop (N96TS), a 12-residue cytoplasmic loop (N203AS), and helix 7 (N276SS) (Fig. 1). In this study, we determined which one of the glycosylation sites in human G6Pase is being utilized in order to put the topology of human G6Pase on a firmer experimental basis. We show that only N96TS is an acceptor for oligosaccharide chains, further validating the nine-transmembrane model for G6Pase.

The glycan moieties of glycoproteins have been shown to play important roles, including the direction of protein folding, regulation of cell-surface expression, maintenance of polypeptide conformation and solubility, and increase in the half-life of proteins (21, 22). The N96A mutant directed the synthesis of a 32-kDa nonglycosylated G6Pase, whereas the WT construct supported the synthesis of a glycoprotein of 36 kDa. However, Western blot analysis showed that similar levels of N96A and WT G6Pase proteins were produced, suggesting that the oligosaccharide chains in G6Pase do not affect the half-life of G6Pase.

At present, over 240 G6Pase alleles have been characterized in GSD-1a patients (reviewed in Ref. 4). It is interesting to note that no missense or codon deletion mutations have been identified in any of the four cytoplasmic loops in human G6Pase. Asn203 is located in a cytoplasmic loop and mutation (N203A) of this residue has no adverse effects on protein synthesis, as similar amount of G6Pase were synthesized by WT or the N203A construct. Moreover, the N203A mutant retained 37% of WT activity, whereas all mutations uncovered in the G6Pase gene of GSD-1a patients abolish or markedly reduce (retaining 18% or less of WT activity) G6Pase activity (7, 14, 15, 23, 24). This suggests that the structural requirement of the cytoplasmic loops is less stringent than other features of human G6Pase.

Eighteen amino acids were shown to be altered by missense and codon deletion mutations uncovered in the G6Pase gene of GSD-1a patients (Fig. 1). Twelve missense mutations and the codon deletion mutation (ΔF327) were further examined for G6Pase activity in transient expression assays (7, 14, 15, 23, 24). These include R83C and R83H, which altered Arg83, an amino acid contributing to the active center of the enzyme (4); D38V, V166G, P178S, G222R, G270V, R295C, ΔF327, and L345R, which are situated in the transmembrane helices 1–9 and E110Q and W236R, which are located in the two large luminal loops. It is not unexpected that R83C and R83H mutations, which account for 40% of all G6Pase mutations identified, abolish G6Pase activity. Whereas the G222R mutation, located in helix 6, greatly reduces G6Pase activity (4% of WT activity), the other eight transmembrane helical mutations, D38V, V166G, P178S, G188S, G270V, R295C, ΔF327, and
L345R, totally abolish enzymatic activity. The two luminal loop mutations, E110Q and W236R, retained 18.2 and 4.2% WT enzymatic activity, respectively. Our data suggest that the structural integrity of transmembrane helices in G6Pase is vital for catalysis.

We demonstrate that mutations of amino acids comprising the transmembrane helices, including N276A, D38V, and P178S, destabilize the enzyme. Significantly lower amounts of N276A, D38V, or P178S mutant proteins were synthesized compared with the WT construct. Furthermore, the total loss of enzymatic activity seen in D38V and P178S mutants (14, 15) suggests that the low levels of mutant enzymes synthesized cannot even function as a phosphohydrolase. It is possible that the structural requirement of the transmembrane helices is so rigid that the mutant enzymes that survive proteolytic degradation cannot fold properly for catalysis.

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