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Functional characterization of MODY2 mutations in the nuclear export signal of glucokinase

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ABSTRACT

Glucokinase (GCK) plays a key role in glucose homeostasis. Heterozygous inactivating mutations in the *GCK* gene cause the familial, mild fasting hyperglycaemia named MODY2. Besides its particular kinetic characteristics, glucokinase is regulated by subcellular compartmentation in hepatocytes. Glucokinase regulatory protein (GKRP) binds to GCK, leading to enzyme inhibition and import into the nucleus at fasting. When glucose concentration increases, GCK-GKRP dissociates and GCK is exported to the cytosol due to a nuclear export signal (NES). With the aim to characterize the GCK-NES, we have functionally analysed nine MODY2 mutations located within the NES sequence.

Recombinant GCK mutants showed reduced catalytic activity and, in most cases, protein instability. Most of the mutants interact normally with GKRP, although mutations L306R and L309P impair GCK nuclear import in cotransfected cells. We demonstrated that GCK-NES function depends on exportin 1. We further showed that none of the mutations fully inactivate the NES, with the exception of mutation L304P, which likely destabilizes its α-helicoidal structure. Finally, we found that residue Glu300 negatively modulates the NES activity, whereas other residues have the opposite effect, thus suggesting that some of the NES spacer residues contribute to the low affinity of the NES for exportin 1, which is required for its proper functioning.

In conclusion, our results have provided functional and structural insights regarding the GCK-NES and contributed to a better knowledge of the molecular mechanisms involved in the nucleo-cytoplasmic shuttling of glucokinase. Impairment of this regulatory mechanism by some MODY2 mutations might contribute to the hyperglycaemia in the patients.

1. Introduction

Glucokinase (GCK) plays a central role in the regulation of glucose metabolism. Its activity is restricted to cells with important roles in whole-body glucose homeostasis [1]. In pancreatic beta-cells GCK acts as glucose sensor by integrating blood glucose levels and glucose metabolism with insulin secretion. In hepatocytes, where glucokinase is also expressed, its activity controls glycogen accumulation, glycolysis and gluconeogenesis rates. Moreover, a role of glucokinase has also been reported in the brain, pancreatic alpha cells and pituitary gonadotropes, for review see [2]. *GCK* mutations can result in monogenic disorders characterized by hyper or hypoglycaemia. Heterozygous activating mutations cause hyperinsulinemic hypoglycaemia. Homozygous inactivating mutations cause permanent neonatal diabetes mellitus, whereas heterozygous inactivating mutations cause maturity-onset diabetes of the young type 2 (MODY2) [3]. The pathophysiological mechanism of GCK associated disorders is a defect in glucose sensing that results in modification of the glucose threshold for beta-cell insulin secretion. Additionally, defects in liver glycogen storage and increased rate of gluconeogenesis have been demonstrated in MODY2 patients [4,5].

Glucokinase belongs to the hexokinase family (GCK is also named

Abbreviations: CRM1, Chromosome Region Maintenance 1; F1P, fructose 1-phosphate; F6P, fructose 6-phosphate; GCK, glucokinase; GFP, green fluorescent protein; GKRP, glucokinase regulatory protein; GST, glutathione *S*-transferase; HA, hemagglutinin; NES, nuclear export signal; RAI, relative activity index; WT, wild type

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hexokinase IV), which converts glucose in glucose-6-phosphate with ATP as second substrate. This reaction is the first limiting step of glucose utilization in hepatocytes and beta-cells. The function of glucokinase is based on its particular kinetic characteristics, mainly low affinity and cooperativity for glucose. These properties are conferred by multiple conformational protein structure states. Briefly, in the absence of glucose, GCK has an inactive super-open conformation. Conversely, the glucose-bound enzyme adopts an active closed conformation [6,7]. In addition, glucokinase can be regulated by tissue-specific post-transcriptional mechanisms [2]. In the liver, glucokinase is also regulated through protein-protein interactions by the glucokinase regulatory protein (GKRP), which inhibits the enzyme and also induces its nuclear retention in hepatocytes [8,9]. GCK-GKRP interaction is strengthened by fructose-6-phosphate (F6P) and counteracted by fructose-1-phosphate (F1P), which bind to the same site of GKRP [10,11]. During fasting, at low glucose concentrations, GKRP-F6P anchors the super-open GCK form, allowing its import into the nucleus. Mutational and structural studies of GKRP-bound GCK have shown that the GKRP-binding surface is located at the allosteric site in the hinge region of GCK, which is exposed in the super-open conformation [11–15]. Upon binding to GKRP, GCK translocates to the nucleus in hepatocytes or in heterologous cells co-transfected with GKRP and GCK, and the absence of GKRP results in exclusive cytoplasmic localization of this enzyme [9,16,17]. After feeding, when glucose (and fructose) concentrations rise, GCK adopts an active glucose-bound closed conformation and dissociates from GKRP-F1P. Substrate binding causes a structural rearrangement of GCK, which results in the dissociation of the complex [15]. The dissociation of GCK from GKRP occurs prior to being exported from the nucleus to the cytoplasm [18,19]. The enzyme nuclear export is mediated by a leucine-rich nuclear export signal (NES) covering aminoacids 300 to 310 of glucokinase (GCK-NES: ^{300}ELVRLVLLKLV^{310}) [17].

Due to the central role of glucokinase in the regulation of glucose homeostasis, this enzyme has been considered as a potential target for the development of new anti-diabetic drugs. However, tackling such a challenge requires a thorough knowledge of GCK regulatory mechanisms [20,21]. The biochemical characterization of *GCK* mutations associated to glycemic disorders has contributed significant insights into the regulatory mechanism of this enzyme [3]. In this work, we carried out a systematic analysis of MODY2 mutations within the GCK-NES sequence.

2. Material and methods

2.1. Selection of MODY2 mutations in the nuclear export signal of glucokinase

We selected a series of GCK mutations covering the NES sequence of this enzyme. Mutations E300Q, E300K, R303W, L304P, L306R, R308W, L309H and L309P had been previously identified [3,22–25]. Mutation V302E is a novel mutation identified in this work.

2.2. Subjects and genetic analysis

The patient bearing V302E mutation was referred to our laboratory at the Hospital Clinic of Barcelona for a molecular diagnosis of MODY. A 27 years old woman of Caucasian descent, presented repetitive mild fasting hyperglycaemia. A 75-g OGTT resulted in blood glucose concentrations of 6.1, 9.3 and 6.4 mmol/l and insulin of 7.18, 18.5, 22.9 and 20.2 mU/l at 0, 60 and 120 min respectively. Her 6 years old daughter and 4 years old son also presented repetitive mild fasting hyperglycaemias. Body mass index was 21.5, 19.5 and 16 kg/m^2 for the patient, her daughter and son, respectively. Informed consent was obtained from the subject or their parents. The study was performed according to the Declaration of Helsinki as revised in 2008 and approved by the local ethical committee. *GCK* genetic analysis was performed as described in [26].

2.3. Plasmid constructs and mutagenesis

Plasmid pGEX-5X.2-GCK express human wild type beta-cell GCK fused to glutathione-*S*-transferase (GST-GCK) [27]. Plasmid *p*-FLAG-ctc-hGKRP-FlagC contains human Flag-tagged GKRP [28]. Plasmid pEGFP-N2-ratGCK$^{(299–359)}$ express rat GCK residues 299 to 359 as an N-terminal enhanced green fluorescent protein (GFP) fusion protein (GCK$^{(299–359)}$-GFP) [17]. Plasmid pYFP-CRM1 contains the human cDNA for CRM1 (Chromosome Region Maintenance 1)/exportin1 [29]. Plasmids pEGFP-C3-GCK (expressing human β-cell GCK as a C-terminal GFP fusion protein GFP-GCK), pGKRP-mCherry (expressing the human GKRP as an N-terminal mCherry fusion protein (GKRP-mCherry)), pmCherry and pCMV-HA-CRM1 (expressing hemagglutinin (HA) tagged-exportin1) were prepared as described in Supplementary Material and Methods. Mutagenesis was performed as previously described [30] by using primers shown on Supplementary Table S1.

2.4. Protein production and purification

Recombinant wild-type and mutant GST-GCK were bacterially expressed, purified and stored as described previously [27]. Flag tagged human GKRP was purified from *E. coli* as in [28] with the modifications detailed in Supplementary Material and Methods.

2.5. GCK enzymatic assays and in silico structural analysis

Determination of kinetic parameters, thermal inactivation, inhibition by GKRP and structural analysis were performed as previously described [25,27,30]. See Supplementary Material and Methods.

2.6. Cell culture, transient transfection and treatment with leptomycin B

HEK293T cells were a gift from Dr. C. Hernández-Sánchez (Centro de Investigaciones Biológicas, Madrid, Spain) and were cultured in DMEM containing glucose 25 mmol/l. HepG2 cells were purchased from the American Type Culture Collection (ATCC HB-8065, Manassas, VA, USA) and cultured in MEM containing glucose 5.5 mmol/l. See Supplementary Material and Methods. Transient transfections of HEK293T were performed with lipofectamine (Invitrogen, Carlsbad, CA, USA) and HepG2 with XtremeGENE HP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, see Supplementary Material and Methods. When indicated, cells were incubated for 6 h with 40 nmol/l of leptomycin B (Sigma-Aldrich, St Louis, MO, USA).

2.7. Western blot analysis

Western blot analysis was performed in transfected cells to detect GFP, mCherry and actin as detailed in Supplementary Material and Methods.

2.8. Inmunocytochemistry, fluorescence microscopy and image analysis

Cells, cultured and transfected on coverslips (Menzel-Gläser, Braunschweig, Germany), were fixed with 4% formaldehyde (Merck Sharp & Dohme International, Whitehouse Station, NJ, USA) 42 h after transfection. Following nuclei staining with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen), coverslips were mounted with *Fluoromount G* (Electron Microscopy Sciences, Hatfield, PA, USA). For inmunocytochemistry, fixed cells were permeabilised with 0.4% Triton X-100 and blocked in 10% normal goat serum (Sigma-Aldrich) with 0.1% Triton X100 in PBS. Cells were incubated 2 h with *Monoclonal Rat anti-HA Tag antibody* (1:100; Roche), washed with blocking buffer and then incubated for 1 h with goat anti rat IgG (H&L): TexasRed (1:200; AbD

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