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Functional analysis of mutations in the glucose-6-phosphate transporter that cause glycogen storage disease type Ib

Shih-Yin Chen, Chi-Jiunn Pan, Soojung Lee, Wentao Peng, and Janice Y. Chou*

Section on Cellular Differentiation, Program on Developmental Endocrinology and Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Abstract

The glucose-6-phosphate transporter (G6PT) deficient in glycogen storage disease type Ib is a phosphate (P_i)-linked antiporter capable of $G6P:P_i$ and $P_i:P_i$ exchanges. We previously characterized G6PT mutations by measuring G6P uptake activities in microsomes co-expressing G6PT and glucose-6-phosphatase- α . Here we report a new assay, based on reconstituted proteoliposomes carrying only G6PT, and characterize G6P and P_i uptake activities of 23 G6PT mutations. We show that co-expression and G6PT-only assays are equivalent in measuring G6PT activity. However, the p.Q133P mutation exhibits differential G6P and P_i transport activities, suggesting that characterizing G6P and P_i transport activities of G6PT mutations may yield insights to this genetic disorder.

Introduction

Glycogen storage disease type Ib (GSD-Ib, (MIM232220)) is an autosomal recessive disorder caused by a deficiency in the endoplasmic reticulum (ER)-bound glucose 6-phosphate transporter (G6PT) [1,2]. The primary function of G6PT is to translocate glucose-6-phosphate (G6P) from the cytoplasm into the lumen of the ER for hydrolysis to glucose and inorganic phosphate (P_i) by one of the two glucose-6-phosphatases (G6Pases), G6Pase- α [1,2] or G6Pase- β [3,4]. The concerted action of G6PT and G6Pase- α is required to maintain glucose homeostasis between meals and a deficiency of either protein results in a phenotype of disturbed glucose homeostasis characterized by fasting hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic acidemia, and growth retardation [1,2]. The concerted action of G6PT and G6Pase- β is vital for normal neutrophil functions and a deficiency of either protein results in a phenotype of myeloid dysfunctions characterized by neutropenia and impaired neutrophil respiratory burst, chemotaxis and calcium flux activities [5,6]. Therefore, understanding of the structure-function requirements of G6PT will provide valuable insight into the functional coupling between G6PT and both G6Pases.

The transport and hydrolysis of G6P are tightly coupled processes and G6Pase- α activity is required for the efficient transport of G6P into the microsomes [7]. Based on this finding, we established a functional assay for the recombinant G6PT by measuring G6P uptake activity

*Address for correspondences: Dr. Janice Chou, Building 10, Room 9D42, NIH, 10 Center Drive, Bethesda, MD 20892-1830, Tel: 301-496-1094, Fax: 301-402-6035, chouja@mail.nih.gov.

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in microsomes isolated from COS-1 cells co-expressing G6PT and G6Pase- α [8]. Using this co-expression assay, we have functionally characterized 28 missense mutations identified in the *G6PT* gene of GSD-Ib patients [8–10]. In sequencing the *G6PT* gene in clinical cases reported to represent GSD type Ic deficient in a putative P_i transporter [1], deleterious *G6PT* mutations found in GSD-Ib patients were identified [11–14], suggesting that G6PT is a G6P and a P_i transporter. However, attempts to measure P_i uptake in microsomes co-expressing G6PT and G6Pase- α have been unsuccessful. Moreover, it would be more desirable to establish a functional assay of G6PT in the absence of a co-expressed G6Pase- α . Using reconstituted proteoliposomes, we recently show that G6PT is a P_i -linked antiporter capable of both homologous ($P_i:P_i$) and heterologous (G6P: P_i) exchange [15], similar to the bacterial hexose-6-phosphate transporter, UhpT [16]. The study establishes that G6PT has a dual role as a G6P and a P_i transporter and that GSD-Ib and GSD-Ic are deficient in the same *G6PT* gene [15]. In this study, we characterize G6P and P_i transport activities of 19 previously characterized and 4 newly identified G6PT mutations in the reconstituted proteoliposomal system, and compare the results to their respective microsomal G6P uptake activity determined by the co-expression assay. Our results show that all three assays yield similar results and accurately determine G6PT activity. However, the p.Q133P mutation exhibits differential G6P and P_i transport activities. Taken together, our results, for the first time, elucidate G6P and P_i transport activities of G6PT mutations that cause GSD-Ib which may yield valuable insights to this genetic disorder characterized by both metabolic and myeloid abnormalities.

Materials and methods

Construction of G6PT Mutants

The template for G6PT mutant construction by PCR was nucleotides 1 to 1286 of the human G6PT cDNA in the pAdlox shuttle vector [10], which contains the entire coding region, with the translation initiation codon, ATG, at nucleotides 1–3. The two outside PCR primers are nucleotides 1 to 20 (sense) and 1270 to 1290 (antisense). The sense and antisense mutant primers are 20 nucleotides in length with the codon to be mutated in the middle. The nucleotide changes in the mutant constructs include: Y24H [17] (nucleotides 70–72, TAT to CAT); R166L [18] (nucleotides 496 to 498, CGC to CTC); L229P [19] (nucleotides 685 to 687, CTC to CCC); and G281R [18] (nucleotides 841 to 843, GGC to CGC). After PCR, the amplified fragment was ligated into the pAdlox vector.

Recombinant Adenoviruses containing mutant G6PT were generated by the Cre-*lox* recombination system [20] as described [10]. The recombinant virus was plaque purified and amplified to produce viral stocks with titers of approximately 1 to 3×10^{10} plaque forming unit (pfu) per ml.

Gene expression in COS-1 cells and microsomal G6P uptake assays

Recombinant adenovirus carrying wild-type G6PT (Ad-G6PT) and G6Pase- α (Ad-G6Pase- α) have been described [10,21]. COS-1 cells were grown at 37 °C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with 4% fetal bovine serum. Cells in 150-cm² flasks were infected with Ad-G6PT or an Ad-G6PT mutant, or co-infected with Ad-G6Pase- α and wild-type or mutant Ad-G6PT. The multiplicity of infection for Ad-G6PT or Ad-G6PT mutant and Ad-G6Pase- α was 50 pfu/cell and 25 pfu/cell, respectively. Mock infected COS-1 cells were used as controls. After incubation at 37 °C for 24 h, the infected cultures were used to isolate microsomes for G6P uptake, proteoliposome reconstitution, and Western-blot analysis.

Microsomal G6P uptake measurements were performed essentially as described previously [8]. Briefly, microsomes (40 μ g) were incubated in a reaction mixture (100 μ l) containing 50 mM sodium cacodylate buffer, pH 6.5, 250 mM sucrose, and 0.2 mM [U- 14 C]G6P (50 μ Ci/ μ mol, American Radiolabeled Chemicals, St Louis, MO). The reaction was stopped at the appropriate time by filtering immediately through a nitrocellulose membrane (0.45 μ m, Millipore Co., Billerica, MA). In microsomes expressing both G6Pase- α and G6PT, the [U- 14 C]G6P taken up by the microsomes is hydrolyzed by G6Pase- α to [U- 14 C]glucose and P_i and the radioactive molecule(s) accumulated inside the microsomes is primarily [U- 14 C]glucose.

Solubilization and reconstitution of membrane proteins

Microsomal membrane protein solubilization and proteoliposome reconstitution were performed as previously described [15,16]. Briefly, membrane proteins were solubilized on ice by mixing 2 mg of microsomes in 1 ml of a solution containing 20 mM Tris-HCl, pH 7.5, 20% glycerol, 1.25% (w/v) *n*-octyl- β -D-glucopyranoside (octylglucoside, Calbiochem, San Diego, CA), 2 mM DTT, protease inhibitors (1% aprotinin, 1 mM AEBSF, 2 μ g/ml pepstatin A, and 2 μ g/ml leupeptin, all from Roche Diagnostics, Indianapolis, IN), and 0.4% (w/v) lipid mixture in 2 mM β -mercaptoethanol that contains the *E coli* polar lipid extract, L- α phosphatidylcholine, L- α phosphatidylserine, and cholesterol (60:17.5:10:12.5 w/w), all from Avanti Polar lipids, Inc., Alabaster, AL.

To reconstitute proteoliposomes, the detergent solubilized microsomal membrane extracts (500 to 700 μ g) were mixed with the sonicated lipid mixture [11] at a ratio of 1:10 protein:lipid (w/w). Proteoliposomes were then formed by placing the reaction inside a dialysis cassette from PIERCE (Rockford, IL) and dialyzing extensively overnight against a phosphate buffer (50 mM KH_2PO_4 , pH 7.0, 1mM DTT, and protease inhibitors) or a MOPS/K buffer (20 mM MOPS, pH 7.5 adjusted with KOH; 75 mM K_2SO_4 ; 2.5 mM $MgSO_4$, 1 mM DTT, and protease inhibitors) at 4 $^{\circ}C$. The resulting P_i -loaded or MOPS-loaded proteoliposomes were pelleted, resuspended in MOPS/K buffer, and used immediately in transport assays. The MOPS-loaded proteoliposomes were used as negative controls. In addition, proteoliposomes prepared from detergent solubilized microsomal membrane extracts of mock-infected cells and liposomes lacking microsomal proteins were prepared in parallel and also used as negative controls. The protein content in microsomes, detergent extract and proteoliposomes was quantified by the Amido black B protein estimation method as described previously [22].

Transport assays

Transport assays were performed at room temperature using reaction mixtures containing 20 mM MOPS/K buffer, 25 μ g/ml of 50 mM P_i -loaded G6PT-proteoliposomes or MOPS-loaded proteoliposomes, 0.1 mM [U- 14 C]G6P or 0.5 mM $^{32}P_i$. The $^{32}P_i$ was prepared by boiling carrier-free $^{32}P_i$ (MP Biochemical, Inc., Irvine, CA) in 1 ml of 1 N HCl for 3 h and diluting with an equal volume of 2 M K_2HPO_4 as described previously [23]. After incubation at room temperature for 9 min [15], the reaction mixture was filtered immediately through presoaked 0.22 μ m nitrocellulose filters (Millipore), and the washed and dried filters were counted in a liquid scintillation counter.

Western blot analysis

For Western-blot analysis, proteins were resolved by electrophoresis through a 10% polyacrylamide-SDS gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore). The membranes were incubated overnight with a rabbit anti-G6PT antibody raised against amino acids 25 to 79 of human G6PT [10]. The membranes were then incubated with a horseradish peroxidase-conjugated second antibody and the

immunocomplex visualized using the Immobilon™ western chemiluminescent HRP substrate (Millipore).

Statistical analysis

The unpaired t test was performed using the GraphPad Prism Program, version 4 (GraphPad Software, San Diego, CA). Values were considered statistically significant at $p < 0.05$.

Results

G6P and P_i transport activities of 19 previously characterized G6PT mutations

The G6PT is a P_i-linked antiporter [11] that is capable of heterologous G6P:P_i and homologous P_i:P_i exchange. We therefore examined G6P and P_i uptake activities of 19 previously characterized, naturally occurring, G6PT missense mutations using P_i-loaded proteoliposomes reconstituted from detergent solubilized microsomal membrane extracts isolated from COS-1 cells infected with a wild-type or a mutant Ad-G6PT construct. The 19 G6PT mutations, including all missense G6PT mutations identified in GSD-I non a patients [11–14], were classified as helical and non-helical based on their predicted locations in human G6PT [24].

Using the co-expression assay, we showed that the G6PT mutations, p.G20D, p.R28H, p.G50R, p.L85P, p.W118R, p.Q133P, p.G149E, p.G150R, p.C176R, p.C183R, p.P191L, p.G339D, and p.A373D were devoid of microsomal G6P uptake activity, while the following retained a percentage of the wild type activity, p.P153L (8.6%), p.I278N (10.4%), p.R300C (5.2%), p.G339C (4.9%), p.A367T (23.1%), and p.G376S (5.6%) [10]. Using the reconstituted proteoliposomal assays, we showed that, with the exception of Q133P, the other null mutants were devoid of both G6P and P_i transport activity (Table 1). Moreover, the P153L, I278N, R300C, G339C, A367T, and G376S mutants retained residual G6P and P_i transport activities (Table 1) similar to the activities obtained by the co-expression assay. Interestingly, the Q133P mutant exhibited differential activity being devoid of microsomal and proteoliposomal G6P uptake activity but retaining 5% of wild-type P_i transport activity (Table 1).

Characterization of newly identified G6PT mutations

To further identify amino acid residues essential for the functions of G6PT, we constructed Ad-G6PT mutants carrying 4 mutations recently identified in the *G6PT* gene of GSD-Ib patients and examined their transport activities using both co-expression and proteoliposomal assays. These include p.Y24H [17] in helix 1, p.R166L [18] in luminal loop 2, p.L229P [19] in helix 5, and p.G281R [18] in helix 6 [24]. The G281R mutant completely abolished G6P and P_i uptake activity and the Y24H, R166L, L229P mutants retained residual G6P and P_i transport activities (Table 2). Western blot analysis of G6PT proteins expressed in COS-1 microsomes showed that the R166L, L229P, and G281R mutants supported the synthesis of wild-type levels of G6PT proteins and the Y24H mutant supported the synthesis of increased levels of G6PT protein, as compared to the wild-type transporter (Fig. 1).

Discussion

Mutations in the gene encoding a glucose-6-phosphate transporter, G6PT, cause GSD-Ib [1,2]. In initial work on GSD-Ia and GSD-Ib, it became clear that mutation of G6PT also impaired the *in vivo* activity of G6Pase-α, the gene underlying GSD-Ia [1,2]. These proteins are codependent for their full activities. Therefore previous work has used a microsomal-based G6PT/G6Pase-α co-expression assay system to study the biological activity of G6PT

[8–10]. More recently, G6PT was shown to be a P_i -linked antiporter that mediates homologous $P_i:P_i$ and heterologous G6P: P_i exchanges [15] similar to the bacterial hexose-6-phosphate transporter, UhpT [15]. We now show that G6PT activity can also be measured accurately in the absence of G6Pase- α if a reconstituted proteoliposomes carrying only G6PT are pre-loaded with P_i . To show the equivalence of this simpler assay, we examined G6P and P_i transport activities of 19 disease-causing G6PT missense mutations previously characterized by the co-expression assays [8–10], and 4 mutations recently identified in the *G6PT* gene of GSD-Ib patients [17–19]. We show that G6P and P_i transport activities of the G6PT mutants determined by the proteoliposomal assays agree with microsomal G6P uptake activities measured by the co-expression assay.

We have previously established a functional assay for the recombinant G6PT by measuring G6P uptake activity in microsomes co-expressing G6PT and G6Pase- α [8–10]. Co-expression of G6Pase- α is necessary because a G6Pase- α -mediated increase in the P_i concentration in the ER lumen may generate a driving P_i gradient that is required for the sensitivity of the assay. However, we were unable to successfully preload microsomes with P_i to measure microsomal P_i uptake making the co-expression assay inadequate to fully characterizing G6PT activity. On the other hand, the P_i -loaded proteoliposomal system enabled us to measure G6P and P_i transport of wild type G6PT and its mutants in the absence of G6Pase- α . Here we show that G6PT activity determined by all three assays are comparable, confirming the validity of the co-expression assays. The only differential activity observed is the p.Q133P mutation identified in a GSD-Ib patient originally classified as GSD-I non a [12] which is devoid of G6P transport activity regardless of the assay methods but retained 5% wild-type P_i transport activity. Interestingly, a D388C mutation in the bacterial antiporter UhpT was devoid of G6P transport activity but displayed considerable P_i exchange activity [25]. D388C in UhpT is also a gain-of-function mutation that biases substrate preference away from G6P [25]. It would be of interest to investigate whether the Q133P mutant exhibited altered substrate specificity.

In summary, we report a reconstituted proteoliposome-based assay for G6PT which is independent of the presence of G6Pase- α . We functionally characterized G6P and P_i transport activity of 19 naturally occurring GSD-Ib mutations, previously characterized by the co-expression assay and 4 newly identified G6PT missense mutations. We show the two assays yield equivalent results and expand the mutation database to 32 missense G6PT mutations.

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Fig. 1.

Western-blot analysis of G6PT synthesis in COS-1 cells. COS-1 cells were infected with a wild-type or mutant Ad-G6PT construct as described under Materials and methods. Each lane contained 20 μ g of microsomal proteins. The G6PT proteins were visualized with an anti-human G6PT luminal loop 1 antibody [10].

Table 1G6P and P_i transport activities of G6PT mutants in reconstituted proteoliposomes

Ad-G6PT	Location	G6P Uptake Activity	P _i Uptake Activity
Mock		0.36 ± 0.05	10.8 ± 0.1
Wild-type		2.37 ± 0.09 (100)	120.0 ± 2.4 (100)
G20D	H1	0.37 ± 0.04	11.1 ± 0.7
R28H	L1	0.38 ± 0.02	10.3 ± 1.2
G50R	L1	0.37 ± 0.01	10.8 ± 1.1
L85P	H2	0.34 ± 0.01	8.9 ± 1.5
W118R	C1	0.27 ± 0.02	9.2 ± 0.7
Q133P	C1	0.34 ± 0.04	16.3 ± 1.4 (5.0)
G149E	H3	0.35 ± 0.02	9.7 ± 0.6
G150R	H3	0.36 ± 0.02	9.3 ± 0.9
P153L	H3	0.61 ± 0.01 (12.4)	17.3 ± 0.2 (6.0)
C176R	H4	0.37 ± 0.01	9.6 ± 0.7
C183R	H4	0.38 ± 0.03	9.4 ± 1.1
P191L	C2	0.31 ± 0.04	9.1 ± 0.7
I278N	H6	0.59 ± 0.06 (11.4)	18.0 ± 0.5 (6.6)
R300C	C3	0.57 ± 0.04 (10.4)	23.7 ± 1.0 (11.8)
G339C	H8	0.49 ± 0.03 (6.5)	16.4 ± 1.0 (5.1)
G339D	H8	0.36 ± 0.02	9.5 ± 0.8
A367T	H9	1.14 ± 0.08 (38.8)	56.8 ± 2.4 (42.1)
A373D	H9	0.35 ± 0.04	8.4 ± 0.7
G376S	H9	0.60 ± 0.05 (11.9)	21.7 ± 0.7 (10.0)

G6P or P_i uptake activity (*nmol/mg proteoliposomal protein/9 min*) was analyzed in proteoliposomes reconstituted from detergent solubilized COS-1 microsomal membrane extracts expressing Ad-G6PT or an Ad-G6PT mutant and were loaded with 50 mM P_i as described under Materials and Methods. Results shown are from 3 independent experiments, each point determined in triplicate. Values represent mean ± SEM. Numbers in parentheses represent the percentage of wild-type transport activity. H, L, and C, the location of each mutant in transmembrane helices, luminal or the cytoplasmic loop, respectively [24].

Table 2G6P and P_i transport activities of 4 newly identified G6PT mutants

Ad-G6PT	Location	Microsomal G6P Uptake Activity	Proteoliposomal G6P Uptake Activity	Proteoliposomal P _i Uptake Activity
Mock		0.085 ± 0.003	0.31 ± 0.04	9.3 ± 0.3
Wild-type		0.332 ± 0.002 (100)	1.52 ± 0.01 (100)	112.7 ± 0.4 (100)
Y24H	H1	0.089 ± 0.006 (1.6)	0.37 ± 0.02 (5.0)	11.2 ± 0.7 (1.9)
R166L	L2	0.093 ± 0.002 (3.2)	0.34 ± 0.03 (2.5)	10.0 ± 0.8 (6.8)
L229P	H5	0.115 ± 0.005 (12.1)	0.35 ± 0.03 (3.3)	17.1 ± 0.3 (7.5)
G281R	H6	0.086 ± 0.003	0.28 ± 0.01	9.5 ± 0.3

Microsomal G6P uptake activity (*nmol/mg microsomal protein/3 min*) was analyzed in microsomes isolated from COS-1 cells co-infected with Ad-G6Pase-α and Ad-G6PT or an Ad-G6PT mutant. Proteoliposomal G6P or P_i uptake activity (*nmol/mg proteoliposomal protein/9 min*) was analyzed in proteoliposomes reconstituted from detergent solubilized COS-1 microsomal membrane extracts expressing a wild-type or a mutant Ad-G6PT and were loaded with 50 mM P_i as described under Materials and Methods. Results shown are from 3 independent experiments, each point determined in triplicate. Values represent mean ± SEM. Numbers in parentheses represent the percentage of wild-type transport activity. H, and L, the location of each mutant in transmembrane helices or luminal loops, respectively [24].