

J.-J. Shieh · C.-J. Pan · B. C. Mansfield · J. Y. Chou

In islet-specific glucose-6-phosphatase-related protein, the beta cell antigenic sequence that is targeted in diabetes is not responsible for the loss of phosphohydrolase activity

Received: 25 October 2004 / Accepted: 28 February 2005 / Published online: 13 July 2005
© Springer-Verlag 2005

Abstract *Aims/hypothesis:* There are three members of the glucose-6-phosphatase (G6Pase) family: (1) the liver/kidney/intestine G6Pase- α (encoded by *G6PC*), which is a key enzyme in glucose homeostasis; (2) the ubiquitous G6Pase- β (encoded by *G6PC3*); and (3) the islet-specific G6Pase-related protein (IGRP, encoded by *G6PC2*). While G6Pase- α and G6Pase- β are functional glucose-6-phosphate hydrolases, IGRP possesses almost no hydrolyase activity. This was unexpected since G6Pase- α is more closely related to IGRP than G6Pase- β . Recently, amino acids 206–214 in IGRP were identified as a beta cell antigen targeted by a prevalent population of pathogenic CD8⁺ T cells in autoimmune diabetes, suggesting that this peptide confers functional specificity to IGRP. We therefore investigated the molecular events that inactivate IGRP activity and the effects of the beta cell antigen sequence on the stability and enzymatic activity of G6Pase- α . *Methods:* Studies were performed using site-directed mutagenesis and transient expression assays. Protein stability was evaluated by Western blotting, proteasome inhibitor studies and in vitro transcription–translation. *Results:* We showed that the residues responsible for G6Pase activity are more extensive than previously recognised. Introducing the IGRP antigenic motif into G6Pase- α does not completely destroy activity, although it does destabilise the protein. The low hydrolytic activity in IGRP is due to the combination of multiple independent mutations. *Conclusions/interpretation:* The loss of catalytic activity in IGRP arises from the sum of many sequence differences.

G6Pase- α mutants containing the beta cell antigen sequence are preferentially degraded in cells, which prevents targeting by pathogenic CD8⁺ T cells. It is possible that IGRP levels in beta cells could dictate susceptibilities to diabetes.

Keywords Beta cell antigen · Diabetes mellitus · Glucose-6-phosphatase · Mutation analysis · Protein stability · Structure-function relationship

Abbreviations G6P: glucose-6-phosphate · G6Pase: glucose-6-phosphatase · IGRP: islet-specific G6Pase-related protein · NOD: non-obese diabetic

Introduction

The concentration of glucose in the blood is highly regulated. During meals, excessive dietary glucose is taken up by tissues, predominantly muscle and liver, converted to glucose-6-phosphate (G6P) and then on to glycogen. Between meals, as the blood glucose level starts to drop, the process is reversed, and the glycogen in the liver is broken down to G6P, which is then dephosphorylated to glucose and released back into the blood [1, 2]. An additional source of blood glucose is derived from the G6P generated by gluconeogenesis in the liver and the kidney [1, 2]. The glucose-6-phosphatase (G6Pase) enzyme family plays a key role in regulating interprandial blood glucose homeostasis [3, 4] by catalysing the hydrolysis of G6P to glucose in the terminal step of gluconeogenesis and glycogenolysis [5].

The G6Pase family has three members. G6Pase- α (G6PC) [6, 7] is expressed primarily in the liver, kidney and intestine [5, 8]. G6Pase- β (G6PC3 or UGRP) [9–12] is expressed ubiquitously, while the islet-specific G6Pase-related protein (G6PC2 or IGRP) [13–16] is restricted to expression in the pancreas. All three proteins are similarly orientated within the membrane of the endoplasmic reticulum and contain nine transmembrane domains [12, 16, 17].

J.-J. Shieh · C.-J. Pan · B. C. Mansfield · J. Y. Chou
Section on Cellular Differentiation,
Heritable Disorders Branch, National Institute of Child Health
and Human Development, National Institutes of Health,
MD USA

J. Y. Chou (✉)
Building 10, Room 9042, NIH, 9000 Rockville Pike,
Bethesda, MD 20892-1830, USA
e-mail: chouja@mail.nih.gov
Tel.: +1-301-4961094
Fax: +1-301-4026035

G6Pase- α is the enzyme most widely recognised for its role in glucose homeostasis. A deficiency in G6Pase, characterised by a failure in the production and release of glucose to the blood, causes the autosomal recessive disorder type Ia glycogen storage disease [3, 4]. G6Pase- β has been implicated in a more limited role of glucose homeostasis within the muscle [18]. However, the biological importance of IGRP had been unclear until a recent report linked it to autoimmune diabetes [19]. The critical finding was that IGRP amino acids 206–214 constituted a beta cell antigen targeted by a prevalent population of pathogenic CD8⁺ T cells [19] in nonobese diabetic (NOD) mice [20], a model of type 1 diabetes [21]. The corresponding peptide in G6Pase- α is not recognised by the pathogenic CD8⁺ T cells [19], suggesting this beta cell antigenic peptide may confer functional specificity to IGRP.

Both the human *G6PC* [22] and *G6PC3* [9] genes map to chromosome 17. The human *G6PC2* gene maps to a diabetes susceptibility locus [23] on chromosome 2 [14], consistent with its role as a beta cell autoantigen in diabetes [19]. Type 1, insulin-dependent, diabetes mellitus, is an autoimmune disorder which is characterised, in part, by an inflammation of the pancreatic islets, which progresses into T-cell-mediated destruction of insulin-producing beta cells [21]. The mechanisms of beta cell destruction in type 1 diabetes and the mechanisms underlying the genetic predisposition and resistance to this autoimmune disease are issues of fundamental importance, which is why IGRP has gained increased attention. The IGRP-reactive T-cell population was shown to constitute the earliest NOD islet infiltrates and the response to IGRP may be one of the first events leading to beta cell destruction by diabetogenic CD8⁺ T lymphocytes [19]. In human and mouse islets, there are several alternatively spliced IGRP transcripts [13, 14], whose roles are unclear. The full-length mRNA is not the most abundant transcript, but it is the only one that contains the antigenic peptide.

G6Pase- α is the most active member of the G6Pase family. Characterisation of the naturally occurring inactivating mutations of G6Pase- α , combined with site directed mutagenesis, has identified 43 key residues required for hydrolytic activity [24]. G6Pase- β , the second most active member of the family, which shares an overall 36% sequence identity [9–11] and retains 35 of the 43 critical residues identified in G6Pase- α [10], possesses ~12% of the G6P hydrolytic activity of G6Pase- α [10]. The third and most closely related family member to G6Pase- α is IGRP. It shares an overall 50% amino acid sequence identity [13, 14] and retains 39 of the 43 residues critical for G6Pase- α activity. Despite this, it is the least active hydrolase in the family, possessing 0% [13, 14] to 5% [15] of the activity of G6Pase- α . Of the four critical residue differences between IGRP and G6Pase- α , three are also altered in G6Pase- β . This suggests that while these three common mutations abrogate activity substantially, it is the fourth difference in IGRP, namely Lys²⁰⁹, which lies in the beta cell antigen motif, is responsible for the near total loss of hydrolytic activity. However, since the backbone residues differ between the three proteins, it is also pos-

sible that other residues, not previously identified, also interact to abolish the hydrolase activity. The only way to address this is to introduce the residues of interest back into the G6Pase- α backbone and monitor activity. We show that, unexpectedly, the four mutations in IGRP do not inactivate G6P hydrolysis. Indeed these four residues in G6Pase- α are more resilient to mutation than the literature suggests. However, G6Pase- α mutants harbouring amino acid substitutions in the motif equivalent to the antigenic nona-peptide in IGRP do have reduced stability. Our subsequent analysis shows that the marked loss of phosphohydrolase activity of IGRP appears to result from a number of independent mutations, some in regions not previously implicated in hydrolytic activity. We discuss the implication of this for diabetes and the evolution of the critical G6Pase activity.

Materials and methods

Construction of IGRP-like G6Pase- α mutants The human G6Pase- α -3FLAG cDNA was used as a template for construction of the G6Pase- α mutants, essentially as described previously [24]. The mutational primers spanned nucleotides 116–136 (T16A, AGG to GCG); nucleotides 227–253 (Q54N, CAG to AAT); nucleotides 437–463 (A124S, GCA to AGC); nucleotides 697–725 (L211K, CTC to AAG); nucleotides 941–973 (P293S, CCA to TCA); and nucleotides 689–736 (LIT211-213KTN, CTC to AAG at position 211, ATT to ACT at position 212 and ACC to AAC at position 213).

The G6Pase- α MEEG1-4del-3FLAG construct [17] was used as a template for construction of MEEG1-4del/SH135ins and MEEG1-4del/GM140ins mutants. The primers for MEEG1-4del/SH135ins were nucleotides 479–514 (5'-ACT CTT AGC CAC TCC ATC TTT CAG GGA AAG ATA AAG CCG ACC-3', sense, inserted sequences underlined) and nucleotides 455–490 (5'-GAT GGA GTG GCT AAG AGT AGA TGT GAC CAT CAC GTA GTA TAC-3', antisense) and the primers for MEEG1-4del/GM140ins were nucleotides 494–529 (5'-CAG GGA GGG ATG AAG ATA AAG CCG ACC TAC AGA TTT CGG TGC-3', sense, inserted sequences underlined) and nucleotides 469–505 (5'-TAT CTT CAT CCC TCC CTG AAA GAT GGA AAG AGT AGA TGT GAC-3', antisense). All constructs were verified by DNA sequencing.

Expression in COS-1 cells, phosphohydrolase assays and western blot analysis COS-1 cells in 25-cm² flasks were transfected with 10 μ g of a construct in the pSVL vector as previously described [24]. After incubation at 37°C for 2 days, the transfected cultures were harvested for phosphohydrolase assay or Western blot analysis.

Phosphohydrolase assays were performed essentially as described previously [24]. Reaction mixtures (100 μ l) containing 50 mmol/l cacodylate buffer, pH 6.5, 10 mmol/l G6P, 2 mmol/l EDTA and appropriate amounts of cell homogenates, were incubated at 30°C for 10 min. Phosphate release was determined by comparing the change in

absorbance at 820 nm with a standard curve constructed from a stock of inorganic phosphate solution. Phosphohydrolase activities were assayed in three separate transfections. Statistical analysis using the unpaired *t*-test was performed with the Prism Program (GraphPad Software, San Diego, CA, USA). Data are presented as means±SEM.

For Western blot analysis of FLAG-tagged constructs, proteins in transfected COS-1 lysates were separated by electrophoresis through a 12% polyacrylamide-SDS gel and trans-blotted onto polyvinylidene fluoride membranes (Millipore Co., MA, USA). The membranes were first incubated with a monoclonal antibody against the FLAG epitope and then with goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The immune complex was detected with the SuperSignal West Pico Chemiluminescent substrate obtained from Pierce (Rockford, IL, USA).

In vitro transcription–translation analysis In vitro transcription–translations of G6Pase- α constructs cloned into the pGEM-11Zf vector were performed using the TnT coupled reticulocyte lysate system (Promega Biotech, Madison, WI, USA) with L-[³⁵S]methionine as the labelled

precursor. The in vitro synthesised proteins were analysed by 12% polyacrylamide-SDS gel electrophoresis and visualised by fluoro-autoradiography.

Results

The conserved residue mutations are insufficient to inactivate G6Pase- α Functional analysis of naturally occurring mutations in the G6PC gene of patients with the glycogen storage disease type Ia, along with active site analysis, has identified 43 amino acid residues essential for G6Pase- α catalysis [24, 25]. Amino acid sequence alignment shows that 39 of the 43 essential amino acids are conserved between mammalian G6Pase- α and IGRP (Fig. 1). Four essential amino acids, Thr¹⁶, Gln⁵⁴, Ala¹²⁴ and Leu²¹¹ in human G6Pase- α , are substituted with non-conserved residues in human and mouse IGRP (Fig. 1). In G6Pase- β , Thr¹⁶, Gln⁵⁴ and Ala¹²⁴ are also substituted relative to G6Pase- α , but Leu²¹¹ is conserved. Since G6Pase- α is a more active phosphohydrolase than G6Pase- β , and IGRP has little [15] or no [13, 14] activity, the Thr¹⁶, Gln⁵⁴ and Ala¹²⁴ substitutions are thought to reduce the activity in G6Pase- β , while the addition of the

Fig. 1 Alignment of the amino acid sequences of mammalian G6Pase- α and IGRP. The conserved amino acids essential for G6Pase- α catalysis [24, 25] are bracketed. The Ile²¹², Lys²¹³ and Pro²⁹³ residues conserved amongst mammalian G6Pase- α but altered in human and mouse IGRP are shaded and shown in boldface letters. The beta cell antigen at amino acids 206–214 in human or mouse IGRP is bracketed. The G6Pase- α sequences are GENBANK accession numbers U01120 (*human*), U00445 (*mouse*), U07933 (*rat*) and U91844 (*dog*); and the IGRP accession numbers are NM021176 (*human*) and NM021331 (*mouse*)

Human G6Pase- α	MEEG M NVLHD FGIQ S THYL Q VNYQDSQDF ILVSVIAD L R NAFYVLFPIW FHI Q EA V G I K	60
Mouse G6Pase- α	---I---R---E---V---K-T---N	
Rat G6Pase- α	---R---R---E---V---I-T---N	
Dog G6Pase- α	---K---D---R---E---V---R	
Human IGRP	****-DF--R N-VL I QH- K D-RAY T F L NFM-NV G -P- I -FIY--LC-Q F N O T--T-	56
Mouse IGRP	****-DF--R S-VL I QH- E D-RTY G F L NFM-NV G -P- I -SIY--L-Q-N O N--T-	
Human G6Pase- α	LL M VA V I G D W LNLV F K W IL F Q R P Y W W V L D TDY S NT S V P LI K Q F P V T C E T G P G S P S H A	120
Mouse G6Pase- α	---V---F---S---I---G---G	
Rat G6Pase- α	---V---F---S---V---V	
Dog G6Pase- α	---V---F---M---G	
Human IGRP	MI---I---H---QE-QI-P-H-S-CLE--T---G	116
Mouse IGRP	MI---F-I---H---IQE-EI-P-H-S-CLE--T---G	
Human G6Pase- α	M G T IG V Y V Y V M VT S T L **S I F Q G K I K P T Y R F R C L N V I L M L G F W A V Q L N V C L S R I Y L A H F E	178
Mouse G6Pase- α	---A---**A---R-K---G---G	
Rat G6Pase- α	---A---**A---R-K-S---G---Y	
Dog G6Pase- α	---A---**A---R-RKR---IL---G	
Human IGRP	---A S C-W---AA-SHT V C G M D K F S I T L H -L T W S F-- S V --LI- I S-- I - V F I - T -- G	176
Mouse IGRP	---S S C-W---AA--Y T -S R M E E S S V T L H -L T W S F-- S V --LI- I S-- I - V F I - T -- G	
Human G6Pase- α	H O V V A G V L S G I A V A E T F S H I H S I Y N A S L K K Y F I T T F L F S F A I G F Y L L K G L G V D L L M T L	238
Mouse G6Pase- α	---R---R---RG---R---C---I---G---L	
Rat G6Pase- α	---R---R---RG---Q R ---C---G---L	
Dog G6Pase- α	---R---R---R---Q---G	
Human IGRP	---I L --I G -- M L--A-E-T P G-Q T -- G T--L K T N L--L--V---R V-NI-- S V	236
Mouse IGRP	---I L --I G -- M L--A-E-T P G V H M -- S V--L K T N V--L--L---R L F -I-- S V	
Human G6Pase- α	E K A Q R W C E Q P E W V H I D T H F A S L L K N L G T L F G L G L A L N S M Y R E S C K G K L S K W L P F R L S	298
Mouse G6Pase- α	---K---R---L---F---I---K---E---S F ---F A C	
Rat G6Pase- α	---K---R---L---F---I---K---E---R-S--- A C	
Dog G6Pase- α	---R---R---V---V---F---E---F---F	
Human IGRP	PI I - K K--AN- D -I---G-VR---V---F-I--E--FLL--R-G N NY T - S ---L C	296
Mouse IGRP	PI I - K K--AN- D -I---S---G-VR---V---F-I--E--F L R--Q-EN G T K P S ---L C	
Human G6Pase- α	I V A S L V L L H V F D S L K P P S Q V E L V F I V L S F C K S A V V P L A S V S V I P Y C L A Q V L G Q **P H K S L	357
Mouse G6Pase- α	---L---K---I---I---T---F---I---R I ---**T---	
Rat G6Pase- α	---G---L---I---S I ---I---T---F---I---R L ---**T---	
Dog G6Pase- α	---V---I---L---T---I---T---A---L---R- F D---**D---	
Human IGRP	ALT-- T I- Q L Y H F - Q I- T H E - H L---S I -- T V A F--- S V H M L M K--** S G-- Q	355
Mouse IGRP	ALT-- T T M Q L Y R F I-I- T H A - P L---L---S I -- M V A L--- V H M L M R P ** G D-- T K	

Leu²¹¹ substitution finally abrogates the activity in IGRP.

To determine the relative importance of each of these four essential amino acid substitutions, we introduced these mutations into human G6Pase- α , tagged with a C-terminal FLAG epitope. Previous studies have shown that a C-terminal FLAG epitope does not interfere with the activity of G6Pase- α [17, 24]. G6P hydrolytic activities of these mutants were examined after transient expression in COS-1 cells. The single mutants, T16I, Q54N, A124S and L211K, retain 75–81% of wild-type G6Pase- α activity, suggesting no single mutation is sufficient to cause the inactivation of IGRP. In combination, the double (T16I/Q54N), triple (T16I/Q54N/A124S), and quadruple (T16I/Q54N/A124S/L211K) mutants all retain 37% of wild-type activity (Table 1), again significantly greater than the residual 5% of activity that is reported for IGRP [15]. To ensure that the lack of activity does not arise from poor expression or stability of the mutants, the cell lysates were also subjected to Western blot analysis using a monoclonal anti-FLAG antibody. Both the single and multiple mutants are expressed to a similar level as wild-type G6Pase- α (Fig. 2). Therefore, inactivation of the phosphohydrolase activity in IGRP is caused, in part, by mutations that lie beyond the 43 residues previously identified as critical to the catalytic activity of G6Pase- α .

G6Pase- α mutants harbouring amino acids 209–211 in the autoantigen in IGRP are unstable In looking for the other residues critical for hydrolytic activity, the sequence context of Leu²¹¹ in G6Pase- α became of particular

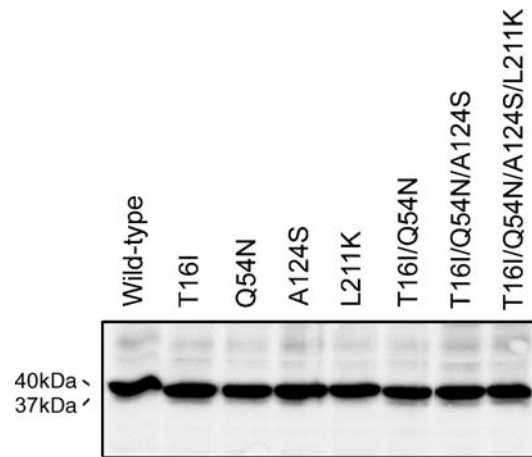


Fig. 2 Western blot analysis of wild-type and mutant G6Pase- α synthesis. COS-1 cells were transfected with FLAG-tagged wild-type or mutant human G6Pase- α cDNA constructs as described under [Materials and methods](#). The G6Pase- α proteins on the western membranes were visualised by an anti-FLAG monoclonal antibody; each lane contained 20 μ g protein

interest because amino acids 204–216 (VYLKTNVFL) in murine IGRP have been identified as a beta cell antigen targeted by a prevalent population of pathogenic CD8⁺ T cells in autoimmune diabetes [19]. Since the homologous residues in human G6Pase- α (amino acids 206–218, KYCLITIFL) are not autoantigenic [19], other residues in this motif might be critical for the differences between G6Pase- α and IGRP. The major difference between this

Table 1 Phosphohydrolase activity of IGRP-like human G6Pase- α mutants

G6Pase- α mutants	Phosphohydrolase activity (nmol min ⁻¹ mg ⁻¹)	Phosphohydrolase activity (% wild-type)
Mock	785±0.25	0.0
Wild-type	48.05±1.98	100.0
T16I	38.27±2.19	75.7
Q54N	40.25±3.99*	80.6
A124S	38.02±2.40*	75.0
L211K	38.26±2.43*	75.6
T16I/Q54N	22.76±0.70	37.1
T16I/Q54N/A124S	22.88±1.71	37.4
T16I/Q54N/A124S/L211K	23.12±1.23	38.0
P293S	35.33±1.53	68.4
L211K/P293S	21.60±1.06	34.2
T16I/Q54N/L211K/P293S	21.75±2.09	34.6
T16I/Q54N/A124S/L211K/P293S	21.59±2.29	34.2
LIT211–213KTN	17.77±1.23	24.7
LIT211–213KTN/P293S	12.40±1.00	11.3
T16I/Q54N/LIT211–213KTN/P293S (M6)	11.88±0.74	10.0
T16I/Q54N/A124S/ LIT211–213KTN/ P293S (M7)	10.72±0.57	7.1
MEEG1-4del	43.81±1.03*	89.4
MEEG1-4del/SH135ins	21.65±1.23	34.3
MEEG1-4del/GM140ins	29.22±1.22	53.1
MEEG1-4del/SH135ins/M7	7.23±0.33	0.0
MEEG1-4del/GM140ins/M7	7.65±0.72	0.0

COS-1 cells were transfected with a FLAG-tagged wild-type or mutant G6Pase- α construct in a pSVL vector. Phosphohydrolase activities were assayed in three separate transfections (see [Materials and methods](#)). Data are means±SEM. The unpaired *t*-test was used to evaluate the statistical difference between mutant and wild-type activities *For Q54N, *p*=0.01; A124S, *p*=0.017; L211K, *p*=0.019; and MEEG1-4del, *p*=0.08. For all other comparisons *p*<0.001

nona-peptide motif between these proteins is that the sequence LIT (amino acids 211–213) in G6Pase- α is substituted by the sequence KTN (amino acids 209–211) in IGRP. We therefore investigated the effects of LIT to KTN mutations on the stability and activity of G6Pase- α . While the L211K mutant retains 75.6% of wild-type activity, the LIT211-213KTN mutants drops to 24.7% of wild-type activity (Table 1). Cell lysates were subjected to Western blot analysis (Fig. 3a) to determine if the mutations lowered the G6P hydrolytic activity by altering the expression or stability of the proteins. They did; the LIT211–213KTN mutant is expressed at a significantly lower level than the wild-type or L211K mutant.

An additional difference between G6Pase- α and IGRP that drew attention was the non-conservative Pro²⁹³ to Ser (P293S) in helix 8 of G6Pase- α (Fig. 1), which lies in a transmembrane domain of the proteins. The stability and enzymatic activity of G6Pase- α depends upon the structural integrity of transmembrane helices [24]. The P293S mutation lies close to the critical Arg²⁹⁵ residue that is conserved among G6Pase- α , G6Pase- β and IGRP [10]. By itself the P293S mutation retains 68.4% of wild-type G6Pase- α activity (Table 1), and is associated with a lower level of expression compared with the wild-type G6Pase- α (Fig. 3a), which could account for the partial loss of enzymatic activity. When the P293S mutation was combined the L211K mutation, the activity of the resulting double mutant dropped to 34.2% of wild-type G6Pase- α , due at least in part to a further loss of expression or stability (Fig. 3a). However, the addition of the other three critical residue changes T16I, Q54N and A124S, to form T16I/Q54N/A124S/L211K/P293S, did not result in a further loss of activity (Table 1), having a similar activity to the L211K/P293S mutant. These data show that while the L211K and

P293S mutations cooperate in an additive manner, neither cooperates further with the T16I, Q54N or A124S mutations (Table 1). Therefore the critical residue mutations are not synergistic.

In contrast, the LIT211–213KTN mutations are cooperative with P293S, the combined mutant (LIT211–213KTN/P293S), exhibiting only 11.3% of wild-type G6Pase- α activity (Table 1)—much less than that seen with any of the critical residue mutations. Again, the loss of activity (Fig. 3a) is associated with a loss of expression and/or stability of the mutant protein. To determine if these mutations, combined with the critical residue changes (T16I, Q54N, A124S), could explain the inactivating mutations, the T16I/Q54N/A124S/LIT211–213KTN/P293S (M7) G6Pase- α mutant was assayed for activity. The combinatorial M7 mutations lost slightly more activity, leaving a residual activity of 7.1% of wild-type G6Pase- α (Table 1). Consistent with the previous observation, this reduction of activity is associated with a loss of expression and/or stability of the protein (Fig. 3a).

To differentiate between the possibilities of a loss of expression, and a loss of stability, we examined expression of the mutant proteins in a cell-free transcription-translation system. The LIT211–213KTN, LIT211–213KTN/P293S and T16I/Q54N/A124S/LIT211–213KTN/P293S mutants, and wild-type G6Pase- α , all direct the synthesis of similar amounts of protein *in vitro* (Fig. 3b). This suggests that the loss of activity reflects reduced protein stability *in vivo*. This leads to lower levels of accumulation *in vivo* and confirms the vital role of the transhelical domains in the structural integrity of G6Pase- α .

Insertions and deletions within G6Pase- α The optimal sequence alignment between G6Pase- α and IGRP starts

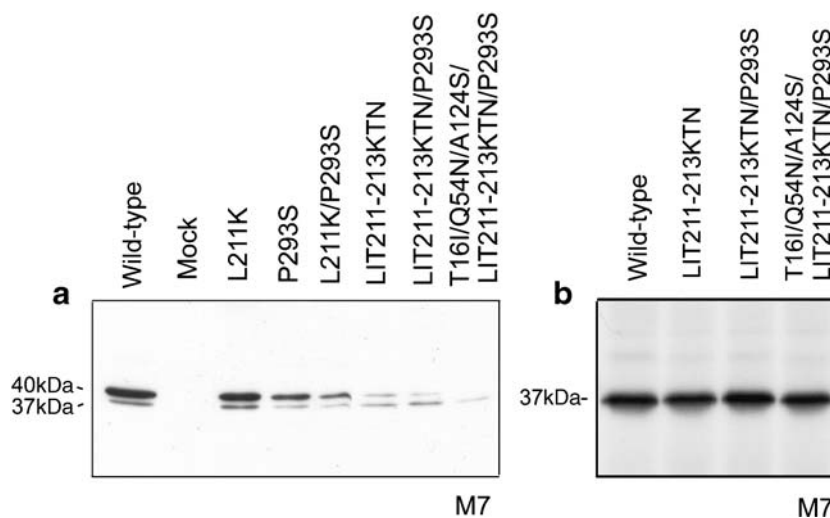


Fig. 3 Comparison of *in vivo* and *in vitro* synthesis of G6Pase- α wild-type and helical mutants. **a** Western blot analysis of *in-vivo*-expressed constructs. COS-1 cells were transfected with FLAG-tagged wild-type or mutant G6Pase- α cDNA constructs (see Materials and methods). Mock transfected cells were used as controls. The G6Pase- α proteins on the western membranes were visualised by an anti-FLAG monoclonal antibody; each lane con-

tained 20 μ g protein. **b** *In vitro* transcription-translation analysis. *In vitro* synthesis of G6Pase- α directed by FLAG-tagged wild-type or mutant G6Pase- α construct in a pGEM-11Zf vector was performed using the TnT coupled reticulocyte lysate system. L-[³⁵S]methionine was used as the labelled precursor and after electrophoresis, the proteins were visualised by fluoro-autoradiography

the alignment of Met¹ of IGRP with the essential residue Met⁵ of G6Pase- α (Fig. 1). The resulting shift requires a subsequent two-amino-acid insertion further down the sequences to align the 357-residue G6Pase- α with the 355-residue IGRP. Two theoretically equivalent alignments place this insertion between either amino acids 135 and 136 (Fig. 1) or amino acids 140 and 141 in human G6Pase- α .

Removing the first four amino acids in G6Pase- α (MEEG1-4del) has little effect on the activity (Table 1) or expression (Fig. 4a) of G6Pase- α . Combining this deletion with either of the two alternative insertions (MEEG1-4del/SH135ins) or (MEEG1-4del/GM140ins) reduces expression a little (Fig. 4a) and reduces activity between 34 and 53% (Table 1), the SH135ins being the slightly more destabilising mutation. Finally, combination of the G6Pase- α insertion/deletion mutations, with the critical residue and helical mutations (MEEG1-4del/SH135ins/M7 or MEEG1-4del/GM140ins/M7) abolishes all detectable activity (Table 1) but also reduces the expression and/or stability of the protein to a level where it is almost not detectable (Fig. 4a). However, when examined in a cell-free transcription-translation system (Fig. 4b), the MEEG1-4del, MEEG1-4del/SH135ins, MEEG1-4del/GM140ins, MEEG1-4del/SH135ins/M7 and MEEG1-4del/GM140ins/M7 mutants all direct the synthesis of similar amounts of proteins. This also suggests that the M7-containing mutants have reduced protein stability in vivo.

G6Pase- α mutants lacking expression have reduced stability G6Pase- α is processed through the proteasome pathway [24]. Lactacystin, a proteasome inhibitor [26, 27] inhibits the pathway by stabilising endoplasmic reticulum membrane proteins against proteolytic turnover. To further distinguish between losses of activity due to protein

instability, and poor expression, we examined G6Pase- α mutant protein accumulation in the absence or presence of lactacystin. In the presence of lactacystin, wild-type G6Pase- α greatly increases the accumulation of both the 37-kDa precursor and 40-kDa post-translationally modified G6Pase- α proteins. The mutant proteins similarly increase in the presence of the inhibitor (Fig. 5), suggesting that the IGRP-like G6Pase- α mutants have reduced stability in vivo. However, despite a marked increase in protein accumulation in the presence of lactacystin, both the wild-type and the mutant G6Pase- α proteins have decreased enzymatic activities. Moreover, the ratios of their relative activities in the absence or presence of lactacystin remain similar (Table 2), suggesting that the additional proteins accumulated in the presence of lactacystin are non-functional, most probably as a result of incorrect folding.

Discussion

Until very recently, blood glucose homeostasis between meals was thought to be regulated primarily by the liver and kidney. Only these tissues were known to express G6Pase- α [5, 8], which is required to convert G6P, generated by gluconeogenesis and glycogenolysis, into glucose for release to the blood. Recently, however, two additional members of the G6Pase family, G6Pase- β [9–12] and IGRP [13–16] were identified. IGRP, which bears the closest protein sequence to G6Pase- α , is expressed solely in the pancreatic beta cells [13, 14], while G6Pase- β , a more distant relative, is expressed ubiquitously [9]. G6Pase- β has an activity very similar to G6Pase- α , although its specific activity is only ~12% that of G6Pase- α [10, 11]. Surprisingly, IGRP, which is more closely relat-

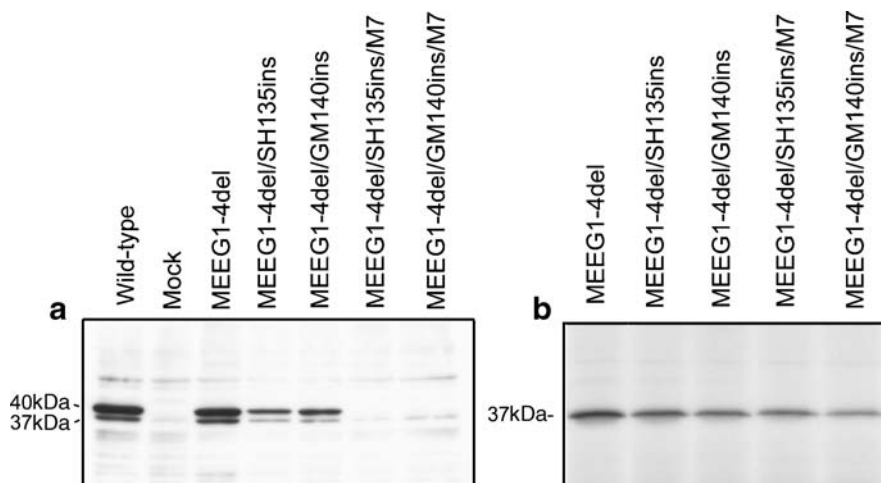


Fig. 4 Comparison of in vivo and in vitro synthesis of G6Pase- α wild-type and insertion/deletion mutants. **a** Western blot analysis of in-vivo-expressed constructs. COS-1 cells were transfected with FLAG-tagged wild-type or mutant human G6Pase- α cDNA constructs (see [Materials and methods](#)). Mock transfected cells were used as controls. The G6Pase- α proteins on the western membranes were visualised by an anti-FLAG monoclonal antibody; each lane

contained 20 μ g protein. **b** In vitro transcription-translation analysis. In vitro synthesis of G6Pase- α directed by FLAG-tagged wild-type or mutant G6Pase- α construct in a pGEM-11Zf vector was performed using the TnT coupled reticulocyte lysate system. L-[³⁵S] methionine was used as the labelled precursor and after electrophoresis, the proteins were visualised by fluoro-autoradiography

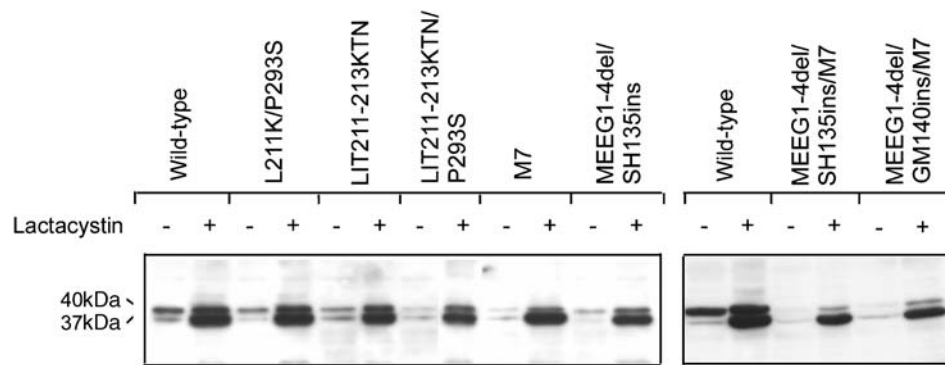


Fig. 5 The effect of the proteasome inhibitor lactacystin on degradation of IGRP-like human G6Pase- α mutants. Two sets of COS-1 cells were transfected with FLAG-tagged wild-type or mutant G6Pase- α constructs. After 34 h incubation at 37°C, 1 μ mol/l lactacystin was added to one set of cultures and the incubation was

continued for an additional 12–14 h. The steady-state levels of protein in transfected cultures were examined by western blot analysis using an anti-FLAG monoclonal antibody; each lane contained 20 μ g protein

ed to G6Pase- α structurally, has appeared to lack activity [13, 14], or at most retain only 5% of the activity of G6Pase- α [15]. An inactivated protein in normal tissue is not usually of immediate interest. However, the recent observation that a nona-peptide in IGRP is a beta cell antigen for autoimmune diabetes prompted us to look more closely at IGRP to understand the basis of its inactivation, and the potential for reactivation in diabetes. Of the 43 amino acids critical for hydrolytic activity of G6Pase- α [24, 25], four are not conserved in IGRP (Fig. 1), and three of the four are also altered in G6Pase- β (10). The initial assumption was that the reduced hydrolytic activity of G6Pase- β was due to these three substitutions and that the loss of the remaining activity in IGRP was due to the fourth mutation, a unique Lys²⁰⁹ (corresponding to Leu²¹¹ in human G6Pase- α). In attempting to confirm this we discovered that these four critical sites in G6Pase- α can tolerate some specific substitutions without a dramatic loss of activity. In turn, we demonstrated that the low hydrolytic activity

in IGRP is due to the combination of multiple independent mutations, which reduces the potential of its reactivation to a phosphohydrolase.

The sequence context of the Lys²⁰⁹ substitution in IGRP attracted our attention. It is part of the sequence lying between amino acids 206 and 214 (VYLKTNVFL) in murine IGRP, which represent the beta cell antigenic peptide targeted by a prevalent population of pathogenic CD8⁺ T cells [19] in NOD mice [20]. The corresponding peptide (KYCLITFL) between amino acids 208 and 216 in murine G6Pase- α is not auto-antigenic [19]. Between the mouse and human IGRP (T/VYLKTNL/VFL) and the mammalian G6Pase- α (K/RYP/CLIT/IFL), the peptides differ at six of the nine positions (Fig. 1). The major difference is the LIT (amino acids 211–213 in G6Pase- α) to KTN (amino acids 209–211 in IGRP) substitutions. We show that the G6Pase- α LIT211-213KTN triple mutant retains 24.7% of wild-type enzymatic activity, mainly as the result of decreased protein stability compared with the

Table 2 Effects of lactacystin on the phosphohydrolase activity of IGRP-like human G6Pase- α mutants

G6Pase- α mutants	Phosphohydrolase activity without lactacystin		Phosphohydrolase activity with lactacystin	
	nmol min ⁻¹ mg ⁻¹	% wild-type	nmol min ⁻¹ mg ⁻¹	% wild-type
Mock	8.60±0.40	0.0	5.11±0.35	0.0
Wild-type	61.68±1.20	100.0	20.29±1.29	100.0
L211K	51.11±2.38*	80.0	15.68±1.11*	69.6
L211K/P293S	31.19±0.75	42.6	10.08±0.49	33.0
LIT211–213KTN	22.92±0.33	27.0	8.78±0.32	24.2
LIT211–213KTN/P293S	15.62±1.21	13.2	6.39±0.06	8.4
T16I/Q54N/A124S/ LIT211–213KTN/P293S (M7)	15.63±0.02	13.2	6.47±0.74	9.0
MEEG1-4del/SH135ins	30.48±0.55	41.2	8.73±0.05	23.8
MEEG1-4del/SH135ins/M7	8.60±0.4	0.0	4.84±0.01	0.0
MEEG1-4del/GM140ins/M7	8.49±0.4	0.0	5.16±0.30	0.0

Two sets of COS-1 cells were transfected with a FLAG-tagged wild-type or mutant G6Pase- α construct. After 34 h incubation at 37°C, 1 μ mol/l lactacystin was added to one set of cultures and the incubation was continued for an additional 12–14 h. Phosphohydrolase activities were assayed in three separate transfections (see [Materials and methods](#)). Data means±SEM. The unpaired *t*-test was used to evaluate the statistical difference between mutant and wild-type activities

*For L211K, *p*=0.008 in the absence of lactacystin and *p*=0.03 in the presence of lactacystin. For all other comparisons *p*<0.001

wild-type G6Pase- α . Our results suggest that a G6Pase harbouring mutations in the IGRP auto-antigenic peptide is preferentially degraded in cells, and thus would not present a risk of diabetes.

The KTN motif, however, is not destabilising in IGRP. This led to the recognition that despite the structural similarity, and the close conservation of the residues previously identified as critical for G6P hydrolytic activity between G6Pase- α and IGRP, the motif must be interacting with backbone residues that differ between G6Pase- α and IGRP. Our identification of a critical residue lying within a transmembrane helix reinforces the view that the transmembrane domains in the G6Pase family are not passive membrane anchors but actively interacting with other sequences to control stability. In the case of IGRP, the stabilising interactions between the KTN motif and other sequences in the protein have left it, as a pancreatic beta cell-specific antigen, open to recognition by diabetogenic CD8⁺ T lymphocytes.

In the pancreatic beta cells, the intracellular concentration of G6P acts as a measure of the blood glucose level and regulates the activation of insulin gene transcription [28, 29]. Any consistent disruption in intracellular G6P, especially a lowering of the concentration via hydrolysis of G6P, would reduce insulin expression and could become a contributing factor to diabetes mellitus. Consistent with this, G6Pase expression is elevated in human diabetic patients [30] and in rodent models of diabetes [31–33]. The expression of a G6Pase-related protein is not favourable to islet G6P metabolism or blood glucose control. This may explain why alternatively spliced IGRP transcripts lacking exon-4, which encodes the catalytic site of the hydrolase [25], and multiple mutations in the full-length transcript, evolved to inactivate the IGRP protein.

To further increase our understanding of the role of IGRP in diabetes, it will be important in future studies to look for mutations within the IGRP protein associated with the disease. Since only the full-length IGRP mRNA, which is not the most abundant transcript, contains the antigenic peptide [13, 14], it will also be important to determine whether the ratio of full-length to truncated IGRP mRNA differs between normal and diabetes-susceptible mammals in order to ascertain whether an increase in the full-length IGRP predicts a risk of developing diabetes.

References

- Gerich J, Meyer C, Woerle HJ, Stumvoll M (2001) Renal gluconeogenesis. *Diabetes Care* 24:382–391
- Cano N (2002) Bench-to-bedside review: glucose production from the kidney. *Critical Care* 6:317–321
- Chou JY, Matern D, Mansfield BC, Chen YT (2002) Type I glycogen storage diseases: disorders of the glucose-6-phosphatase complex. *Curr Mol Med* 2:121–143
- Chen Y-T (2001) Glycogen storage diseases. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B (eds) *The metabolic and molecular bases of inherited disease*, 8th edn. McGraw-Hill Inc., New York, pp 1521–1551
- Nordlie RC, Sukalski KA (1985) Multifunctional glucose-6-phosphatase: a critical review. In: Martonosi AN (ed) *The enzymes of biological membrane*, 2nd edn. Plenum Press, New York, pp 349–398
- Lei K-J, Shelly LL, Pan C-J, Sidbury JB, Chou JY (1993) Mutations in the glucose-6-phosphatase gene that cause glycogen storage disease type 1a. *Science* 262:580–583
- Shelly LL, Lei K-J, Pan C-J et al (1993) Isolation of the gene for murine glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1a. *J Biol Chem* 268:21482–21485
- Pan C-J, Kei K-J, Chen H, Ward JM, Chou JY (1998) Ontogeny of the murine glucose-6-phosphatase system. *Arch Biochem Biophys* 358:17–24
- Martin CC, Oeser JK, Svitek CA, Hunter SI, Hutton JC, O'Brien RM (2002) Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. *J Mol Endocrinol* 29:205–222
- Shieh J-J, Pan C-J, Mansfield BC, Chou JY (2003) A glucose-6-phosphate hydrolase, widely expressed outside the liver, can explain age-dependent resolution of hypoglycemia in glycogen storage disease type 1a. *J Biol Chem* 278:47098–47103
- Guionie O, Clottes E, Stafford K, Burchell A (2003) Identification and characterisation of a new human glucose-6-phosphatase isoform. *FEBS Lett* 551:159–164
- Ghosh A, Shieh J-J, Pan C-J, Chou JY (2004) Histidine-167 is the phosphate acceptor in glucose-6-phosphatase- β forming a phosphohistidine-enzyme intermediate during catalysis. *J Biol Chem* 279:12479–12483
- Arden SD, Zahn T, Steegers S et al (1999) Molecular cloning of a pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein. *Diabetes* 48:531–542
- Martin CC, Bischof LJ, Bergman B et al (2001) Cloning and characterization of the human and rat islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) genes. *J Biol Chem* 276:25197–25207
- Petrolonis AJ, Yang Q, Tummino PJ et al (2004) Enzymatic characterization of the pancreatic islet-specific glucose-6-phosphatase-related protein (IGRP). *J Biol Chem* 279:13976–13983
- J-J Shieh, C-J Pan, BC Mansfield, JY Chou (2004) The islet-specific glucose-6-phosphatase-related protein, implicated in diabetes, is a glycoprotein embedded in the endoplasmic reticulum membrane. *FEBS Lett* 562:160–164
- Pan C-J, Lei K-J, Annabi B, Hemrika W, Chou JY (1998) Transmembrane topology of glucose-6-phosphatase. *J Biol Chem* 273:6144–6148
- Shieh J-J, Pan C-J, Mansfield BC, Chou JY (2004) A potential new role for muscle in blood glucose homeostasis. *J Biol Chem* 279:26215–26219
- Lieberman SM, Evans AM, Han B et al (2003) Identification of the β cell antigen targeted by a prevalent population of pathogenic CD8⁺ T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A* 100:8384–8388
- Serreze DV, Leiter EH (2001) Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice. *Curr Dir Autoimmun* 4:31–67
- Castano L, Eisenbarth GS (1990) Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu Rev Immunol* 8:647–679
- Lei K-J, Pan C-J, Shelly LL, Liu J-L, Chou JY (1994) Identification of mutations in the gene for glucose-6-phosphatase, the enzyme deficient in glycogen storage disease type 1a. *J Clin Invest* 93:1994–1999
- Pociot F, McDermott MF (2002) Genetics of type 1 diabetes mellitus. *Genes Immun* 3:235–249
- Shieh J-J, Terzioglu M, Hiraiwa H et al (2002) The molecular basis of glycogen storage disease type 1a: structure and function analysis of mutations in glucose-6-phosphatase. *J Biol Chem* 277:5047–5053

25. Ghosh A, Shieh J-J, Pan C-J, Sun M-S, Chou JY (2002) The catalytic center of glucose-6-phosphatase: His¹⁷⁶ is the nucleophile forming the phosphohistidine-enzyme intermediate during catalysis. *J Biol Chem* 277:32837–32842
26. Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 268:726–731
27. Fenteany G, Schreiber SL (1998) Lactacystin, proteasome function, and cell fate. *J Biol Chem* 273:8545–8548
28. Vaulont S, Vasseur-Cognet M, Kahn A (2000) Glucose regulation of gene transcription. *J Biol Chem* 275:31555–31558
29. Matschinsky FM, Glaser B, Magnuson MA (1998) Perspective in diabetes. Pancreatic β -cell glucokinase: closing the gap between theoretical concepts and experimental realities. *Diabetes* 47:307–315
30. Clore JN, Stillman J, Sugerma H (2000) Glucose-6-phosphatase flux in vitro is increased in type 2 diabetes. *Diabetes* 49:969–974
31. Liu Z, Barrett EJ, Dalkin AC, Zwart AD, Chou JY (1994) Effect of acute diabetes on rat hepatic glucose-6-phosphatase activity and its messenger RNA level. *Biochem Biophys Res Commun* 205:680–686
32. Haber BA, Chin S, Chuang E, Buikhuisen W, Najji A, Taub R (1995) High levels of glucose-6-phosphatase gene and protein expression reflect an adaptive response in proliferating liver and diabetes. *J Clin Invest* 95:832–841
33. Massillon D, Barzilai N, Chen W, Hu M, Rossetti L (1996) Glucose regulates in vivo glucose-6-phosphatase gene expression in the liver of diabetic rats. *J Biol Chem* 271:9871–9874