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## Functional analysis of mutations in a severe congenital neutropenia syndrome caused by glucose-6-phosphatase- $\beta$ deficiency

Su Ru Lin<sup>1</sup>, Chi-Jiunn Pan<sup>1</sup>, Brian C. Mansfield<sup>1,2</sup>, and Janice Yang Chou<sup>1,\*</sup>

<sup>1</sup>Section on Cellular Differentiation, Program on Developmental Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

<sup>2</sup>Foundation Fighting Blindness, Columbia, MD 21046

### Abstract

Glucose-6-phosphatase- $\beta$  (G6Pase- $\beta$  or G6PC3) deficiency is characterized by neutropenia and dysfunction in both neutrophils and macrophages. G6Pase- $\beta$  is an enzyme embedded in the endoplasmic reticulum membrane that catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate. To date, 33 separate *G6PC3* mutations have been identified in G6Pase- $\beta$ -deficient patients but only the p.R253H and p.G260R missense mutations have been characterized functionally for pathogenicity. Here we functionally characterize 16 of the 19 known missense mutations using a sensitive assay, based on a recombinant adenoviral vector-mediated expression system, to demonstrate pathogenicity. Fourteen missense mutations completely abolish G6Pase- $\beta$  enzymatic activity while the p.S139I and p.R189Q mutations retain 49% and 45%, respectively of wild type G6Pase- $\beta$  activity. A database of residual enzymatic activity retained by the G6Pase- $\beta$  mutations will serve as a reference for evaluating genotype-phenotype relationships.

### Keywords

G6PC3; mutation analysis; recombinant adenoviral vector

### 1. Introduction

Glucose-6-phosphatase- $\beta$  (G6Pase- $\beta$  or G6PC3) deficiency also known as severe congenital neutropenia syndrome type 4 (SCN4, MIM 612541) is a rare autosomal recessive disorder characterized by neutropenia and dysfunction of both neutrophils and macrophages [1-7]. The dysfunctions include impairments in respiratory burst, chemotaxis, calcium mobilization, and phagocytic activities [2-4, 6]. G6Pase- $\beta$  deficiency also underlies Dursun

\*Correspondence should be addressed to: Janice Y. 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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syndrome [8]. There are two enzymatically active G6Pases, the ubiquitously expressed G6Pase- $\beta$  [9] and the liver/kidney/intestine-restricted G6Pase- $\alpha$  (also known as G6PC) [10]. Both phosphatases catalyze the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate and both are key enzymes for intracellular glucose production. However, G6Pase- $\alpha$  is eight times more active than G6Pase- $\beta$  [9]. Topological analyses showed that both G6Pase- $\alpha$  [11] and G6Pase- $\beta$  [12] span the endoplasmic reticulum (ER) membrane, with multiple domains, their active sites lying inside the ER lumen. For G6P catalysis, both enzymes depend on two critical steps: the translocation of G6P from the cytoplasm into the ER lumen by the transmembrane protein G6P transporter (G6PT) and functional coupling of G6Pase with G6PT to form an active G6Pase/G6PT complex [3]. The liver/kidney/intestine-specific G6Pase- $\alpha$ /G6PT complex maintains interprandial blood glucose homeostasis while the ubiquitous G6Pase- $\beta$ /G6PT complex maintains energy homeostasis and functionality in neutrophils and macrophages [3, 4, 6]. Deficiencies in G6Pase- $\alpha$  cause glycogen storage disease type-Ia (GSD-Ia) characterized by impaired glucose homeostasis [3]. Deficiencies in G6PT cause GSD-Ib characterized by impaired glucose homeostasis and neutropenia/myeloid cell dysfunction typical of G6Pase- $\beta$  deficiency [3]. Recent studies have shown that enhanced neutrophil apoptosis underlies neutropenia [1-5, 13] and impaired neutrophil energy homeostasis underlies neutrophil dysfunction [4, 14] in both G6Pase- $\beta$  deficiency and GSD-Ib. G6Pase- $\beta$ -deficiency also presents with nonhematological defects, including prominent superficial venous pattern, congenital cardiac anomaly, urogenital malformations, and thrombocytopenia [1, 5, 7, 8] not reported in GSD-Ib, which points to additional roles for G6Pase- $\beta$  that are not yet characterized.

Human *G6PC3* is a single copy gene mapping to human chromosome 17q21 and consisting of 6 exons [15]. Thirty-three separate mutations, including 19 missense, 4 nonsense, 3 splicing, and 7 insertions and/or deletions, have been identified [1, 7, 8, 16-24]. To date, only the p.R253H [1] and p.G260R [5] mutations have been characterized functionally and shown to be pathogenic. However, the yeast assay system used previously [1] has a high phosphatase background activity which is sub-optimal for assaying the low activity expected for pathogenic mutations. The Epstein-Barr virus-transformed lymphoblastoid cell line assay system used previously [5] is also sub-optimal because the lines express very low G6Pase- $\beta$  activity, which also limit the assay sensitivity. Functional characterization in a more sensitive, low background assay should give more definitive results [9]. In this study, we adapt the recombinant adenovirus (rAd) vector-mediated expression system to increase the levels of expression of G6Pase- $\beta$  mutants, enhance the sensitivity of the phosphohydrolase activity assay, and analyze functionally 16 naturally occurring *G6PC3* missense mutations, yielding valuable information on functionally important residues of the G6Pase- $\beta$  protein.

## 2. Materials and methods

### 2.1. Construction of G6Pase- $\beta$ mutants

To construct G6Pase- $\beta$  mutants, nucleotides 1 to 1041 of human G6Pase- $\beta$  cDNA in the pAdlox shuttle vector [9], which contains the entire coding region, with the translation initiation codon, ATG, at nucleotides 1-3 was used as a template. For PCR-directed mutagenesis, the template was amplified using two outside PCR primers matching

nucleotides 1 to 20 (sense) and 1022 to 1041 (antisense) that flanked the 20 nucleotide long sense and antisense mutant primers. The mutated sequences were cloned in pAdlox and verified by DNA sequencing. The rAd vectors expressing G6Pase- $\beta$  mutants were then generated using the Cre-*lox* recombination system as described previously [9, 25]. The rAd vector carrying wild-type G6Pase- $\beta$  has been described previously [9]. The recombinant virus was plaque purified and amplified [26] to produce viral stocks with titers of approximately 1 to  $3 \times 10^{10}$  plaque forming unit (pfu) per ml.

## 2.2. Expression in COS-1 cells, phosphohydrolase, and Western-blot analysis

For activity assays, COS-1 cells in 25-cm<sup>2</sup> flasks were grown at 37 °C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with 4% fetal bovine serum. The cells were then infected with the appropriate rAd-G6Pase- $\beta$  wild type or mutant at 100 pfu/cell and incubated at 37 °C for 48 h. Mock infected COS-1 cells were used as controls. Phosphohydrolase activity was determined essentially as described previously [9]. Briefly, reaction mixtures (50  $\mu$ l) contained 50 mM cacodylate buffer, pH 6.5, 10 mM G6P and appropriate amounts of cell homogenates were incubated at 37 °C for 10 min [9].

The antibody against human G6Pase- $\beta$  was generated against a chimeric protein consisting of an N-terminal glutathione S-transferase (GST) fused to amino acids 77 to 114 of human G6Pase- $\beta$ , expressed in the pGEM4T-1 vector (Promega, Madison, WI). The GST-G6Pase- $\beta$  (amino acids 77-114) fusion protein was purified on a GStrap FF column (GE Healthcare Bio-Sciences, Pittsburgh, PA) and then used to generate a polyclonal antibody in rabbits.

For Western-blot analysis, proteins were resolved by electrophoresis through a 12% polyacrylamide-SDS gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore Co., Billerica, MA). The membranes were incubated overnight with a rabbit polyclonal antibody against amino acids 77 to 114 of human G6Pase- $\beta$  or a mouse monoclonal antibody against  $\beta$ -actin (Santa Cruz Biotechnology, Dallas, TX), washed, then incubated with the appropriate species of horseradish peroxidase-conjugated second antibody. The immunocomplex was visualized using the Immobilon<sup>TM</sup> western chemiluminescent HRP substrate (Millipore).

## 2.3. 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  $p < 0.05$ .

## 3. Results and discussion

G6Pase- $\beta$  is a hydrophobic protein anchored in the ER membrane by 9 helices, H1 to H9 creating 4 cytoplasmic loops (C1 to C4) [12] (Fig. 1). We constructed rAd vectors carrying 16 of the 19 known missense mutations, including 12 helical and 4 cytoplasmic-loop mutations that alter a total of 11 codons (Fig. 1). The mutations are: p.P44L and p.P44S in C1; p.M116I, p.M116IK, p.M116T, p.M116IV, and p.T118R in H3; p.S139I in C2; p.L154P and p.R161Q in H4; p.L185P in H5; p.R189Q in C3; p.L208R in H6; and p.G260D, in H7 (Fig. 1B). To provide cross-correlation to the previously used assays we included p.R253H [1] and p.G260R [5] in H7 as positive controls for the pathogenic mutations. The yeast

expression system, previously used to characterize the p.R253H mutant is sub-optimal because the background activity was 40% of wild type activity [1]. Similarly, the assay system using Epstein-Barr virus-transformed lymphoblastoid cell lines derived from healthy donors and G6Pase- $\beta$ -deficient patients previously used for the G260R mutant is also sub-optimal because normal lymphoblastoid cell line expresses very low G6Pase- $\beta$  activity averaging only 2-3 nmol/min/mg [5]. Therefore in this study the mutants were assayed in a rAd vector-mediated expression system with the wild type construct yielded an activity of  $107.5 \pm 5.1$  nmol/min/mg and a low background activity of only 7.4% of wild type activity (Table 1).

Ten of the 12 naturally occurring helical G6Pase- $\beta$  mutants, p.M116I, p.M116IK, p.M116T, p.L154P, p.R161Q, p.L185P, p.L208R, p.R253H, p.G260D, and p.G260R lacked G6P hydrolytic activity above background (Table 1). While G6Pase- $\beta$  activities of the p.M116V and p.T118R mutants were above background activity, the increases were not statistically significant (Table 1). These data suggest that the structural integrity, at least the H3 to H7 transmembrane helices, is critical for enzymatic activity of G6Pase- $\beta$ , similar to the finding that transmembrane integrity is important for G6Pase- $\alpha$  activity [27]. Among the 4 cytoplasmic loop mutations, p.P44L and p.P44S mutants at C1 had undetectable enzymatic activity (Table 1). The p.S139I in C2 [19] and p.R189Q in C3 [17] retained 49% and 45%, respectively of wild type G6Pase- $\beta$  activity (Table 1), suggesting they were more tolerant of these mutations. Western blot analysis showed that at the protein level, all mutants were expressed as efficiently as the wild-type G6Pase- $\beta$  protein (Fig. 2). Interestingly the two C1 null mutants, p.P44L and p.P44S migrated as two bands on SDS gel electrophoresis. Since G6Pase- $\beta$  is not a glycoprotein, the modification causing this change is unknown and the significance of this finding is unclear.

The c.416G>T/p.S139I mutation has been identified in two patients [7, 19]. One patient carrying the homozygous p.S139I mutations manifests neutropenia and arterial septal defect and died at age 9 months [19], suggesting that the p.S139I mutant would retain little or no enzymatic activity. Using the NNSplice software, Banka and Newman predicted that the splice donor site in the wild-type sequence is lost with c.416G > T and suggested that c.416G>T is a splice-site mutation [7]. The 49% of wild type G6Pase- $\beta$  activity retained by the p.S139I mutant indicates that this missense mutation is not pathogenic. Since the affected patient manifest syndromes characteristic of G6Pase- $\beta$  deficiency, the results are consistent with c.416G>T as a splice-site mutation [7]. The paternally inherited p.R189Q mutation, which is predicted to be tolerated (SIFT score of 0.41), was identified in a neutropenic patient who was also carrying deleterious heterozygous mutations in the *HAXI* gene [17]. The 45% of wild type G6Pase- $\beta$  activity retained by this mutant is consistent with the view that p.R189Q is not the disease-causing mutation.

G6Pase- $\beta$  (346 amino acid residues) and G6Pase- $\alpha$  (357 amino acid residues) share structural and functional similarities, including their active centers [9] and this is further borne out by our activity assays that show mutations of conserved residues lead to similar losses in activity (Table 1). These include: the M116I G6Pase- $\beta$  mutation that corresponds to the null p.M121I G6Pase- $\alpha$  mutation identified in the Maltase GSD-Ia dog [28] and the G260R G6Pase- $\beta$  mutation that corresponds to a null p.G270R G6Pase- $\alpha$  mutation identified

in human GSD-Ia patients [27]. Both residues are conserved among mammalian G6Pase- $\beta$  and G6Pase- $\alpha$  [9]. The p.G270V is another null G6Pase- $\alpha$  mutation identified in human GSD-Ia [27].

Since this work was undertaken, three new missense G6Pase- $\beta$  mutations, p.W59R in H2 [24], p.R253C in H7 [7], and p.L325R in H9 [23] (Fig. 1) have been identified. There 3 residues are conserved among mammalian G6Pase- $\beta$  [9]. The corresponding residues in G6Pase- $\alpha$  are p.W63, p.K263, and p.V338 which are conserved among mammalian G6Pase- $\alpha$  [9]. The p.R253H is a null G6Pase- $\beta$  mutation [1 and this study] and p.W63R and p.V338F are two null G6Pase- $\alpha$  mutations identified in human GSD-Ia patients [27]. The structural and functional similarities between the two phosphatases would suggest that W59R, p.R253C, and p.L325R mutations are pathogenic.

Several neutropenic G6Pase- $\beta$ -deficient patients do not manifest non-hematological abnormalities, including 3 patients carrying the homozygous p.P44S mutation, 1 patient carrying the homozygous p.M116T mutation, and 2 patients carrying compound heterozygous c.1000\_1001delAT/p.R253H mutations [7]. The c.1000\_1001delAT (M334fs) [7] and p.R253H [1] mutations are null and we now show that the p.P44S and p.M116T mutations also lack detectable G6Pase- $\beta$  activity. Therefore, the lack of non-hematological abnormalities in G6Pase- $\beta$ -deficient patients does not correlate with leakiness of the mutation. While mutational analysis has clearly established that inactivating G6Pase- $\beta$  mutations cause this genetic disorder, the information contained in this database cannot explain the observed phenotypic variations seen in G6Pase- $\beta$ -deficient patients carrying the same genetic mutation. Studies have shown that modifier genes play a considerable role in the phenotypic heterogeneity of genetic disorders [29], raising the possibility that phenotypic variations seen in G6Pase- $\beta$ -deficient patients could be caused by the existence of a G6Pase- $\beta$  modifier.

The G6PT/G6Pase- $\beta$  complex plays a key role in meeting the increased demand in energy in neutrophils and macrophages [3, 4, 6, 14]. Consequently, G6Pase- $\beta$ -deficient and GSD-Ib patients manifest a near identical abnormal myeloid phenotype of neutropenia and neutrophil/macrophage dysfunction. However, the non-hematological abnormalities seen in G6Pase- $\beta$  deficiency have not been reported for GSD-Ib patients. In addition to G6PT, there are two additional ER-associated G6P transporters, SLC37A1 and SLC37A2 [30]. Notably, SLC37A1 and SLC37A2 do not form functional complexes with either G6Pase- $\alpha$  or G6Pase- $\beta$  [30]. It is possible that in some non-gluconeogenic tissues, the G6P substrate could be translocated from the cytoplasm into the ER lumen by either SLC37A1 or SLC37A2, providing one explanation why the non-hematological abnormalities seen in G6Pase- $\beta$ -deficient patients have not been observed in GSD-Ib patients. In summary, using a sensitive assay for G6Pase- $\beta$ , we have generated a database of residual G6P hydrolytic activity retained by 16-codon mutations to serve as a reference for evaluating genotype-phenotype relationships and the many aspects of phenotypic variability seen in G6Pase- $\beta$  deficiency. This database will also help understanding the syndromic elements of G6Pase- $\beta$  deficiency such as bone marrow variability [1, 5] and Dursun syndrome [8].

## Abbreviations

|               |                                 |
|---------------|---------------------------------|
| <b>ER</b>     | endoplasmic reticulum           |
| <b>GSD-I</b>  | glycogen storage disease type I |
| <b>G6Pase</b> | glucose-6-phosphatase           |
| <b>G6P</b>    | glucose-6-phosphate             |
| <b>G6PT</b>   | glucose-6-phosphate transporter |
| <b>GST</b>    | glutathione S-transferase       |
| <b>rAd</b>    | recombinant adenovirus          |

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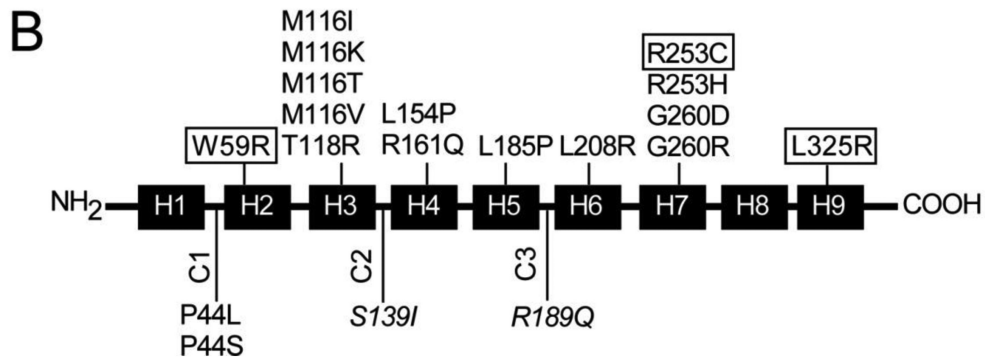
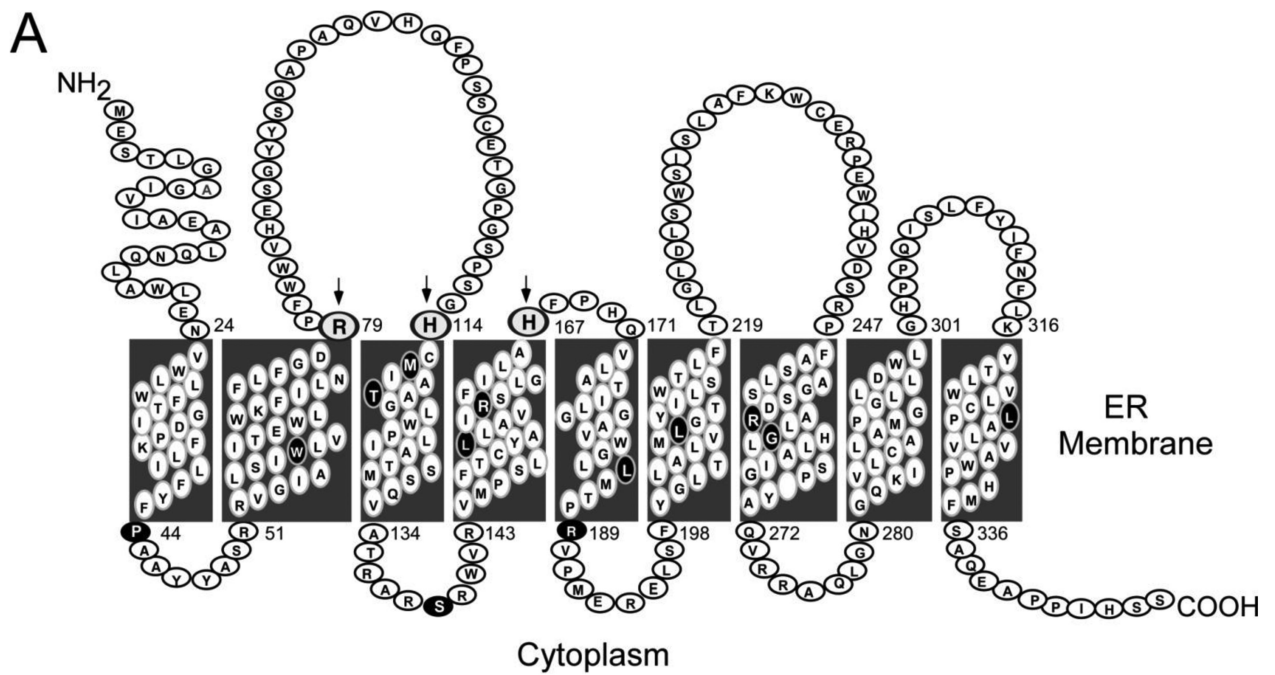
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### Highlights

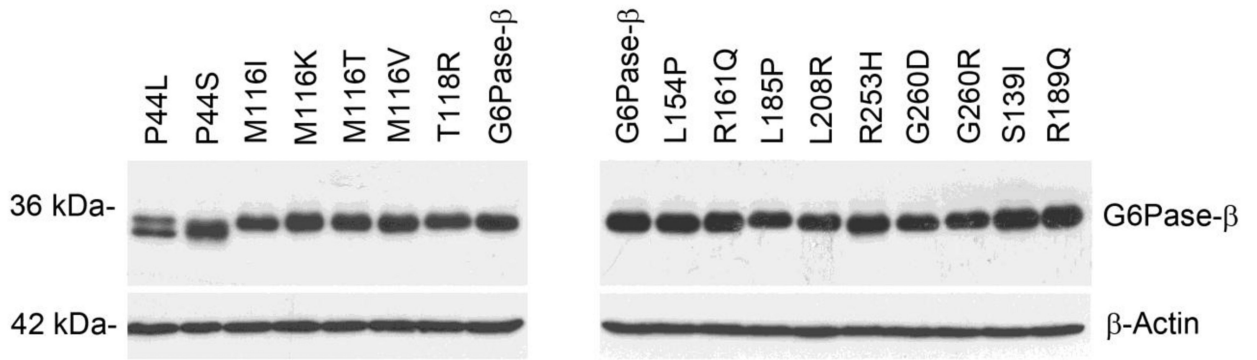
- Establish a sensitive, low background assay and characterize functionally 16 missense *G6PC3* mutations
- Generate a database of residual enzymatic activity retained by *G6PC3* mutations that serve as a reference for future evaluating genotype-phenotype relationships in glucose-6-phosphatase- $\beta$  deficiency





**Fig. 1.**

Missense G6Pase- $\beta$  mutations identified in G6Pase- $\beta$ -deficient patients. (A) The G6Pase- $\beta$  protein is shown to anchor in the ER membrane by 9 helices, H1 to H9. The 346 amino-acid residues are denoted by circles. The 13 amino-acid residues altered by the 19 missense mutations identified in G6Pase- $\beta$ -deficient patients are shown in black. The active site residues, R79, H114 and H167 [9], are indicated by arrows. (B) A summary of missense G6Pase- $\beta$  mutations. The 9-helical transmembrane domains of G6Pase- $\beta$  are marked as boxes H1 to H9 and the cytoplasmic loops are marked as C1 to C3. Mutations that have not been functionally characterized are boxed and the 2 mutations, p.S139I and p.R189Q, that retained significant activity are shown in italic.



**Fig. 2.** Western-blot analysis of G6Pase- $\beta$  in COS-1 cells transduced with a rAd vector containing wild type or mutant G6Pase- $\beta$ . Each lane contained 20  $\mu$ g proteins.

**Table 1**Phosphohydrolase activity of G6Pase- $\beta$  mutants

| Mutations         | Locations | Phosphoryhydrolase activity nmol/min/mg | Corresponding codons/mutations in G6Pase- $\alpha$ | References |
|-------------------|-----------|---|--|------------|
| Mock              |           | 8.0 $\pm$ 0.8 (0%)                      |  |            |
| WT                |           | 107.5 $\pm$ 5.1 (100%)                  |  |            |
| c.131C>T/p.P44L   | C1        | 8.4 $\pm$ 0.8                           | p.P48  | [20]       |
| c.130C>T/p.P44S   | C1        | 6.8 $\pm$ 0.3                           | p.P48  | [18]       |
| c.348G>A/p.M116I  | H3        | 7.5 $\pm$ 0.6                           | p.M121I  | [20]       |
| c.347T>A/p.M116K  | H3        | 7.0 $\pm$ 0.6                           | p.M121I  | [16]       |
| c.347T>C/ p.M116T | H3        | 7.9 $\pm$ 0.5                           | p.M121I  | [18]       |
| c.346A>G/p.M116V  | H3        | 9.1 $\pm$ 0.5                           | p.M121I  | [17]       |
| c.353C>G/p.T118R  | H3        | 9.3 $\pm$ 0.6                           | p.T123   | [15]       |
| c.416G>T/p.S139I* | C2        | 56.4 $\pm$ 5.1 (48.6%)                  | p.R147   | [19]       |
| c.461T>C/p.L154P  | H4        | 7.8 $\pm$ 1.2                           | p.Q163   | [22]       |
| c.482G>A/p.R161Q  | H4        | 8.8 $\pm$ 1.2                           | p.R169   | [20]       |
| c.554T>C/p.L185P  | H5        | 8.1 $\pm$ 0.3                           | p.F195   | [1]        |
| c.566G>A/p.R189Q  | C3        | 52.7 $\pm$ 3.0 (45.0%)                  | p.H199   | [16]       |
| c.623T>G/p.L208R  | H6        | 7.4 $\pm$ 0.5                           | p.S218   | [21]       |
| c.758G>A/p.R253H  | H7        | 7.9 $\pm$ 1.1                           | p.K263   | [1]        |
| c.779G>A/p.G260D  | H7        | 7.8 $\pm$ 1.2                           | p.G270V  | [20]       |
| c.778G>C/p.G260R  | H7        | 7.2 $\pm$ 0.7                           | p.G270R  | [1]        |

Phosphohydrolase activity of COS-1 cells infected with wild type or mutant rAd-G6Pase- $\beta$  and is presented as the mean  $\pm$  SEM. Mock infected cells are used as controls. H and C denote the locations of the mutants in helices 1 to 9 or cytoplasmic loops 1 to 3, respectively. Numbers in parentheses are % of wild type G6Pase- $\beta$  activity calculated as follows:  $100 \times (\text{mutant activity} - \text{mock activity}) / (\text{wild type activity} - \text{mock activity})$ . The phosphohydrolase activities of the p.M116V and p.T118R mutants were above background activity but the increases were not statistically significant. The p.M121I is a null G6Pase- $\alpha$  mutation identified in the Maltese GSD-Ia dog [28]. The p.G270V and p.G270R were null G6Pase- $\alpha$  mutations identified in human GSD-Ia patients [27]. To date, the G6Pase- $\beta$  mutations identified in codons p.P48, p.T123, p.R147, p.Q163, p.R169, p.F195, p.H199, p.S218, and p.R263 have not been reported in G6Pase- $\alpha$  of human GSD-Ia patients.

\* The c.416G>T/p.S139I mutation [19] is most likely a splicing mutation [7].