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GCK-MODY diabetes as a protein misfolding disease: The mutation R275C promotes protein misfolding, self-association and cellular degradation

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ABSTRACT

GCK-MODY, dominantly inherited mild hyperglycemia, is associated with more than 600 mutations in the glucokinase gene. Different molecular mechanisms have been shown to explain GCK-MODY. Here, we report a Pakistani family harboring the glucokinase mutation c.823C > *T* (p.R275C). The recombinant and *in cellulo* expressed mutant pancreatic enzyme revealed slightly increased enzyme activity (k_{cat}) and normal affinity for α-D-glucose, and resistance to limited proteolysis by trypsin comparable with wild-type. When stably expressed in HEK293 cells and MIN6 β-cells (at different levels), the mutant protein appeared misfolded and unstable with a propensity to form dimers and aggregates. Its degradation rate was increased, involving the lysosomal and proteasomal quality control systems. On mutation, a hydrogen bond between the R275 side-chain and the carbonyl oxygen of D267 is broken, destabilizing the F260-L271 loop structure and the protein. This promotes the formation of dimers/aggregates and suggests that an increased cellular degradation is the molecular mechanism by which R275C causes GCK-MODY.

1. Introduction

Metabolic homeostatic mechanisms in mammals respond to hormones and nutrients in order to maintain blood glucose levels within a narrow range (4–8 mM). Among the major hormones, a balance between insulin and glucagon is important in controlling circulating levels of glucose. Insulin has the most critical metabolic effects, and involves a complex interplay between pancreas, liver and peripheral tissues. Its regulated secretion from pancreatic β-cells by exocytosis represents an important control mechanism.

The pancreatic β-cell and the hepatocytes, as well as endocrine cells in the gut, share the key glycolytic enzyme glucokinase (GK, EC 2.7.1.1, OMIM *138079) (Jetton et al., 1994; Katzen and Schimke, 1965; Magnuson and Shelton, 1989; Meglasson et al., 1983). GK is catalytically activated by glucose binding, which in the hepatocytes (GK isoform 2) results in a stimulation of glucose uptake, glycolysis and glycogen synthesis (Agius et al., 1996; Agius, 2008; Matschinsky and Ellerman, 1968; Matschinsky, 2009), and in the β-cells (GK isoform 1) glucose-stimulated insulin secretion (GSIS) (Matschinsky and Ellerman, 1968; Matschinsky, 1990). GK is a monomeric enzyme (52 kDa) composed of the two domains S (small) and L (large), linked by connecting regions I–III, and the

Abbreviations: BCA, bicinchoninic acid; DMEM, dulbecco's modified eagles medium; *GCK*, glucokinase gene; Glc, α-D-glucose; GKRP, glucokinase regulatory protein; HEK, human embryonic kidney; hGK, human glucokinase; IFG, impaired fasting glucose; MIN, mouse insulinoma; MODY, maturity-onset diabetes of the young; PFK-2/FBPase, bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PNS, post nuclear supernatant; PDB, protein data bank; RRL, rabbit reticulocyte lysate.

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active site resides in a cleft between the two domains (Kamata et al., 2004). The enzyme catalyzes the phosphorylation of α-D-glucose (Glc) to form glucose-6-phosphate, which is the entry point into glycolysis. GK displays a positive cooperativity with respect to Glc ($n_H \sim 1.8$) and is not inhibited by the product glucose-6-phosphate.

GK is differentially regulated in hepatocytes and β-cells by a complex network of posttranslational mechanisms. In hepatocytes, its activity and cytoplasmic ↔ nuclear transport are controlled by the GK regulatory protein (GKRP) (Shiota et al., 1999; Van Schaftingen et al., 1994) and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFK-2/FBPase-2). The latter enzyme is expressed in both hepatocytes and pancreatic β-cells and plays a role in GK activation (Baltrusch et al., 2001; Massa et al., 2004). Moreover, both pancreatic and liver recombinant human GK (hGK) is poly/multi-ubiquitinated, which in part promote proteasomal degradation of at least the pancreatic isoform (Bjørkhaug et al., 2007).

One of the most common forms of maturity-onset diabetes of the young (MODY, #125851) is caused by mutations in the glucokinase gene (*GCK*). GCK-MODY is characterized by chronic, non-progressive mild fasting hyperglycemia, which is usually treated with diet alone (Froguel et al., 1993; Timsit et al., 2005), and with a very low risk for diabetes-associated late complications. Among the >600 different *GCK* mutations identified to date, around 80 have been functionally characterized *in vitro*, most of them as pancreatic recombinant glutathione S-transferase (GST)-GK fusion proteins (Osbak et al., 2009). The majority of these mutations are associated with altered enzyme kinetics and/or by a change in the overall phosphorylating capacity due to altered regulation by allosteric effector molecules (Osbak et al., 2009). In some cases, the *GCK* mutations do not affect the steady-state kinetic parameters nor their regulation by allosteric effectors, and the molecular mechanism leading to diabetes remains unexplained (Davis et al., 1999; Gloyn, 2003; Sagen et al., 2006; Zelent et al., 2011).

Here, we report clinical and molecular investigations on a Pakistani family expressing the GCK-MODY missense mutation c.823C > *T* (p.R275C). The family was followed for ten years. The mutation resulted in a recombinant mutant pancreatic GK enzyme with a slightly increased k_{cat} and Glc-induced fluorescence enhancement, related to substrate activation (Gill and von Hippel, 1989; Molnes et al., 2008). Although the soluble recombinant enzyme demonstrated wild type-like resistance to limited proteolysis by trypsin, the mutation generated a misfolded and unstable protein, with a propensity to form dimers and aggregates when stably expressed at different levels in HEK293 cells and MIN6 β-cells. Moreover, the cellular enzyme demonstrated an increased rate of degradation involving the lysosomal and possibly the proteasomal quality control systems. Based on *in silico* 3D structural analysis, a molecular mechanism for the oligomerization is proposed.

2. Materials and methods

2.1. Subjects and genetic screening

We screened 340 probands referred to the Norwegian MODY Registry for mutations in the *GCK* gene. The probands had diabetes in at least two generations, onset of disease before 30 years of age, pancreatic β-cell dysfunction together with an HbA1c less than 7.5% (normal range 4.0–5.2%). We obtained informed consent from all participants or their guardians. The investigations were performed in accordance with the Declaration of Helsinki. As controls, we screened 100 anonymous blood donors. Exon 1a and 2–10 of *GCK* were screened as described (Sagen et al., 2008).

2.2. Materials

We obtained the QuikChange® site-directed mutagenesis kit from Stratagene (La Jolla, CA, USA). All cell culture media as well as trypsin, soybean trypsin inhibitor, and factor Xa were from Sigma Aldrich (St. Louis, MO, USA). Transfection reagents Metafectene Pro was from Biontex (Martinsried, Planegg, Germany) and Lipofectamine 2000 was from Invitrogen (Grand Island, NY, USA). TnT® T7 Quick Coupled Transcription/Translation System, the MagneHis Protein Purification System and FastBreak lysis buffer were purchased from Promega (Madison, WI, USA). Proteasomal inhibitor MG132 and lysosomal protease inhibitor leupeptin were from Biomol (Cambridge, MA, USA) and Sigma-Aldrich, respectively. We acquired protease inhibitor cocktail from F. Hoffmann-La Roche (Mannheim, Germany) and the bicinchoninic acid (BCA) protein assay kit from Pierce (Rockford, IL, USA). The protein G-sepharose beads were purchased from GE Healthcare (Buckinghamshire, UK). All PAGE running equipment and homogenization (HS) medium were from Invitrogen.

2.3. Plasmid constructs

For mammalian expression of His-tagged, or both V5- and His-tagged pancreatic hGK we used the pcDNA3.1/HisC vector (His-tag N-terminal) for *in vitro* expression in the RRL system and pcDNA3.1V5/His vector (V5- and His-tag C-terminal) for expression in all cell studies (both vectors from Invitrogen). For expression in *E. coli* (BL21 cells), pancreatic WT hGK in pGEX-3X was used (kind gift from Prof. F.M. Matschinsky, University of Pennsylvania, PA, USA). The GK variant R275C was introduced into the WT human *GCK* cDNA sequence using the QuikChange® site-directed-mutagenesis kit. We verified all constructs by DNA sequencing.

2.4. Expression and purification of recombinant hGK

We expressed recombinant pancreatic WT and mutant (R275C) hGK as glutathione-S-transferase (GST) fusion proteins in *E. coli* and purified proteins by Glutathione Sepharose 4B affinity chromatography as previously described (Bjørkhaug et al., 2007). Protein concentrations were determined on the basis of A_{280} (1 mg ml^{-1} cm^{-1}) of 1.05 (Gill and von Hippel, 1989; Molnes et al., 2008). Purified protein yield per liter culture amounted to ~4.5 mg for GST-WT hGK and ~4 mg for GST-R275C hGK. Samples were aliquoted and stored in liquid nitrogen in the absence of glucose. All recombinant fusion proteins (76 kDa) were found to be essentially pure (>95%) by SDS/PAGE.

2.5. Assay of catalytic activity

We measured the steady-state kinetic properties of both GST-tagged and tag-free pancreatic enzyme by a glucose-6-phosphate dehydrogenase coupled assay as previously described (Molnes et al., 2008), using Glc and ATP as the variable substrates (Negahdar et al., 2012). The GST-tag was cleaved off using restriction protease factor Xa (protein to substrate ratio of 1:25 (by mass)). The time course of the NADH formation was measured at 340 nm, at 30 and 37 °C, and the steady-state kinetic parameters were determined by nonlinear regression analyses using the Hill equation (Molnes et al., 2008). The kinetic properties were determined from three different protein preparations.

2.6. Intrinsic tryptophan fluorescence (ITF) measurements

Fluorescence measurements were performed on a Perkin-Elmer LS-50B instrument (1 cm path-length quartz cell with maximal