Structural Requirements for the Stability and Microsomal Transport Activity of the Human Glucose 6-Phosphate Transporter*

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Deficiencies in glucose 6-phosphate (G6P) transporter (G6PT), a 10-helical endoplasmic reticulum transmembrane protein of 429 amino acids, cause glycogen storage disease type 1b. To date, only three missense mutations in G6PT have been shown to abolish microsomal G6P transport activity. Here, we report the results of structure-function studies on human G6PT and demonstrate that 15 missense mutations and a codon deletion (AF93) mutation abolish microsomal G6P uptake activity and that two splicing mutations cause exon skipping. While most missense mutants support the synthesis of G6PT protein similar to that of the wild-type transporter, immunoblot analysis shows that G20D, ΔF93, and I278N mutations, located in helix 1, 2, and 6, respectively, destabilize the G6PT. Further, we demonstrate that G6PT mutants lacking an intact helix 10 are misfolded and undergo degradation within cells. Moreover, amino acids 415–417 in the cytoplasmic tail of the carboxyl-domain, extending from helix 10, also play a critical role in the correct folding of the transporter. However, the last 12 amino acids of the cytoplasmic tail play no essential role(s) in functional integrity of the G6PT. Our results, for the first time, elucidate the structural requirements for the stability and transport activity of the G6PT protein.

Glycogen storage disease type 1 (GSD-1), also known as von Gierke disease, is a group of autosomal recessive disorders that occurs approximately once in every 100,000 live births (1, 2). Deficiencies in the glucose 6-phosphate transporter (G6PT) cause GSD type 1b (GSD-1b), the second most prevalent form of GSD-1. G6PT translocates glucose 6-phosphate (G6P), the product of gluconeogenesis and glycogenolysis, from cytoplasm to the lumen of the endoplasmic reticulum (ER) (3, 4), where the active site of glucose-6-phosphatase (G6Pase) is positioned (5). Inside the ER, G6Pase catalyzes the conversion of G6P to glucose and phosphate (4). Therefore, G6PT and G6Pase work in concert to maintain glucose homeostasis. GSD-1b patients display symptoms associated with functional G6Pase deficiency, including hypoglycemia, hepatomegaly, kidney enlargement, growth retardation, lactacidemia, hyperlipemia, and hyperuricemia (1, 2). These patients also suffer from complications resulting from infections due to chronic neutropenia and functional deficiencies of neutrophils and monocytes (6, 7). Thus, GSD-1b offers a potential model for exploring the molecular mechanism(s) of neutropenia, neutrophil/monocyte dysfunction, and its relationship to G6Pase deficiency.

Complementary DNAs encoding human (8), mouse (9), and rat (9) G6PT have been isolated and characterized. Two adjacent lysine residues at the C termini of mammalian G6PT proteins conform to an ER membrane protein retention motif, consistent with the cellular location of the transporter. Recently, the orientation of human G6PT in the ER has been resolved by protease protection and glycosylation scanning analyses (10). Those studies showed that the G6PT protein is anchored to the ER by 10 transmembrane helices with both N and C termini facing the cytoplasm.

Human G6PT, a single copy gene located on chromosome 11q23 (11), spans approximately 5.5 kilobases and is composed of 9 exons (12, 13). Using G6Pase-deficient mice, we have shown that the transport and hydrolysis of G6P are tightly coupled processes and that G6Pase activity is required for efficient G6P transport into the microsomes (14). Based on that finding, a functional assay for the recombinant G6PT protein was established (12), which showed that G6PT can function minimally as a G6P transporter in the absence of G6Pase. However, microsomal G6P uptake activity was markedly enhanced in the simultaneous presence of G6PT and G6Pase (12). To date, a large number of mutations have been identified in the G6PT gene of GSD-1b patients (8, 12, 15–22). However, only three disease-causing missense mutations have been functionally characterized (12). The resulting three amino acid substitutions, R28H, G149E, and C183R, abolish microsomal G6P transport activity as shown by heterologous expression of mutant proteins in COS-1 cells. Here we report the identification of four novel G6PT mutations among 14 GSD-1b patients. Fifteen missense mutations and a codon deletion mutation completely abolish microsomal G6P transport activity, and two splicing mutations cause exon skipping. Our studies establish that mutations in the G6PT gene cause GSD-1b.

At present, very little is known about the structural requirements for the correct folding and G6P transport activity of the G6PT protein. In this study, we undertake structure-function studies and demonstrate that G20D, ΔF93, and I278N mutations, located in helix 1, 2, and 6, respectively, destabilize the transporter. Further, we show that intactness of helix 10 in G6PT is essential for proper folding and stability of the transporter. However, the last 12 amino acids in the cytoplasmic tail of the C-domain in G6PT, including the ER transmembrane protein retention motif, are not essential for membrane insertion, stability, or microsomal G6P transport activity. Finally, we establish the minimal length of the cytoplasmic tail of the C-domain essential for functional integrity of the G6PT.
MATERIALS AND METHODS

Mutational Analysis—We have analyzed the G6PT gene of 13 GSD-1b patients that presented with hypoglycemia, hepatomegaly, growth retardation, neutropenia, neutrophil dysfunction, and recurrent bacterial infections; each also lacks a genetic defect in the G6Pase gene. We have also analyzed the G6PT gene of one GSD-1 patient diagnosed clinically as GSD-1a who proved to have no mutation in the G6Pase gene. The lymphoblast line of GSD-1b patient 1 reported by Beaudet et al. (6) was obtained from the National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository (Camden, NJ). Genomic DNA preparations were extracted from lymphoblasts or blood samples using a Nucleon II kit (Scotlab Bioscience). All peripheral blood samples were obtained with the informed consent of the patients and/or parents.

The G6PT gene in GSD-1 patients was characterized by single strand conformation polymorphism analysis (23) on MDE (mutation-detection-enhancement nondenaturing) gels (AT Biochem, Malvern, PA) containing 5% glycerol. Exon-containing fragments were amplified by polymerase chain reaction using primers containing intronic, 5’-untranslated, and 3’-untranslated sequences of the human G6PT gene as described previously (12). The mutation-containing fragments identified by single strand conformation polymorphism were subcloned and characterized by DNA sequencing.

Construction of G6PT Mutants and Expression in COS-1 Cells—The template for G6PT mutant construction by PCR was nucleotides 166–1496 of the human G6PT cDNA in a pSVL vector (Amersham Pharmacia Biotech), which contains the entire coding region at nucleotides 170–172 with the translation initiation codon, ATG, at nucleotides 170–172. Fourteen G6PT cDNA clones were subcloned to the BstEII site at nucleotides 869–871 (8). The PCR primers for mutants that contained mutations located upstream of the BstEII site are nucleotides 166–186 (sense) and 851–878 (antisense) in the G6PT cDNA. The primers for mutants that contained mutations located downstream of the BstEII site are nucleotides 851–878 (sense) and 1476–1496 (antisense). The amplified fragments were ligated into either the pSVLhG6PT-BstEII-3’ or the pSVLhG6PT-BstEII-5’ fragment.

The mutant primers are as follows: G20D (nucleotides 218–237), GGC → GAC at position 20; R28C (nucleotides 241–260), GGC → TGC at position 28; S55R (nucleotides 323–342), AGC → CGC at position 55; G68R (nucleotides 361–380), GGG → AGG at position 68; L85P (nucleotides 413–432), CTC → CCC at position 85; G88D (nucleotides 422–441), GCC → GAC at position 88; ΔF93 (nucleotides 436–457), deleting TCT at nucleotides 446–448; W118R (nucleotides 511–530), TGG → CGG at position 118; G150R (nucleotides 607–626), GGG → AGG at position 150; N198I (nucleotides 752–771), AAC → ATC at position 198; I278N (nucleotides 992–1011), ATC → AAC at position 278; R300H (nucleotides 1058–1077), CGC → CAC at position 300; G339C (nucleotides 1174–1193), GCT → TGT at position 339; A373D (nucleotides 1277–1296), GCC → GAC at position 373. The antisense primer for each change contains a corresponding complementary sequence. Boldface letters indicate nucleotide changes. The W393X mutant cDNA clone was obtained by reverse transcriptase-PCR amplification of RNA isolated from lymphoblasts of patient 5.

The C-terminal deletion mutants were constructed by PCR amplification using a sense primer containing nucleotides 851–878 and an antisense primer (20 bases) containing a termination codon at position 401 (GAA → TAA), 408 (AGC → TAG), 415 (CGA → TGA), 416 (AAC → TAA), 417 (ATC → TAA), 418 (CGC → TAA), 419 (ACC → TAA), 420 (AAG → TAG), 426 (AAG → TAG), or 427 (AAG → TAG).

The G6PT-5’FLAG and G6PT-3’FLAG constructs (10) containing the 5’- and 3’-end of the G6PT coding region were used as templates for constructing the FLAG-tagged mutants. The nucleotide sequence in all constructs was verified by DNA sequencing.

COS-1 cells were grown at 37 °C in HEPES-buffered Dulbecco’s modified minimal essential medium supplemented with 4% fetal bovine serum. The G6PT construct in a pSVL vector was transfected into COS-1 cells by the DEAE-dextran/chloroquine method (24) in the absence, respectively, of the co-transfected G6Pase cDNA as described previously (12) (12). After incubation at 37 °C for 2 days, the transfected cultures were harvested for microsomal G6P uptake assay, Western blot analysis, or RNA isolation.

Northern Blot, Western Blot, and in Vitro Transcription-Translation Analyses—Total RNA was isolated by the guanidinium thiocyanate/ CsCl method (25), fractionated by electrophoresis through a 1.2% agarose gel containing 2.2 M formaldehyde, and transferred to a Nitran membrane by electrophoretic transfer. The membranes were hybridized with either a uniformly labeled G6PT or β-actin riboprobe. For Western blot analysis of FLAG-tagged G6PT, microsomal proteins were separated by electrophoresis through a 12% polyacrylamide-SDS gel and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were incubated with a monoclonal antibody against the FLAG epitope (Sigma). The immunocomplex was detected with the horseradish peroxidase-linked chemiluminescent system containing the SuperSignal West Pico Chemiluminescent substrate obtained from Pierce.

In vitro transcription of G6PT cDNA constructs in a pGEM-7Zf + vector was performed using the T7oupled reticulocyte lysate system obtained from Promega Biotech (Madison, WI). 1.5 mM ATP, 5 mM GTP, 5 mM CTP, 5 mM UTP, and 100 ng of each RNA polymerase were used as the labeled precursor. The in vitro synthesized proteins were analyzed by 12% polyacrylamide-SDS gel electrophoresis and fluorography.

G6P Uptake Assays—G6P uptake measurements were performed essentially as described previously (12). Briefly, microsomes (40 μg) were incubated in a reaction mixture (100 μl) containing 50 mM sodium cacodylate buffer, pH 6.5, 250 mM sucrose, and 0.2 mM [14]C(G6P) (50 μCi/μmol). The reaction was stopped at the appropriate time by filtering immediately through a nitrocellulose membrane (BA85, Schleicher & Schuell) and washed with an ice-cold solution containing 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 three G6P uptake studies were performed for each microsome preparation. We have previously shown that microsomal G6P uptake in COS-1 cells transfected with a G6PT and a G6Pase cDNA reached a plateau at 3 min of incubation (12). Therefore, the 3-min G6P uptake values were used to compare microsomal G6P uptake activities of wild-type (WT) and mutant G6PT proteins. Statistical analysis using the unpaired t test was performed with The GraphPad Prism Program (GraphPad Software, San Diego, CA). Data are presented as the mean ± S.E.

RESULTS

Mutations Identified in the G6PT Gene of GSD-1b Patients—Single strand conformation polymorphism and DNA sequencing analyses were used to identify mutations in the G6PT gene of 13 GSD-1b patients and one putative GSD-1a patient, although the G6Pase gene had no identifiable mutation in the coding region. G6PT mutations were identified in all 14 patients, indicating that the GSD-1a patient was misdiagnosed. Fourteen different mutations were identified, including nine missense (G20D, R28C, L85P, W118R, G149E, G150R, I278N, G339C, and A393D), two nonsense (W96X and W393X), one splicing (550+2T → g), and two deletion (1211delCT and ΔF93) mutations (Table I). Four novel mutations identified in this study are L85P, ΔF93, I278N, and A373D.

G6PT Mutations That Cause GSD-1b—To date, 59 different mutations, including 23 missense, have been identified in the G6PT gene of GSD-1b patients (Refs. 8, 12, and 15–22 and this study). However, only three missense mutations, R28H, G149E, and C183R, uncovered in this laboratory were shown to abolish microsomal G6P transport activity (12). To demonstrate that other missense mutations uncovered in GSD-1b patients cause the disease, we constructed a series of G6PT mutants and examined the ability of each mutant to transport G6P in transient expression studies. We also examined G6P transport activity of the ΔF93 and N198I mutants; the latter was identified as a possible polymorphic marker in a GSD-1b patient homozygous for a splicing mutation (15).

The combined transfection of the G6Pase and WT G6PT cDNAs into COS-1 cells showed that G6P was efficiently taken up by intact microsomes isolated from these cells (Table II). In contrast, microsomal G6P transport activity was undetectable when G20D, R28C, S55R, G68R, ΔF93, L85P, G88D, W118R, G150R, I278N, R300H, G339C, or A393D mutant G6PT cDNA was co-transfected with the G6Pase cDNA (Table II). The N198I construct retained at least 95% of WT G6P transport activity.

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activity (Table II), indicating that it represents a sequence polymorphism in the G6PT gene.

The G20D, ΔF93, and I278N Mutations Destabilize the Transporter—Northern blot analysis confirmed that similar levels of G6PT transcripts were expressed in WT or mutant G6PT-transfected COS-1 cells (Fig. 1A). Our data, therefore, demonstrate that the decrease in G6P uptake was due to a defective G6PT protein and not due to a decrease in efficiency of expression of the transfected cDNA construct.

In earlier studies (10), we have shown that G6PT-WT and G6PT-WT-5'FLAG constructs had similar microsomal G6P uptake activities, indicating that the 8-amino acid FLAG tag at the N terminus of G6PT did not interfere with its G6P transport function. We therefore constructed a series of 5'-FLAG-tagged G6PT mutants and examined their biosynthesis in COS-1 cells by Western blot analysis using a monoclonal antibody against the FLAG epitope. WT, R28C, R28H, S55R, G68R, G88D, W118R, G149E, G150R, C183R, R300H, G339C, and A373D mutant constructs supported the synthesis of similar amounts of G6PT proteins that were approximately 30, 40, and 20%, respectively, of that in WT G6PT-transfected cells (Fig. 1B). Our data indicate that G20D, ΔF93, and I278N mutants, located in helix 1, 2, and 6 (Fig. 2), respectively, destabilize the transporter.

We also constructed G6PT mutants containing the FLAG tag at the C terminus and examined their biosynthesis after transient expression in COS-1 cells (Fig. 3A). Again G20D-3'FLAG, ΔF93-3'FLAG, and I278N-3'FLAG constructs supported the synthesis of markedly reduced amounts of G6PT proteins as compared with the WT-3'FLAG as well as the R28H-3'FLAG constructs.

### Table I

| Patient | Exon | Mutation | Effect on coding sequence | Comments |
|---------|------|----------|---------------------------|----------|
| 1       | VIII | 1211delCT | Frameshift at 348, stop at 400 | Homozygote |
| 2       | I    | 251C→T/R28C | Arg→Cys at 28 | Compound heterozygote |
| 3       | VIII | 1184G→T/G339C | Gly→Cys at 339 | Homozygote |
| 4       | VIII | 1211delCT | Frameshift at 348, stop at 400 | Homozygote |
| 5       | IX   | 1348G→A/W393X | Trp→Stop at 393 | Compound heterozygote |
| 6       | II   | 521T→G/W118R | Trp→Arg at 118 | Homozygote |
| 7       | VIII | 1287C→A/A373D | Ala→Asp at 373 | Homozygote |
| 8       | I    | 228G→A/G20D | Gly→Asp at 20 | Compound heterozygote |
| 9       | II   | 550+2t→g | AGgt→AGgg at 550 + 2, 5' splice site | Compound heterozygote |
| 10      | VIII | 1211delCT | Frameshift at 348, stop at 400 | Homozygote |
| 11      | III  | 615G→A/G150R | Gly→Arg at 150 | Compound heterozygote |
| 12      | VIII | 1211delCT | Frameshift at 348, stop at 400 | Homozygote |
| 13      | II   | 446delTTC/ΔF93 | del Phe at 93 | Compound heterozygote |
| 14*     | VIII | 1211delCT | Frameshift at 348, stop at 400 | Compound heterozygote |

* Patient 14 was initially diagnosed as GSD-1a but contains no G6Pase mutations. The translation initiation codon ATG is located at nucleotides 170–172.

### Table II

| Constructs | G6P uptake activitya | Constructs | G6P uptake activity |
|------------|----------------------|------------|--------------------|
| G6Pase     | 0.019 ± 0.0021       | ΔF93/G6Pase | 0.016 ± 0.0011     |
| WT/G6Pase  | 0.095 ± 0.0053       | W118R/G6Pase | 0.017 ± 0.0010    |
| G20D/G6Pase| 0.020 ± 0.0111       | G150R/G6Pase | 0.016 ± 0.0005    |
| R28C/G6Pase| 0.021 ± 0.0030       | I278N/G6Pase | 0.020 ± 0.0017    |
| S55R/G6Pase| 0.020 ± 0.0020       | R300H/G6Pase | 0.018 ± 0.0011    |
| G88D/G6Pase| 0.018 ± 0.0006       | G339C/G6Pase | 0.022 ± 0.0017    |
| L85P/G6Pase| 0.019 ± 0.0017       | A373D/G6Pase | 0.022 ± 0.0013    |
| G88D/G6Pase| 0.021 ± 0.0018       | N198I/G6Pase | 0.090 ± 0.0057    |

* Microsomal G6P uptake activity from WT or mutant G6PT cDNA-transfected cells in the presence of a co-transfected G6Pase cDNA was measured after a 3-min incubation and is presented as the mean ± S.E. Statistical analysis was performed using the unpaired t test.

![Fig. 1. Northern blot (A) and Western blot (B) analyses of G6PT in COS-1 cells transfected with WT, missense, or ΔF93 mutant cDNA construct containing a 5’FLAG tag. Mock-transfected cells were used as controls. The Northern membranes were hybridized with a uniformly labeled G6PT or β-actin riboprobe; each lane contained 10 μg of total RNA. The G6PT proteins on the Western membranes were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg of proteins.](image-url)
In vitro transcription-translation assays showed that WT, R28H, G2OD, ΔF93, and I278N constructs directed the synthesis of similar amounts of G6PT proteins (Fig. 3B). Therefore, the observed decrease in the synthesis of G2OD, ΔF93, and I278N mutants may result from misfolding and rapid degradation of mutant proteins in a heterologous expression system.

The 550+2t→g and 550+1g→t Mutations Cause Exon Skipping—The 550+2t→g splicing mutation (Table I) is likely to cause exon-2 skipping from the G6PT transcript. Sequencing of six G6PT cDNA clones obtained by reverse transcriptase-PCR of RNA isolated from lymphoblasts of patient 7 showed that exon 2 (233-bp) sequences were absent from half of the cDNA clones. The other half contained the 228G→A/G20D mutation.

We have previously identified a splicing mutation (550+2t→g) in a compound heterozygous patient also harboring a 716T→C/C183R mutation (12). To demonstrate that the 550+1g→t mutation also causes exon 2 skipping from the G6PT transcript, we sequenced five G6PT cDNA clones obtained by reverse transcriptase-PCR of RNA isolated from lymphoblasts of the patient. While three of the cDNA clones contained the 716T→C/C183R mutation, the other two clones lacked exon 2 sequences, demonstrating that the 550+1g→t mutation also causes exon-2 skipping.

The cytoplasmic tail at amino acids 415–429 in the C-domain of human G6PT is extended directly from helix 10, which encompasses amino acids 396–414 (Fig. 2). Two nonsense mutations, W393X and R415X (19), lacking helix 10 as well as the cytoplasmic tail and the entire cytoplasmic tail, respectively, were identified in the G6PT gene of GSD-1b patients. To investigate the molecular basis of G6PT deficiency caused by these mutations, we examined G6PT synthesis directed by W393X and R415X constructs (Fig. 4A). While the W393X-5FLAG construct supported little or no G6PT synthesis following transfection into COS-1 cells (Fig. 4A), the R415X-5FLAG construct supported the synthesis of a significant amount of G6PT, approximating 30–40% of the levels in WT-5FLAG-transfected cells. This suggests that helix 10 is required for the correct folding and stability of the transporter. We further examined G6PT synthesis directed by two additional mutants, E401X-5FLAG and T408X-5FLAG, that disrupt helix 10. Again, we observed little or no G6PT synthesis following transfection of these mutants into COS-1 cells (Fig. 4A).
Northern blot analysis showed that similar levels of G6PT transcripts were expressed in WT-, W393X-, E401X-, T408X-, and R415X-transfected cells (Fig. 4B). Moreover, the four mutant constructs, like the WT G6PT, directed the synthesis of similar amounts of G6PT proteins in a cell-free transcription-translation system (Fig. 4C). Our data indicate that helix 10 in G6PT also plays a vital role for correct folding of the transporter and that the incorrectly folded mutants were degraded in the cell.

G6PT contains an ER transmembrane protein retention signal, KKAE, at the C terminus (Fig. 2). The observed reduction in the synthesis of the R415X mutant in COS-1 cells prompted us to investigate the role of this signal on folding and stability of G6PT. Interestingly, K420X-5'FLAG, K426X-5'FLAG, and K427X-5'FLAG mutants lacking this signal supported the synthesis of similar amounts of G6PT protein as the WT construct (Fig. 4A). This was supported by Northern blot analysis showing that similar levels of G6PT transcripts were expressed in WT as well as mutant constructs transfected with COS-1 cells (Fig. 4B). Again, WT as well as mutant constructs directed the synthesis of similar amounts of G6PT proteins in a cell-free transcription-translation system (Fig. 4C).

The Length of the Cytoplasmic Tail Essential for Folding and Stability of the Transporter—Our results show that the R415X mutant lacking the entire 15-amino acid cytoplasmic tail in the C-domain was degraded more rapidly than the WT construct in the COS-1 heterologous expression system. On the other hand, the K420X construct lacking the last 10 amino acids in the cytoplasmic tail had similar stability as the WT G6PT. To determine the minimal length of cytoplasmic tail required for G6PT folding and/or stability, we examined G6PT synthesis in transient expression studies directed by 5'FLAG-tagged G6PT mutants that lack the last 14 (N416X), 13 (I417X), 12 (R418X), and 11 (T419X) amino acids in the tail (Fig. 5A). While N416X and I417X supported the synthesis of reduced levels of G6PT proteins compared with the WT construct, R418X, T419X, like K420X, supported the synthesis of similar amounts of proteins as the WT G6PT (Fig. 5A). The decrease in G6PT synthesis by R415X, N416X, and I417X constructs appeared to correlate to the length of the cytoplasmic tail retained by these mutants.

Northern blot analysis showed that similar levels of G6PT transcripts were expressed in WT as well as in N416X-, I417X-, R418X-, and T419X-transfected COS-1 cells (Fig. 5B). Likewise, all directed the synthesis of similar amounts of G6PT proteins in a cell-free transcription-translation system (Fig. 5C).

**G6P Uptake Activity of G6PT Mutants**—The lack of detectable amounts of mutant G6PT in W393X-transfected COS-1 cells could explain functional G6PT deficiencies manifested by GSD-1b patients harboring this mutation. On the other hand, a significant amount of mutant protein was found in R415X-transfected cells. We therefore examined the ability of the various G6PT mutants to transport G6P in transient expression assays in the presence of a co-transfected G6Pase cDNA (Table III). W393X, E401X, and T408X mutants were devoid of microsomal G6P uptake activity, reflecting the lack of G6PT protein in mutant-transfected cells. On the other hand, microsomal G6P transport activity in R415X-, N416X-, and I417X-transfected cells were approximately 47, 78, and 80% of the activity in G6PT-WT-transfected cells (Table III), indicating that these three G6PT mutants are fully functional in microsomal G6P uptake. As expected, microsomal G6P transport activities of R418X, T419X, K420X, K426X, and K427X mutants were indistinguishable from that of the WT construct (Table III).
In this report, we have undertaken structure-function studies of the G6PT, and our results show that G20D, ΔF93, and I278N mutations, located in helix 1, 2, and 6, respectively, destabilize the transporter. Further, we demonstrate that helix 10, encompassing amino acids 396–414 in G6PT and amino acids 415–417 in the cytoplasmic tail of the C-domain extending directly from helix 10, also play important roles in folding and stability of the transporter. On the other hand, the last 12 amino acids of the cytoplasmic tail, including the ER transmembrane retention signal, are not required for the correct amino acids of the cytoplasmic tail, including the ER transmembrane protein retention signal, had no effect on the functional integrity of the transporter. We have previously shown that truncations of amino acids 415–417 in the cytoplasmic tail of G6PT that extends directly from helix 10 lead to a decrease in the amounts of mutant proteins synthesized in COS-1 cells and a corresponding reduction in microsomal G6P transport activity. The similar amounts of mutant and WT proteins synthesized in a cell-free system indicate that amino acids 415–417 may also play a role in the correct folding and/or stability of the G6PT. On the other hand, truncations of any of the last 12 amino acids in the cytoplasmic tail, including the ER transmembrane protein retention signal, had no effect on synthesis and microsomal G6P transport activity of the transporter, indicating that these residues are not essential for functional integrity of the transporter. We have previously shown that G6Pase, another ER transmembrane protein, remains enzymatically active and associated with the microsomes following truncations of its C-terminal 8 amino acids, including the ER retention signal (30). It appears that the intactness of their transmembrane helices is vital for membrane insertion and functions of G6PT as well as G6Pase.

The R415X mutation was identified in a heterozygous state in two GSD-1b patients of Afro-Caribbean origin (19). No additional mutation was found in any of the exons, intron 1, and the 500-bp promoter region of the G6PT gene. The authors speculated that the putative second mutation, if it exists, must greatly reduce the expression of that copy of the G6PT gene (19). Although it is possible that the R415X mutant protein is less stable in vitro than our in vitro expression system, the synthesis of significant amounts of a functional mutant G6PT following transfecting the R415X construct into COS-1 cells strongly argues that the G6PT genes of these two patients warrant further investigation.

In summary, we have elucidated a number of the structural requirements for the stability and microsomal transport function of the transporter and demonstrated that 15 missense mutations, the ΔF93 deletion mutation, and two splicing G6PT mutations cause GSD-1b.

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