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The islet-specific glucose-6-phosphatase-related protein, implicated in diabetes, is a glycoprotein embedded in the endoplasmic reticulum membrane

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Abstract The islet-specific glucose-6-phosphatase-related protein (IGRP) has no known catalytic activity, but is of interest because it is the source of the peptide autoantigen targeted by a prevalent population of pathogenic CD8$^+$ T cells in non-obese diabetic mice. To better understand the potential roles of this protein in diabetes mellitus, we examine the subcellular localization and membrane topography of human IGRP. We show that IGRP is a glycoprotein, held in the endoplasmic reticulum by nine transmembrane domains, which is degraded in cells predominantly through the proteasome pathway that generates the major histocompatibility complex class I-presented peptides.

Key words: Glucose-6-phosphatase; Diabetes mellitus; Membrane topography; Endoplasmic reticulum; Proteasome; Transmembrane domain

1. Introduction

The glucose-6-phosphatase (G6Pase) family is composed of three proteins: G6Pase-α [1,2], G6Pase-β, also known as ubiquitously expressed G6Pase-related protein [3–5], and IGRP, the islet-specific G6Pase-related protein [6,7]. IGRP was initially identified by its ~50% amino acid sequence identity to G6Pase-α as a putative islet-specific phosphohydrolase [6,7]. Given the critical role of G6Pase-α in the control of blood glucose homeostasis between meals [8,9], the discovery of IGRP appeared to provide a missing link between G6Pase activity and diabetes. But despite sharing the same active site structure as G6Pase [8], IGRP fails to exhibit phosphohydrolase activity and had appeared to be an inactive member of the G6Pase family [6,7]. Recently, however, Lieberman and coworkers [10] revived interest when they identified IGRP amino acids 206–214 as a source of the nonapeptide recognized by a prevalent population of pathogenic CD8$^+$ T cells in non-obese diabetic (NOD) mice [11], a model of type 1 diabetes [12]. We have now investigated if IGRP is, as its homology suggests, a multiple transmembrane domain endoplasmic reticulum (ER)-associated protein, with an intact active site facing into the ER lumen, or whether there is something different between IGRP and G6Pase that can explain the lack of activity yet its unique role in diabetes.

We show that IGRP is localized physically within the membranes of the ER. Using protease protection and glycosylation scanning experiments we demonstrate that IGRP is a glycoprotein anchored in the ER by multiple transmembrane helices and oriented in a similar manner to G6Pase-α [13,14] with the N-terminus in the lumen of the ER and the C-terminus in the cytoplasm. Of the three potential Asn-linked glycosylation sites, only the site at amino acids 92–94, situated in the large luminal loop 1, is glycosylated, consistent with the 'active site' being oriented inside the ER lumen. Processing of IGRP is also shown to proceed via the proteasome [15–17], an intracellular proteolytic pathway that generates antigenic peptides presented by the major histocompatibility complex (MHC) class I molecules [18].

2. Materials and methods

2.1. Isolation of human IGRP cDNA and construction of IGRP mutants

The coding region of human IGRP was isolated by reverse transcription polymerase chain reaction (PCR) amplification of human pancreas mRNA (Invitrogen, Carlsbad, CA, USA) using oligonucleotide primers derived from nucleotides 1–18 (sense) and 1049–1068 (antisense) of human IGRP mRNA [7]. The PCR product was cloned into the pSVL vector and verified by DNA sequencing. Human IGRP containing an N- or C-terminal FLAG (DYKDDDDK) tag was constructed as previously described [13] using IGRP as a template.

Human IGRP containing a C-terminal FLAG (IGRP-3FLAG) was used as a template for the construction of glycosylation mutants. The sense and antisense mutant primers were: N50A (nucleotides 136–162), AAT to GCT at position 50; N92A (nucleotides 262–288), AAT to GCT at position 92; N287A (nucleotides 850–870), AAC to GCC at position 287. The human G6Pase-3FLAG cDNA has been described [13].

2.2. Immunofluorescence microscopy

COS-1 cells were grown at 37°C in HEPES-buffered Dulbecco's modified minimal essential medium supplemented with 4% fetal bovine serum. Cells (5×10^4/chamber) in two-well-chambered coverglasses (Nalge Nunc International, Naperville, IL, USA) were transfected with 2 μg of IGRP-3FLAG or G6Pase-3FLAG construct, in the pSVL vector, using SuperFect transfection reagent according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). After incubation at 37°C for 2 days, the transfected cells were fixed on the coverglass for 10 min at 25°C in 3.7% formaldehyde in phosphate-buffered saline (PBS).

For intracellular staining, the cells were blocked with 2% normal

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Abbreviations: IGRP, islet-specific glucose-6-phosphatase-related protein; G6Pase, glucose-6-phosphatase; G6P, glucose-6-phosphate; ER, endoplasmic reticulum; MHC, major histocompatibility complex

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horse serum in PBS for 20 min at room temperature, incubated with a monoclonal anti-FLAG antibody (Sigma, St. Louis, MO, USA) in PBS containing 0.1% saponin for 1 h, followed by goat fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma) in PBS containing 0.1% saponin for 1 h. Following washing in PBS containing 0.1% saponin, the cells were finally mounted with an anti-fade, water-based mounting medium (Vector Lab, Burlingame, CA, USA) and analyzed under a laser scanning confocal fluorescence microscope (Leica TCS-4D DMIRBE, Heidelberg, Germany). Staining of the calreticulin ER marker [19] was performed similarly using rabbit anti-calreticulin antibody (Affinity BioReagents, Golden, CO, USA) and tetrarhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (Sigma). Excitation wavelengths of 488 (for FITC), and 568 (for TRITC) nm were used to generate fluorescence emission in green and red respectively. Co-localization of green fluorescent IGRP or G6Pase and red fluorescent calreticulin is reflected by yellow fluorescence.

2.3. Protease protection and Western blot analyses

COS-1 cells in 25-cm^2 flasks were transfected with 10 μg of either IGRP-5FLAG or IGRP-3FLAG, in the pSVL vector as previously described [13]. After incubation at 37°C for 2 days, cell homogenates in 100 μl of buffer A (0.25 M sucrose and 5 mM HEPES, pH 7.4) were treated with 250 μg/ml DNase I for 30 min at 30 °C, followed with trypsin (type XIII, 500 μg/mg protein) for 30 min at room temperature. Then, phenylmethylsulfonyl fluoride (final concentration of 5 mM) and trypsin inhibitor (final concentration of 6 mg/mg protein) were added to inactivate trypsin. The resulting digests were diluted 100-fold with cold buffer A and centrifuged at 100 000 × g for 1 h at 4°C to pellet the microsomes. The microsomal pellets were resuspended in buffer A for Western blot analysis. As controls, cell homogenates were prepared as described above, with the addition of 0.5% deoxycholate to disrupt the microsomes.

For Western blot analysis of FLAG-tagged IGRP, proteins in transfected COS-1 lysates were separated by electrophoresis through a 12% polyacrylamide–sodium dodecyl sulfate gel, blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), incubated with a monoclonal antibody against the FLAG epitope and the antigen–antibody complex visualized as described previously [4].

3. Results

3.1. IGRP is localized in the ER

To determine the subcellular localization of IGRP, a FLAG-tagged construct of IGRP was transfected into COS-1 cells and visualized by double immunostaining of the IGRP and calreticulin [19], an ER marker protein. IGRP co-localized with calreticulin, with no plasma membrane staining (Fig. 1), confirming that IGRP is retained within the ER of the cell. As expected, G6Pase-α also co-localized with calreticulin (Fig. 1).

3.2. Membrane topography of IGRP

Since ER microsomes are closed vesicles with defined cytoplasmic and luminal surfaces [20], protease protection assays, using epitope-tagged constructs, can identify the cytoplasmic or luminal exposure of the protein's N- and C-termini and reveal whether there are an even or odd number of transmembrane domains. Intact microsomes, isolated from IGRP-5FLAG- or IGRP-3FLAG-transfected COS-1 cells, were subjected to digestion by trypsin and the presence of the FLAG epitope was visualized by Western blot analysis. The FLAG tag of IGRP-5FLAG-transfected cells was resistant to trypsin digestion while the FLAG tag of IGRP-3FLAG-transfected cells was cleaved by this protease (Fig. 2A). In parallel experiments in which the microsomes were permeabilized by detergent, both N- and C-terminal FLAG tags were cleaved by trypsin (Fig. 2A). Therefore the N- and C-termini of IGRP

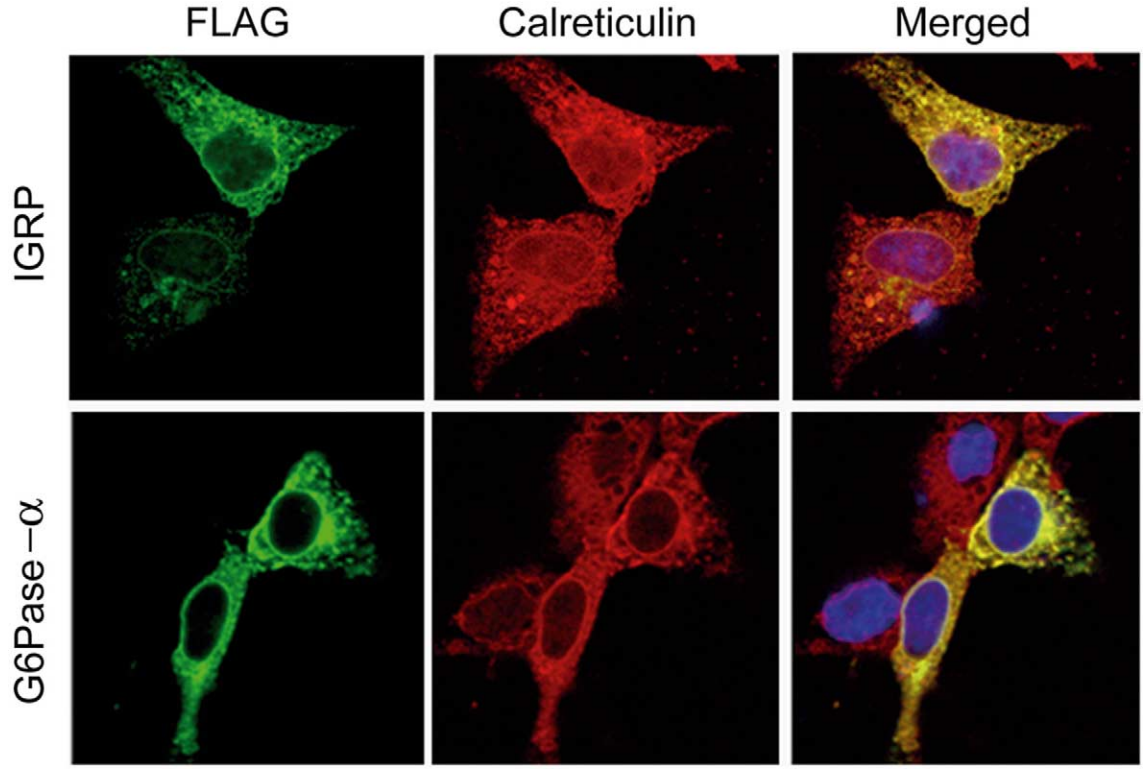

Fig. 1. IGRP is localized in the ER. Double immunofluorescence staining for recombinant IGRP (green fluorescence), recombinant G6Pase-α (green fluorescence), and endogenous calreticulin (red fluorescence) in COS-1 cells expressing human IGRP-3FLAG or human G6Pase-α-3FLAG. Note the intracellular co-localization of IGRP or G6Pase-α with the ER marker calreticulin, indicated by yellow in the merged image.

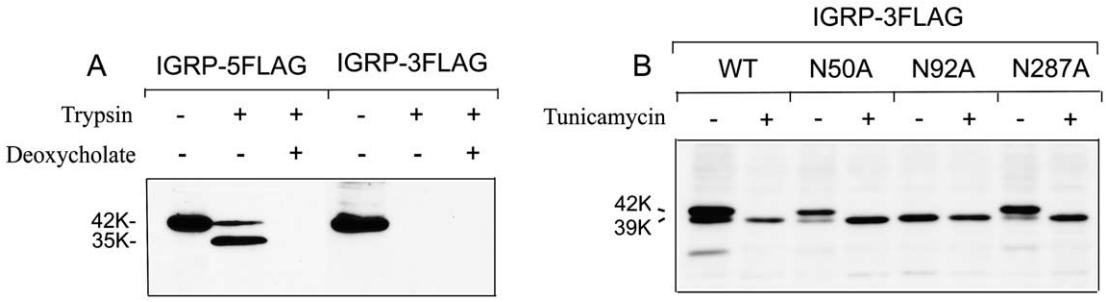

Fig. 2. IGRP is a nine-domain transmembrane protein. A: Sensitivity of N- and C-termini of human IGRP to proteolytic digestion. Intact microsomes isolated from IGRP-5FLAG- or IGRP-3FLAG-expressing COS-1 cells were subjected to digestion by trypsin in the absence or presence of deoxycholate as described in Section 2. The IGRP proteins on the Western membrane were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg proteins. B: Western blot analysis of wild-type and mutant IGRP synthesis. COS-1 cells were transfected with 3FLAG-tagged wild-type or mutant IGRP construct in the absence or presence of tunicamycin (1 μg/ml) as described in Section 2. The IGRP proteins on the Western membrane were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg proteins.

are on opposite sides of the microsomal membrane and there must be an odd number of transmembrane domains.

3.3. Human IGRP contains nine transmembrane helices

Based on its homology to G6Pase-α, IGRP can be fitted to a nine-transmembrane helical structure (Fig. 3). However, other theoretical modeling, using the TMpred program [21] predicts that both human and mouse IGRP could be anchored in the ER by seven putative transmembrane helices. Glycosylation analysis can differentiate these models. IGRP contains three consensus sequences for Asn-linked glycosylation at residues 50–52 ($N^{50}QT$), 92–94 ($N^{92}HS$), and 287–289 ($N^{287}YT$) (Fig. 3). The seven-transmembrane model of IGRP [21] predicts that $N^{50}QT$, residing within the 56-residue N-terminal domain, is the only site that can be glycosylated [22,23]. In contrast, the nine-transmembrane model of IGRP predicts that $N^{92}HS$, situated within the 37-residue luminal loop 1, is glycosylated (Fig. 3). We therefore constructed three IGRP-3FLAG-tagged mutants, each disrupting one of the Asn-linked glycosylation consensus sequences, transfected COS-1 cells and analyzed the biosynthesis of IGRP, in the absence or presence of tunicamycin [24], by Western blot. The wild-type IGRP-3FLAG construct is expressed as polypeptides of 42 kDa and 39 kDa in the absence tunicamycin

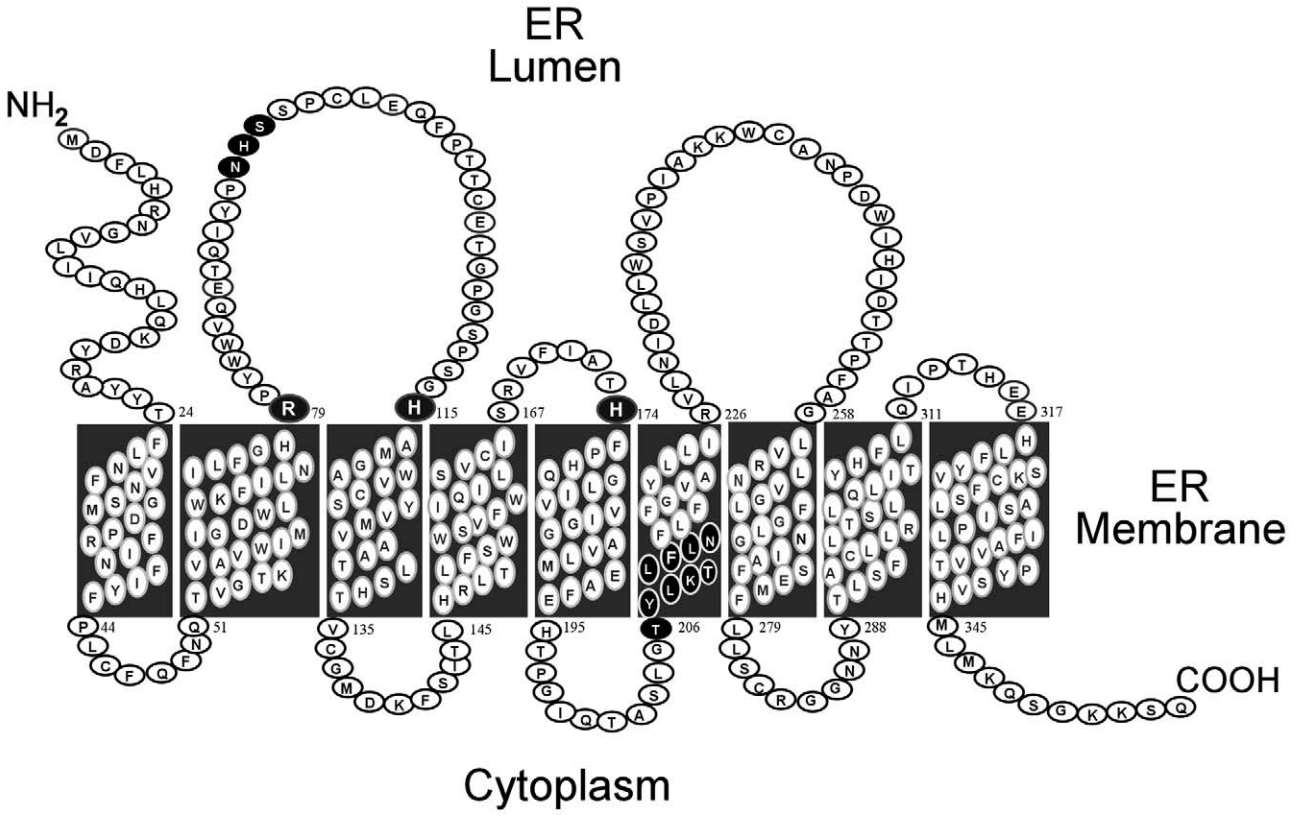

Fig. 3. The predicted nine-transmembrane helical structure of human IGRP. The transmembrane helices are identified based on sequence and topographical similarities to G6Pase-α [13,14]. Amino acid residues predicted to comprise the active center are denoted by large black circles. The Asn-linked glycosylation site at residues 92–94 that acts as an acceptor for oligosaccharides, and amino acids at 206–214 corresponding to the natural peptide antigen are highlighted.

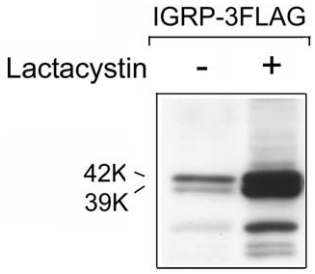

Fig. 4. The effect of proteasome inhibitor lactacystin on degradation of IGRP. Two sets of COS-1 cells were transfected with the IGRP-3FLAG construct. After 34 h incubation at 37°C, 1 μM lactacystin was added to one set of cultures and the incubation was continued for an additional 14 h. The steady-state levels of IGRP protein on the Western membrane were visualized by an anti-FLAG monoclonal antibody; each lane contained 20 μg proteins.

and as a single polypeptide of 39 kDa in the presence of tunicamycin (Fig. 2B), consistent with the Asn glycosylation of human IGRP. When mutated, the N50A and N287A constructs are also expressed as 42-kDa/39-kDa polypeptides in the absence of tunicamycin and as a 39-kDa polypeptide in the presence of tunicamycin (Fig. 2B), consistent with the pattern of the native construct. Therefore N^{50}QT and N^{287}YT are not sites of glycosylation. In contrast, the N92A construct is only expressed as a 39-kDa polypeptide in the absence or presence of tunicamycin (Fig. 2B), consistent with N^{92}HS being the site of glycosylation and supporting the nine-transmembrane domain model.

3.4. *The proteasome inhibitor lactacystin induces the accumulation of IGRP*

If human IGRP is degraded in cells predominantly through the proteasome pathway [15–17], turnover of the protein should be inhibited by the proteasome inhibitor lactacystin [25,26]. In the absence of lactacystin IGRP-transfected COS-1 cells express the 42-kDa glycosylated IGRP two-fold more abundantly than the 39-kDa non-glycosylated IGRP (Fig. 4). In the presence of lactacystin, the steady-state levels of both forms of IGRP increase, although unequally, with both forms being expressed at equivalent levels, suggesting that the 39-kDa non-glycosylated IGRP is normally less stable than the processed form.

4. Discussion

The IGRPs are hydrophobic proteins, which share many of the sequence characteristics of mammalian G6Pases, including a phosphatase signature motif, and a C-terminal ER membrane protein retention motif [6,7]. However, IGRP and the G6Pases differ in their tissue specificity and physiological functions. IGRP has a highly restricted expression profile, primarily limited to pancreatic β-cells [6,7], while G6Pase-α is expressed in the gluconeogenic tissues, liver, kidney, and intestine [1,2,27] and G6Pase-β [3–5] is ubiquitous. Furthermore, while both G6Pase-α and G6Pase-β possess a phosphohydrolase activity, IGRP has shown no enzymatic activity on a variety of phosphorylated substrates, despite extensive study [6,7]. G6Pase is a key enzyme in the regulation of interprandial glucose homeostasis [8,9] and hepatic G6Pase expression is elevated in human patients [28] and in rodent models of non-insulin-dependent diabetes mellitus (NIDDM) [29,30]. Although it was speculated that G6Pase over-expression may contribute to the pathophysiology of NIDDM, there has not been any direct evidence linking G6Pase to diabetes. It is therefore surprising that IGRP, an enzymatically inactive member of the G6Pase family, has been identified as the source of the natural peptide autoantigen targeted by a prevalent population of pathogenic $CD8^+$ T cells in NOD mice [10].

One explanation for the antigenicity and lack of phosphohydrolase activity of IGRP is that it is not processed in the cell in the same manner as the G6Pases. To this end we have examined the post-translational modification, subcellular localization and topography of IGRP.

Using immunofluorescence microscopy, we have demonstrated that IGRP co-localizes in cells with the ER marker protein calreticulin [19], confirming that IGRP is an ER-associated protein. Moreover, IGRP is a transmembrane protein. Protease protection mapping clearly shows IGRP oriented with the N-terminus facing into the lumen of the ER and the C-terminus facing into the cellular cytoplasm, consistent with an odd number of transmembrane domains and with the orientation of G6Pase-α [13,14]. Theoretical modeling of the topography of IGRP [21] predicts that IGRP is anchored in the ER membrane by either seven or eight transmembrane helices. If this is correct, a topographical difference, splitting the conserved active site residues of G6Pase between loops, or placing them on the cytoplasmic side of the ER membrane in IGRP could account for the lack of phosphohydrolase activity in IGRP. However, this is not the case. By individually mutating each of the three potential Asn-linked glycosylation sites in IGRP we showed that only mutation of Asn^{92} changes the natural electrophoretic profile of the protein, identifying Asn^{92} as the sole *N*-linked glycosylation site in vivo. Since potential Asn-linked glycosylation sites are localized on the luminal side of the ER membrane [22,23], N^{92}HS must reside on a luminal loop, which is inconsistent with the seven-transmembrane domain model, but supports the nine-transmembrane domain model for IGRP predicted by sequence alignment of the members of the G6Pase family.

The cellular immune response leading to diabetes in the NOD mouse is executed by cytotoxic and helper T lymphocytes, which recognize small antigenic peptide fragments associated with MHC class I molecules [18]. Most MHC-associated peptide antigens are generated from protein degradation by the proteasomes [15–17] and consistent with this we find that IGRP degradation is also proteasome-mediated, like G6Pase-α [31]. The divergence between amino acids 206–214, the source of the natural peptide antigen in IGRP and the corresponding sequence in mammalian G6Pase may explain why G6Pase-α, processed by the same pathway, is not presented to the immune system in an antigenic manner.

The lack of phosphohydrolase activity associated with IGRP raises the question of the role of IGRP in vivo. Glucose is the primary physiological stimulus for activation of *insulin* gene transcription (reviewed in [32]). In pancreatic β-cells G6Pase functionally antagonizes the glucokinase sensor activity, critical to glucose utilization and glucose-stimulated insulin secretion [33]. Therefore, an active G6Pase within the pancreatic β-cells would be predicted to disrupt the expression of insulin and contribute to diabetes. One possibility is that multiple mutations evolved to inactivate G6Pase activity and avoid the disruption of insulin expression. Consistent with

this is the finding that the rat IGRP gene contains a series of deletions and insertions in the coding sequence, lacks a TATA box in its promoter, and is not transcribed in rat islets [7]. In human and mouse islets, IGRP is transcribed well but there are several alternative spliced transcripts [6,7]. The full-length mRNA was studied here because it encodes the protein most similar to G6Pase-α, but it is not the most abundant transcript. The most abundant transcript is an exon-4-deleted mRNA that encodes a truncated IGRP lacking the peptide antigen at amino acids 206–214 and in an evolutionary sense does not present a risk to the pancreatic β-cells [6,7]. It will be of interest to determine if the ratio of full-length to truncated IGRP mRNA differs between normal and diabetes-susceptible mammals.

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