Transmembrane Topology of Human Glucose 6-Phosphate Transporter*

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Glycogen storage disease type 1b is caused by a deficiency in a glucose 6-phosphate transporter (G6PT) that translocates glucose 6-phosphate from the cytoplasm to the endoplasmic reticulum lumen where the active site of glucose 6-phosphatase is situated. Using amino- and carboxyl-terminal tagged G6PT, we demonstrate that proteolytic digestion of intact microsomes resulted in the cleavage of both tags, indicating that both termini of G6PT face the cytoplasm. This is consistent with ten and twelve transmembrane domain models for G6PT predicted by hydropathy analyses. A region of G6PT corresponding to amino acid residues 50–71, which constitute a transmembrane segment in the twelve-domain model, are situated in a 51-residue luminal loop in the ten-domain model. To determine which of these two models is correct, we generated two G6PT mutants, T53N and S55N, that created a potential Asn-linked glycosylation site at residues 53–55 (N\(^{\text{S53S}}\)) or 55–57 (N\(^{\text{S55Q}}\)), respectively. N\(^{\text{S53S}}\) or N\(^{\text{S55Q}}\) would be glycosylated only if it is situated in a luminal loop larger than 33 residues as predicted by the ten-domain model. Whereas wild-type G6PT is not a glycoprotein, both T53N and S55N mutants are glycosylated, strongly supporting the ten-helical model for G6PT.

Glycogen storage disease type 1 (GSD-1),\(^*\) also known as von Gierke disease, is caused by a deficiency in microsomal glucose 6-phosphatase (G6Pase) activity (1). The disease presents with both clinical and biochemical heterogeneity consistent with the existence of two major subgroups, GSD-1a and GSD-1b. GSD-1a, the most prevalent form of GSD-1 (1), is caused by mutations in the G6Pase gene that abolish or greatly reduce G6Pase activity (2). The active site of G6Pase is situated inside the lumen of the endoplasmic reticulum (ER) (3). Therefore, the glucose 6-phosphate (G6P) substrate, which is present in the cytoplasm, must be translocated across the ER membrane to be converted to glucose and phosphate by G6Pase, a key enzyme in glycogen homeostasis (4). This led Arion et al. (5, 6) to postulate that hydrolysis of G6P requires the participation of at least two membrane proteins, a G6P transporter (G6PT) and a G6Pase catalytic unit. Based on this concept, Narisawa et al. (7) and Lange et al. (8) proposed that GSD-1b is caused by a defect in the microsomal G6P transport system. Both GSD-1a and GSD-1b are characterized by hypoglycemia, hepatomegaly, kidney enlargement, growth retardation, lactic acidemia, hyperlipidemia, and hyperuricemia, consequences of a functional G6Pase deficiency (1). Additionally, GSD-1b patients also manifest infectious complications because of a heritable neutropenia and functional deficiencies of neutrophils and monocytes (9, 10).

Recently, cDNA encoding human (11), mouse (12), and rat (12) G6PT have been isolated and characterized. Mammalian G6PTs are membrane-associated proteins of 429 amino acids, each of which contains an ER transmembrane protein retention signal at its carboxyl (COOH) terminus (11, 12). The human G6PT gene consists of 9 exons (13, 14), spans approximately 5.3 kilobases (14), and maps to chromosome 11q23 (15–17). Mutations in the G6PT gene that segregated with the GSD-1b disorder have been identified in over forty GSD-1b families (11, 13, 14, 16, 17). More recently, we have developed a functional assay for the recombinant G6PT protein and shown that mutations uncovered in the G6PT gene of GSD-1b patients abolish G6P transport activity (14), thus establishing the molecular basis of the GSD-1b disorder.

Hydropathy profile analysis of the G6PT amino acid sequence predicts that this transporter is anchored in the ER membrane by either ten (18) or twelve (11, 19) putative transmembrane helices. The major difference between the two G6PT models is that amino acid residues 50–71, which constitute the transmembrane segment-2 in the twelve-domain model (11), are part of a 51-residue loop in the ten-domain model. A survey of mammalian multi-span membrane proteins has revealed that for a potential Asn-linked glycosylation site to be used, it must be situated inside the ER lumen, and the size of the hydrophilic loop must be at least 33-amino acids in length (20, 21). Therefore, protein glycosylation can provide useful topological information for membrane proteins.

In this report, we have analyzed the orientation of human G6PT in the ER membrane. We used the 8 amino acid FLAG marker peptide to tag the amino (NH\(_2\)) or COOH termini of G6PT and inserted two factor Xa protease cleavage sites (22) between G6PT and its terminal tag, which allows the in situ removal of the tag without affecting the G6PT protein. Our results show that human G6PT contains an even number of transmembrane helices with both NH\(_2\) and COOH termini facing the cytoplasm. To distinguish between the ten- and twelve-domain models of G6PT, we constructed two G6PT mutants, T53N and S55N, which create potential Asn-linked glycosylation sites at residues 53–55 and 55–57, respectively, and examined G6PT synthesis by transient expression and in vitro translation studies. Our data show that although the wild-type (WT) G6PT protein is nonglycosylated, the newly introduced glycosylation sites at either residues 53–55 (NSS) or 55–57 (NQS) are used, confirming the ten transmembrane helical model of G6PT.
In Vitro Transcription and Translation—In vitro transcription-translation of G6PT cDNA constructs, in a pGEM-7Zf(+) vector, was performed using the transcription and translation-coupled reticulocyte lysate system obtained from Promega Biotech (Madison, WI). t-[35S]methionine was used as the labeled precursor. The in vitro synthesized proteins were analyzed by 10% polyacrylamide-SDS gel electrophoresis and Coomassie blue staining.

Generation of Mutant G6PT Constructs—Nucleotides 166–1486 of the human G6PT cDNA (11), which contains the entire coding region (nucleotides 170–1459), were used as a template for mutant construction by polymerase chain reaction. The 8-amino acid FLAG marker peptide, DYKDDDDK (Scientific Imaging Systems, Eastman Kodak, CT) was used to tag the NH2 and COOH termini of G6PT. The 5'-primer for the NH2-terminal FLAG G6PT (G6PT-5'-FLAG) contained the last coding nucleotides (1439–1459) of human G6PT, the 3'-primer for the COOH-terminal FLAG G6PT (G6PT-3'-FLAG) contained nucleotides 166–187 of human G6PT; the 3'-primer contained the last coding nucleotides (1439–1459) of human G6PT, followed by the 24-bp FLAG coding sequence and a termination codon. The amplified fragments were ligated into the pSVL vector (Amersham Pharmacia Biotech).

The G6PT-FLAG plasmids with two factor Xa tetrapeptide recognition motifs (EIGGR) (22) located between the G6PT coding region and the terminal FLAG tag were also constructed by polymerase chain reaction. The 5'-primer for the NH2-terminal FLAG-G6PT (G6PT-5’-FLAG) contained an ATG initiation codon followed by the 24-bp FLAG coding sequence, two Xa recognition sequence (ATCGAGGGTA-5’), and nucleotides 170–190 of human G6PT; the 3'-primer contained nucleotides 1439–1459 of human G6PT. The 5'-primer for the COOH-terminal FLAG-G6PT (G6PT-3’-FLAG) contained nucleotides 166–187 of human G6PT; the 3'-primer contained the last coding nucleotides (1439–1459) of human G6PT, followed by the 24-bp FLAG coding sequence and a termination codon. The amplified fragments were ligated into the pSVL vector.

The two outside polymerase chain reaction primers for codon 53 (nucleotides 326–328) and codon 55 (nucleotides 332–334) mutants are nucleotides 164–187 (sense) and nucleotides 851–878 (antisense) of human G6PT. The antisense primer contains a BstEII site, located at nucleotides 863–869 in human G6PT (11). Codon 53 (ACC, Thr) mutant primers (nucleotides 317–337) are (AAC, Asn) or (CAG, Ser) and codon 55 (AGC, Ser) mutant primers (nucleotides 323–343) are (AAG, Arg); mutant bases are denoted in boldface letters. The amplified fragments were ligated into the pGEM3ZfG6PT-BstEII-3’ or pSVL/G6PT-3’FLAG-BstEII-3’ fragment. All constructs were verified by DNA sequencing.

Expression in COS-1 Cells and Western Blot 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 G6PT construct in a pSVL vector was transfected into COS-1 cells by the DEAE-dextran/chloroquine method (23) in each transfection to a final concentration of 60 μg plasmid DNA per g). After incubation at 37° C for 16–18 h, the microsomal pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.4, and 250 mM sucrose). Microsomes permeabilized with 0.2% deoxycholate, which abolished G6P uptake, were used as negative controls. Two to three independent experiments were conducted, and at least two G6P uptake studies were performed for each microsomal preparation. Data are presented as the mean ± S.E.

Proteinase Protection Assays—G6PT-5’/XaFLAG-, G6PT-3’/XaFLAG-, G6PT-5’-FLAG-, or G6PT-3’-FLAG-transfected COS-1 cultures were used for protease protection assays. Cell homogenates (100 μg of protein) were treated with 7.5 μg of factor Xa protease (New England Biolabs, Beverly, MA) for 2 h at room temperature in a reaction mixture (75 μl) containing 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 2.5 mM CaCl2. Then, dansyl-Glu-Gly-Arg-chloromethylketone (Calbiochem) was added to a final concentration of 10 μM to inactivate factor Xa. The reaction mixture was then diluted 100-fold to 7.5 ml with cold buffer A (0.25 μM 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 Western blot analysis.

RESULTS

Mammalian G6PTs Are Nonglycoproteins—Sequence analysis predicts the presence of a potential Asn-linked glycosylation site at amino acids 354–356, conserved among human, mouse, and rat G6PT proteins (11, 12). This site is predicted to be located in a 17-amino acid loop in the ten-domain model (Fig. 1) or in helix 10 in the twelve-domain model, thus would not satisfy the criteria as an acceptor for oligosaccharides in either model. In vitro translation-translation assays showed that human, mouse, or rat G6P cDNA supported the synthesis of a 37-kDa polypeptide both in the absence or presence of canine microsomal membranes (Fig. 2), confirming that this glycosylation site is not normally used.

Membrane Topology of G6PT—Sequence analysis predicts that G6PT is anchored in the ER membrane by ten (18) or twelve (11, 19) transmembrane helices. Because microsomes are closed vesicles with a defined cytoplasmic-side-out orientation (24), protease protection assays using NH2- and COOH-terminal tagged G6PT constructs should allow us to assess the location of its NH2 and COOH termini with respect to the ER lumen. To tag G6PT, we used the 8-amino acid FLAG marker peptide, DYKDDDDK (Scientific Imaging Systems, Eastman Kodak, CT) or (CAG, Ser) which has been successfully used to tag human G6Pase (3). To demonstrate that the FLAG-tagged G6PT proteins are targeted to the ER membrane and retain G6P transport activity, we examined microsomal G6P uptake in COS-1 cells transfected with the WT or a FLAG-tagged G6PT construct. In earlier studies, we have demonstrated that G6Pase greatly facilitates G6P transport into the ER lumen by the G6PT protein (14). In the presence of a co-transfected G6Pase cDNA, G6P was efficiently taken up by intact microsomes isolated from COS-1 cells transfected with the G6PT-WT cDNA (Fig. 3A). Microsomal G6P uptake activity in G6PT-5’FLAG/G6Pase-transfected cells (Fig. 3B) was indistinguishable from that of G6PT-WT-transfected cells (Fig. 3A), suggesting that a small 5’FLAG tag did not interfere with G6P transport function of the G6PT protein. Significant levels of microsomal G6P uptake activity (approximately 71% of G6PT-WT activity) was also observed in COS-1 cells transfected with G6PT-3’FLAG/G6Pase cDNAs (Fig. 3B), suggesting that G6PT is less tolerant of a small COOH-terminal tag.

Both the ten and the twelve transmembrane helical models predict that NLS and COOH terminal of G6PT are situated at the same side of the ER membrane. The presence of an ER transmembrane retention signal at the COOH termini of mammalian G6PT proteins suggests that COOH terminus of G6PT faces the cytoplasm (25). Thus, in intact microsomes, both NH2- and COOH-terminal FLAG tags in G6PT should be sensitive to proteolytic digestion. However, unlike G6Pase, which is resistant to limited proteolytic digestion (3), the G6PT protein is sensitive to proteolysis as trypsin at 10 μg/ml microsomal protein rapidly abolishes G6P transport activity of the G6PT.

HCl, pH 7.4, and 250 mM sucrose. Microsomes permeabilized with 0.2% deoxycholate, which abolished G6P uptake, were used as negative controls. Two to three independent experiments were conducted, and at least two G6P uptake studies were performed for each microsomal preparation. Data are presented as the mean ± S.E.
protein both in the absence or presence of G6Pase. To accurately determine the topology of G6PT, we inserted two factor Xa protease recognition motifs (22) between the G6PT coding region and the FLAG tag to yield G6PT-5\textsuperscript{fXaFLAG} and G6PT-3\textsuperscript{fXaFLAG} constructs, and examined G6P transport by transient expression assays. Significant G6P uptake activities were observed in microsomes from either G6PT-5\textsuperscript{fXaFLAG}/G6Pase or G6PT-3\textsuperscript{fXaFLAG}/G6Pase-transfected cells (Fig. 3C), albeit at reduced efficiencies. Our data suggest that both G6PT-5\textsuperscript{fXaFLAG} and G6PT-3\textsuperscript{fXaFLAG} constructs were anchored in the ER membrane and functioned as a G6P transporter.

Intact microsomes, isolated from G6PT-5\textsuperscript{fXaFLAG} or G6PT-3\textsuperscript{fXaFLAG} transfected COS-1 cells, were then subjected to digestion by factor Xa and the presence of the FLAG epitope was visualized by Western blot analysis (Fig. 4). Microsomes isolated from G6PT-5\textsuperscript{F}LAG or G6PT-3\textsuperscript{fFLAG} transfected cells (Fig. 3C), albeit at reduced efficiencies. Our data suggest that both G6PT-5\textsuperscript{fXaFLAG} and G6PT-3\textsuperscript{fXaFLAG} constructs were anchored in the ER membrane and functioned as a G6P transporter.

Intact microsomes, isolated from G6PT-5\textsuperscript{fXaFLAG} or G6PT-3\textsuperscript{fXaFLAG} transfected COS-1 cells, were then subjected to digestion by factor Xa and the presence of the FLAG epitope was visualized by Western blot analysis (Fig. 4). Microsomes isolated from G6PT-5\textsuperscript{fFLAG} or G6PT-3\textsuperscript{fFLAG} transfected cells were used as controls. The FLAG tag in microsomes of G6PT-5\textsuperscript{fXaFLAG} or G6PT-3\textsuperscript{fXaFLAG}-transfected cells was cleaved by factor Xa protease (Fig. 4), indicating that both NH\textsubscript{2} and COOH termini of human G6PT face the cytoplasm and that human G6PT possesses an even number of transmembrane helices. Amino acid sequence analysis of G6PT predicts that the native protein contains no factor Xa recognition motifs. The FLAG tag in microsomes of either G6PT-5\textsuperscript{fFLAG} or G6PT-3\textsuperscript{fFLAG} transfected cells was resistant to factor Xa digestion (Fig. 4), as expected.

**Human G6PT Contains Ten-transmembrane Helices**—The major difference between the two topological models of G6PT is that residues 50–71, which constitute helix 2 in the twelve-helical model of G6PT (11), are situated in a 51-residue luminal loop 1 in the ten-domain model (Fig. 1). Whereas a potential Asn-linked glycosylation site within a transmembrane helix would not be used as an acceptor for oligosaccharides, such a site in a luminal loop larger than 33 residues would be used (20, 21). To distinguish between these two models, we constructed two mutants, G6PT-T53N and G6PT-S55N, that created a potential glycosylation site at residue 53-55 (N\textsuperscript{53}SS) or residues 55-57 (N\textsuperscript{55}QS), respectively. Synthesis and processing of WT or mutant G6PT were examined in transfected COS-1 cells and by in vitro translation assays.

Biosynthesis of G6PT in COS-1 cells transfected with a FLAG-tagged G6PT-WT, G6PT-T53N, or G6PT-S55N construct, in the absence or presence of a protein glycosylation inhibitor, tunicamycin (26), was analyzed by Western blot assays. Both G6PT-WT/FLAG and G6PT-WT/3\textsuperscript{FLAG} constructs supported the synthesis of a 37-kDa polypeptide in the absence or presence of tunicamycin, respectively (Fig. 5A), confirming that G6PT is not a glycoprotein. In contrast, G6PT-T53N/FLAG as well as G6PT-S55N3/FLAG constructs supported the synthesis of polypeptides of 41- and 37-kDa in the absence or presence of tunicamycin, respectively (Fig. 5A). Our data show that the introduced glycosylation sites, N\textsuperscript{53}SS and N\textsuperscript{55}QS, were used as acceptors for oligosaccharides.

In vitro translation assays showed that G6PT-T53N and G6PT-S55N mutant mRNAs directed the synthesis of a polypeptide of 37 kDa, which was processed to a glycopolyptide of 41 kDa in the presence of canine microsomal membranes (Fig. 5B). This is in contrast to the G6PT-WT mRNA which directed the synthesis of a 37-kDa protein both in the absence.

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proteins.

The G6PT-5 proteins were analyzed by Western blot hybridization and visualized by an anti-FLAG monoclonal antibody as described under “Materials and Methods.” Cells transfected with the pSVL vector (mock) or a G6Pase cDNA alone were used as controls. A, uptake of G6P into microsomes of G6PT-WT/G6Pase- (○) G6Pase- (■), or mock- (●) transfected cells. B, uptake of G6P into microsomes of G6PT-3’FLAG/G6Pase- (△) or G6PT-3’FLAG/G6Pase- (▲) transfected cells. C, uptake of G6P into microsomes of G6PT-5’XaFLAG/G6Pase- (△) or G6PT-3’XaFLAG/G6Pase- (▲) transfected cells. Data are presented as the mean ± S.E.

The presence of a co-transfected G6Pase cDNA, was nondetectable in intact microsomes isolated from COS-1 cells transfected with the G6PT-S55R, G6PT-T53N, or G6PT-S55N cDNA, in the presence of canine microsomal membranes (Fig. 5B). Our data support the ten transmembrane helical model for G6PT.

A number of missense mutations identified in the G6PT gene of GSD-1b patients are located in the 51 amino acid luminal loop (14, 16, 17) in G6PT, predicted by the ten-helical model (Fig. 1). Using transient expression studies, we have previously shown that G6PT harboring one of these mutations, R28H, was unable to transport G6P into the microsomes (14). In the present study, we examined microsomal G6P transport activity of another naturally occurring luminal loop mutant, G6PT-S55R (17), as well as our glycosylated G6PT constructs, G6PT-T53N and G6PT-S55R. Our results showed that G6P uptake activity in intact microsomes isolated from COS-1 cells transfected with the G6PT-S55R, G6PT-T53N, or G6PT-S55N cDNA, in the presence of a co-transfected G6Pase cDNA, was nondetectable (data not shown).

**DISCUSSION**

It has been proposed that hydrolysis of G6P requires the participation of at least two ER-associated membrane proteins, a G6P transporter, G6PT, that translocates G6P from the cytoplasm to the ER lumen, and a catalytic unit, G6Pase, that hydrolyzes G6P to glucose and phosphate (5). In earlier studies (3, 27), we have defined the transmembrane topology of G6Pase and shown that the active site of this enzyme faces the ER lumen. In this study, we characterized the orientation of the G6PT protein in the ER and showed that both the NH₂ and COOH termini of G6PT reside in the cytoplasm, indicating that G6PT contains an even number of transmembrane helices. We further show that mammalian G6PTs are nonglycoproteins and a conserved Asn-linked glycosylation site at residues 354–356 (11, 12) is not used as an acceptor for oligosaccharides.

Hydropathy profile analysis of bacterial transporters/receptors predicts that the sugar-phosphate transporter, uhpT, the G6P receptor, uhpc, and the glycerol-3-P transporter, GlycerT, contain twelve transmembrane segments (28, 29). Sequence alignment of G6PT with uhpT, uhpc, and GlycerT suggests that human G6PT may also contain twelve transmembrane helices (11). The topology of uhpT has been examined using fusion proteins containing uhpT and a topological reporter, alkaline phosphatase (PhoA) (29). It was assumed that fusion of PhoA to a periplasm-facing segment of a membrane protein would allow export of the PhoA moiety, resulting in high phosphatase activity. On the other hand, fusion of PhoA to a cytoplasm-facing segment or to a transmembrane domain of a membrane protein would exhibit lower phosphatase activity. A total of fifteen uhpT-PhoA fusion proteins were analyzed and the results, in general, supported the proposed twelve helical model for uhpT (29). However, fusion of PhoA to residue 79 in uhpT yielded a fusion protein exhibiting 100% of phosphatase activity (29). According to the twelve-domain model of uhpT, residue 79 is situated within helix 2, and thus should exhibit low phosphatase activity. This raised the possibility that amino acid 79 in uhpT actually faces the periplasm. The hydropathy profiles analyzed by newly developed algorithms (the TMPred program and Ref. 18) predict that uhpT, uhpc, and G6PT contains nine, ten, and ten transmembrane helices, respectively. To distinguish between the ten versus twelve transmembrane helical models of G6PT, we employed glycosylation scanning assays. Protein glycosylation provides a useful topological marker for membrane proteins. We created a potential Asn-linked glycosylation site in the region spanning amino acid residues 50–71 in G6PT, which would be in either helix 2, twelve-helical model) or a 51-residue luminal loop 1 (ten-helical model). We showed that the newly introduced glycosylation sites, N³³SS and N⁵⁶QS, were used as acceptors for oligosaccharides. Our data support the ten transmembrane helical model for the G6PT protein (Fig. 1).

To date, twelve missense mutations, G20D, R28C, R28H, S55R, G68R, G88D, W118R, G149E, G150R, C183R, R300H, and G339C, have been uncovered in the G6PT gene of GSD-1b patients (11, 13, 14, 16, 17). It is interesting to note that four of these mutations, R28C, R28H, S55R, and G68R, are in the
Mock transfected cells were used as controls. Each lane contained 20 μg proteins. B, analysis of G6PT synthesis and processing by in vitro transcription-translation of the G6PT-WT, G6PT-T53N, or G6PT-S55N construct in the absence or presence of canine microsomal membranes. L-[35S]methionine was used as the labeled precursor and after electrophoresis, the proteins were visualized by fluoro-autoradiography. Arrows indicate the nonglycosylated and glycosylated G6PT.

51-residue luminal loop 1 in G6PT (Fig. 1). In an earlier study (14), we showed that G6P uptake by the G6PT protein is greatly enhanced by G6Pase, and that G6PT harboring a R28H mutation (G6PT-R28H) was unable to transport G6P both in the absence or presence of G6Pase. In this study, we show that the G6PT-T53N and G6PT-S55N mutants as well as a naturally occurring G6PT-S55R mutant, each of which altered an amino acid within luminal loop 1, also lost their ability to transport G6P. Because this loop is located inside the ER lumen, it is tempting to speculate that luminal loop 1 in G6PT plays an important role in facilitating hydrolysis of G6P by G6Pase and is not involved in recruiting or binding of G6P in the cytoplasm.

In summary, our data strongly support the ten transmembrane helical model of human G6PT. We have previously shown that G6Pase is anchored in the ER membrane by nine transmembrane helices (3). Understanding of the orientations of both proteins in the ER should facilitate studies of the interrelationship between G6PT and G6Pase, two major players of the G6Pase system.

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