Effects of novel maturity-onset diabetes of the young (MODY)-associated mutations on glucokinase activity and protein stability

María GALÁN*, Olivier VINCENT†, Isabel RONCERO*, Sharona AZRIEL‡, Pedro BOIX-PALLARES§, Elías DELGADO-ALVAREZ§, Francisco DÍAZ-CADÓRNIGA§, Enrique BLÁZQUEZ* and María-Angeles NAVAS*¹

*Departamento de Bioquímica y Biología Molecular III, Facultad de Medicina, Universidad Complutense de Madrid, Ciudad Universitaria, Madrid 28040, Spain, †Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas del CSIC, Madrid 28040, Spain, ‡Servicio de Endocrinología, Hospital 12 de Octubre, Av. de Córdoba s/n, Madrid 28041, Spain, and §Servicio de Endocrinología, Hospital General de Asturias, Julián Clavería s/n, Oviedo 33006, Spain

Glucokinase acts as the pancreatic glucose sensor and plays a critical role in the regulation of insulin secretion by the β -cell. Heterozygous mutations in the glucokinase-encoding *GCK* gene, which result in a reduction of the enzymatic activity, cause the monogenic form of diabetes, MODY2 (maturity-onset diabetes of the young 2). We have identified and functionally characterized missense mutations in the *GCK* gene in diabetic families that result in protein mutations Leu¹⁶⁵ → Phe, Glu²⁶⁵ → Lys and Thr²⁰⁶ → Met. The first two are novel *GCK* mutations that co-segregate with the diabetes phenotype in their respective families and are not found in more than 50 healthy control individuals. In order to measure the biochemical effects of these missense mutations on glucokinase activity, we bacterially expressed and affinity-purified islet human glucokinase proteins carrying the respective mutations and fused to GST (glutathione S-transferase). En-

zymatic assays on the recombinant proteins revealed that mutations Thr²⁰⁶ → Met and Leu¹⁶⁵ → Phe strongly affect the kinetic parameters of glucokinase, in agreement with the localization of both residues close to the active site of the enzyme. In contrast, mutation Glu²⁶⁵ → Lys, which has a weaker effect on the kinetics of glucokinase, strongly affects the protein stability, suggesting a possible structural defect of this mutant protein. Finally, none of the mutations tested appears to affect the interaction of glucokinase with the glucokinase regulatory protein in the yeast two-hybrid system.

Key words: diabetes mellitus, enzyme kinetics, *GCK* gene, glucokinase, inactivating mutation, maturity-onset diabetes of the young (MODY).

INTRODUCTION

GK (glucokinase) (ATP:D-glucose-6-phosphotransferase; EC 2.7.1.2), also known as hexokinase D or hexokinase IV, is one of the four glucose-phosphorylating isoenzymes present in vertebrates. This metabolic enzyme is highly expressed in pancreatic β -cells, hepatocytes and brain, and it catalyses the first reaction of glycolysis by converting glucose into G6P (glucose 6-phosphate), with Mg-ATP as a second substrate. This reaction is the first rate-limiting step in glucose metabolism that is necessary for insulin release in the β -cell. GK plays a key role as a pancreatic β -cell glucose sensor by integrating blood glucose levels and glucose metabolism with insulin secretion [1,2]. This specific function of GK is based on the particular kinetic characteristics of this enzyme as compared with the other hexokinase isoforms, which include a low affinity for glucose ($S_{0.5}$ 7–9 mmol/l), a cooperativity with this substrate (Hill coefficient close to 1.7) and a lack of end-product inhibition at physiological concentrations of G6P. In addition, in contrast with the activity of the other hexokinase isoforms, GK activity is regulated through protein–protein interactions by the GKR (GK regulatory protein), which acts as a competitive inhibitor with respect to glucose and also regulates the nucleocytoplasmic localization of the enzyme [3–5].

The correct functioning of GK in β -cells is essential for maintaining glucose homeostasis in the organism, and mutations in the *GCK* gene may cause different monogenic glycaemic disorders in humans [6]. In general terms, GK mutations that in-

crease GK activity produce hypoglycaemia due to congenital hyperinsulinism, HI (hyperinsulinaemia of infancy) (OMIM #601820; [7]). In contrast, *GCK* mutations that decrease enzyme activity produce hypoinsulinism and hyperglycaemia. *GCK*-inactivating mutations carried out in both alleles produce PNDM (permanent neonatal diabetes mellitus) (OMIM #606176; [8]) and, in one allele, MODY (maturity-onset diabetes of the young) type 2 (OMIM #125851 [9]). MODY is a form of diabetes mellitus characterized by an onset that usually appears before 25 years of age, often in childhood and adolescence, and abnormal β -cell function and insulin secretion [10]. The MODY2 phenotype is characterized by a mild form of hyperglycaemia present from birth, but often asymptomatic and only detected later in life [11]. MODY2 patients are usually treated with diet alone and rarely develop diabetic-associated complications [12].

Nearly 200 mutations spanning the complete *GCK* gene have been described as being involved in GK-associated pathologies (for a review, see [6]). These mutations, which are located along the ten exons of the gene expressing the pancreatic β -cell isoform of GK, include nonsense, missense and frameshift mutations produced by deletions or insertions. However, only less than one-fifth of these mutations have been functionally characterized [6]. The structural model and the crystal structure of human GK have been determined in both its active liganded and inactive free forms [13,14], and the functional characterization of naturally occurring *GCK* missense mutations involved in glycaemic disorders has provided further clues to the basic biochemistry and biophysics of

Abbreviations used: DTT, dithiothreitol; GK, glucokinase; GKR, GK regulatory protein; GlcNAc, N-acetylglucosamine; G6P, glucose 6-phosphate; GST, glutathione S-transferase; I_a , activity index; MH, mannoheptulose; MODY, maturity-onset diabetes of the young; OGTT, oral glucose tolerance test; SD medium, synthetic dextrose minimal; SSCP, single-strand conformation polymorphism.

¹ To whom correspondence should be addressed (email manavas@med.ucm.es).

this enzyme. These studies have contributed to a better knowledge of the mechanisms by which GK acts as a glucose sensor and integrates blood glucose levels and insulin release. The GK-activating mutations involved in hyperinsulinism increase substrate affinity and are clustered at the allosteric site of the enzyme [14]. In contrast, MODY2 mutations are located throughout the gene, and most of them are inactivating mutations that affect one or several kinetic parameters of the enzyme (decreased substrate affinities, combined or not with reduced specific activity) [6]. These effects, which can be translated into a lower activity index, lead to an increased threshold for glucose-stimulated insulin release [15]. However, some *GCK* mutations found in MODY families do not appear to affect the enzymatic activity of GK as presumed. An example is the *GCK* mutation Val⁶² → Met, found in two MODY2 families, which results in a kinetically activated enzyme [16]. Its implication in MODY has been explained by other mechanisms, such as protein instability and the loss of regulation by associated proteins and allosteric activators.

In this study, we report the identification and functional analysis of *GCK* missense mutations found in Spanish MODY families and we show that they co-segregate with diabetes. These mutations include two novel GK mutations, Leu¹⁶⁵ → Phe, which is located in proximity to the active site, and Glu²⁶⁵ → Lys, which is located in a more external position that is not physically associated with the glucose-binding site, together with a third, Thr²⁰⁶ → Met, that has been reported previously [17–20] but has not yet been characterized biochemically. In the present study, we provide a functional characterization of these mutations by measuring their effect on the kinetic parameters of the enzyme, on protein stability and on the interaction of GK with the GKRP. Our data indicate that, whereas mutations Leu¹⁶⁵ → Phe and Thr²⁰⁶ → Met affect the catalytic activity of the enzyme directly, mutation Glu²⁶⁵ → Lys has a weaker effect on GK kinetics, but strongly affects protein stability.

EXPERIMENTAL

Patients

The probands of families P5, P6 and P7 were referred to our laboratory by P. B.-P., E. D.-A. and F. D.-C., and the one for family P22 by S. A., for a molecular diagnosis of MODY. The clinical diagnosis of MODY was made using the classical criteria: impaired fasting glucose or development of diabetes before age 25, negative search for the markers of Type I diabetes [ICA (islet cell antibodies), GAD (glutamic acid decarboxylase antibodies), IA2 (tyrosine phosphatase antibodies) and IAA (insulin auto-antibodies)] and a family history of diabetes for at least two consecutive generations. Informed consent was obtained from the subjects or from their parents. The studies were performed according to the Declaration of Helsinki and approved by the corresponding ethical committees.

SSCP (single-strand conformation polymorphism) analysis of the *GCK* gene

Genomic DNA was isolated from human leucocytes using standard methods [21]. PCR of the ten exons of the *GCK* gene expressed in β -cells (GenBank[®] accession number AH005826) was accomplished using previously described primer sequences [22]. SSCP analysis was performed with two electrophoretic conditions, as described in [23]. Genomic DNA from patients showing abnormal SSCP conformers were re-amplified and sequenced on a 3730 DNA analyser (Applied Biosystems) at the sequencing facility of the Complutense University.

Production of recombinant wild-type and mutant GST (glutathione S-transferase)–GK

The recombinant human wild-type β -cell GK fused to GST (GST-GK) was prepared as described previously [5]. MODY-associated mutations were introduced into the GST-GK construct by PCR. To generate mutation Leu¹⁶⁵ → Phe, two first PCR fragments, A and B, were produced using primers hGK379, 5'-AGCAGATCCTGGCAGAGTTC-3', and hGK827r, 5'-GTT-GAAAAGGATCCCCTTATCGATGTCTTC-3', for A, and hGK798, 5'-GAAGACATCGATAAGGGGATCCTTTTCAAC-3', and hGK1222r, ATGTACTTGCCACCTATGAG-3', for B. PCR fragments A and B were used as a template in a new PCR reaction with primers hGK379 and hGK1222r. The resulting PCR fragment was digested with PstI and SacI, and was introduced into the wild-type GST-GK construct by replacing the wild-type fragment. Using the same method, mutation Thr²⁰⁶ → Met was generated using primers hGK379 and hGK956r, 5'-CG-TGGCCACCATGTCATTCACCATGGCCAC-3', for A, and hGK927, 5'-GTGGCCATGGTGAATGACATGGTGGCCACG-3', and hGK1222r for B. Finally, mutation Glu²⁶⁵ → Lys was generated similarly using primers hGK379 and hGK1142r, 5'-CAGCAGGAACCTCGTCAAGCTTGCCGGAGTC-3', for A, and hGK1113, 5'-GACTCCGGCAAGCTTGACGAGTTCTCTGCTG-3', and hGK1389r, 5'-TGCTCAGGATGTTGTAGATC-3' for B. Constructs carrying mutations Leu¹⁶⁵ → Phe, Thr²⁰⁶ → Met and Glu²⁶⁵ → Lys were checked by sequencing and digestion with BamHI, NcoI and HindIII respectively. *Escherichia coli* BL21 strain was transformed with the indicated plasmids and grown at 37°C to mid-exponential phase. Expression of fusion proteins was induced by adding IPTG (isopropyl β -D-thiogalactoside) to a final concentration of 0.2 mM, and cultures were incubated with orbital shaking for 16 h at 22°C. GST-GK fusion proteins were purified from crude *E. coli* extracts by single-step affinity chromatography using glutathione-agarose (Sigma-Aldrich), essentially as described in [24]. Briefly, 200 ml of bacterial culture was pelleted and resuspended in 25 ml of 0.1 M PBS containing 5 mM DTT (dithiothreitol). Cells were lysed by using a French Pressure cell press (Thermo IEC) after adding Triton X-100 to a final concentration of 1%. The supernatant of the lysate was mixed with 50% glutathione-agarose beads, which were collected by centrifugation at 500 g for 5 min before being washed twice with the same buffer. Fusion proteins were eluted with 50 mM Tris/HCl, pH 8.0, 200 mM KCl, 10 mM glutathione and 5 mM DTT, and were stored at a concentration of approx. 1 mg/ml at –80°C in 30% (v/v) glycerol, 50 mM glucose, 10 mM glutathione, 5 mM DTT, 200 mM KCl and 50 mM Tris/HCl, pH 8.0, as indicated in [25]. Protein concentrations were determined using the Bio-Rad protein assay kit with BSA as standard. GST-GK purification resulted in a single band on Coomassie-Blue-stained SDS/10% PAGE gels.

Kinetic parameters

GK activity was measured spectrophotometrically on a Kontron 930 spectrophotometer, using an NADP⁺-coupled assay with glucose-6-phosphate dehydrogenase, as described in [26], with the modifications introduced in [24]. One unit of GK is defined as the amount of enzyme that phosphorylates 1 μ mol of glucose per min at 30°C. In order to determine the kinetic variables for glucose, 15 different concentrations of the substrate were used (0.1–200 mM) with 5 mM ATP plus 1 mM MgCl₂ in excess. For the second substrate, ATP, nine different concentrations were used (0.02–5 mM) with 100 mM glucose or with the corresponding glucose concentration (*S*_{0.5}), as indicated in the Results section.

Table 1 Phenotypic characteristics of index patients at the actual age

BMI, body mass index (normal range, 19–25 kg/m²; overweight, 25–30 kg/m²); FPG, fasting plasma glucose (normal range, 4.0–6.1 mM); OGTT, plasma glucose at 120 min after a 75 g OGTT; HbA_{1c} (normal range, 4.0–5.2%); basal C-peptide (normal range, 1–4 nM); M, mother; F, father; B, brother; S, sister; Sn, son; pA, paternal aunt; pU, paternal uncle; pGF/pGM, paternal grandfather/grandmother. N.A., not analysed. Underlined are those affected family members where mutation was checked by genotyping.

Family	Age at diagnosis (years)	Age now (years)	Patient sex	BMI (kg/m ²)	FPG (mM)	OGTT (mM)	HbA _{1c} (%)	Basal C-peptide (nM)	Affected family members
P5	23	33	Male	26	7.1	6.4	6.3	1.5	M, <u>B</u>
P6	20	50	Male	30	6.6	11.4	6.7	3.5	M, B, <u>two Sn</u>
P7	20	32	Female	21	6.8	12.2	6.2	2.1	pGF, F
P22	14	34	Female	19	8.0	N.A.	6.5	1.2	pGM, <u>E</u> , pA, <u>two pU</u> , <u>S</u>

The Hanes–Woolf plot was used to calculate the GK $V_{\max}$ and the K_m values for ATP. The Hill plot was applied to estimate the Hill coefficient of GK towards glucose and the $S_{0.5}$ for this substrate. The concentration of glucose at which the inflection point occurs was calculated as indicated in [25]. The k_{cat} was calculated as $V_{\max}$ /mol of GK using glucose as variable substrate. To give k_{cat} values, the results were extrapolated to 37°C, as indicated in [15,27]. The activity index (I_a), derived from the kinetic variables, k_{cat} , $S_{0.5}$, h , ATP concentration and the K_m for ATP, was calculated as indicated in [15]. The sensitivity of wild-type and mutant GK to inhibition by competitive inhibitors was determined at a glucose concentration close to the $S_{0.5}$ value or below. IC₅₀ values were derived from Dixon plots [28], using three concentrations of glucose (3, 5 and 7.5 mM for the wild-type protein, and 25, 50 and 75 mM or 7.5, 10 and 15 mM for proteins carrying mutations Leu¹⁶⁵ → Phe and Glu²⁶⁵ → Lys respectively), and five concentrations of inhibitors [0.05–1 mM for GlcNAc (*N*-acetylglucosamine) and 0.5–10 mM for MH (mannoheptulose)]. Thermal stability tests were performed as in [25]. Results are shown as the means ± S.E.M., and statistical significance was analysed by the unpaired Student's *t* test. *P* values of <0.01 were considered to be statistically significant.

Yeast two-hybrid protein–protein interaction test

The *Saccharomyces cerevisiae* strain used for two-hybrid studies was Y187 (MAT α , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4 Δ* , *met⁻*, *gal80 Δ* , *URA3::GAL1_{UAS}-GAL1_{TATA}-LacZ*; Clontech). Standard genetic methods were used. Yeast cells were grown in SD (synthetic dextrose minimal) medium lacking appropriate supplements to maintain selection for plasmids [29]. For β -galactosidase assays, transformants were patched on to selective SD medium and were grown for 2 days at 30°C. Filter lift assays for blue colour were performed as described previously [30] and developed for 1 h. A plasmid encoding a GKRFP fusion protein to the Gal4-binding domain was constructed by inserting a BamHI/XhoI fragment from pGEX-5X-2-GKRFP [5] containing the GKRFP coding sequence between the BamHI and SalI sites of the polylinker of pGBKT7 (Clontech). A construct encoding a fusion of GK and mutant derivatives to the Gal4-activating domain was derived from pACTII (Clontech) by inserting a BamHI/XhoI fragment from the respective GST–GK constructs containing the GK coding sequence between the BamHI and XhoI sites of the polylinker.

RESULTS

Clinical features of the patients

All the patients studied as probands for genetic diagnosis of MODY2 had a clinical history of at least two consecutive gene-

Table 2 *GCK* mutations in MODY families

Nucleotide numbering uses +1 as the adenine of the ATG initiation codon, based on the GenBank® sequence AF041021. Novel mutations are indicated in bold.

Family	Gene exon	Nucleotide change	Amino acid change
P6	GCK 5	c.493C > T	Leu¹⁶⁵ → Phe
P5	GCK 6	c.617C > T	Thr ²⁰⁶ → Met
P7	GCK 6	c.617C > T	Thr ²⁰⁶ → Met
P22	GCK 7	c.793G > A	Glu²⁶⁵ → Lys

rations and age of diagnosis below 25 (Table 1). These patients had fasting hyperglycaemia values ranging from 6.6 to 8.0 mM, which are in the normal range for MODY2 patients [6]. The OGTT (oral glucose tolerance test) in families P6 and P7 gave increment values above 3 mM, which is the average found in MODY2 families [31]. HbA_{1c} values were above the normal range, and fasting serum C-peptide levels were normal. Probands of families P5 and P6 were overweight. All of these patients were treated with diet alone and did not show any chronic diabetic complications.

Identification of novel missense mutations in the *GCK* gene

The ten exons of the *GCK* gene expressed in β -cells were scanned for mutations using SSCP on the probands from each of the affected families. Sequencing of abnormal migrating bands revealed the heterozygous mutations shown in Table 2. The novel missense mutations in *GCK* exon 5 of family P6 (codon 165 CTC → TTC) and exon 7 of family P22 (codon 265 GAG → AAG) result in the GK mutations Leu¹⁶⁵ → Phe and Glu²⁶⁵ → Lys respectively. In addition, the apparently unrelated families P5 and P7 showed the same mutation in exon 6 (codon 206 ACG → ATG), resulting in GK mutation Thr²⁰⁶ → Met. The presence of this last mutation has also been reported in three unrelated Italian families [17–19] and one German family [20]. These mutations co-segregate with the MODY phenotype in the available individuals in their respective families (Table 1). Moreover, the novel missense mutations in codons 165 and 265 were not found in 55 unrelated healthy individuals used as controls, who showed normal conformers of SSCP as compared to the probands and their affected relatives (results not shown).

Based on the model of the protein structure of GK [13,14], Thr²⁰⁶ and Leu¹⁶⁵ are located in the substrate-binding cleft or very close to the active site (Figure 1A). Glu²⁶⁵ is located on a more external position of the enzyme and not in the immediate vicinity of the substrate-binding site (Figure 1A). On the other hand, as shown in Figure 1(B), Leu¹⁶⁵ and Thr²⁰⁶ are highly conserved among GKs and hexokinases, and Glu²⁶⁵ is only conserved among GKs.

Figure 1 Localization of the mutated residues Leu¹⁶⁵, Thr²⁰⁶ and Glu²⁶⁵ on the structural model for the β -cell GK and multiple sequences alignment of GKs and hexokinases from different species at the positions where mutations were identified

(A) Closed conformation (PDB code 1V4S) of wild-type GK [14] is represented using the RasMol program [48]. Mutated residues in the MODY families are indicated as black sticks and ribbons. The position of glucose is indicated as a black sphere. (B) Alignment of human (Hum) GK (GenBank[®] accession number NP_277042), mouse liver GK (GenBank[®] accession number NP_034422), rat liver GK (GenBank[®] accession number NP_036697), *Xenopus* (Xen) liver GK (GenBank[®] accession number X93494), rat hexokinase I (GenBank[®] accession number NP_036866) and rat hexokinase II (GenBank[®] accession number NP_036867) using the ClustalW program. Numbers indicate amino acid positions in the proteins. Boxes indicate the conservation of the corresponding residues.

Table 3 Kinetic constants of human recombinant wild-type and MODY2 mutant β -cell GST-GK fusion proteins

Results are means $\pm$ S.E.M. of *n* separate enzyme expressions. IC₅₀ was measured in three separate enzyme expressions of each mutant. Note that the Hill coefficient (*h*) and the relative activity index (*I*_a) are unitless. **P* < 0.01; ***P* < 0.001.

Preparation	Protein yield (mg/l)	Kcat (s ⁻¹)	S _{0.5} for glucose (mmol/l)	<i>h</i>	Inflection point (mmol/l)	K _m for ATP (mmol/l)	<i>I</i> _a	IC ₅₀ GlcNAc (mmol/l)	IC ₅₀ MH (mmol/l)
Wild-type GST-GK (<i>n</i> = 6)	9.2 $\pm$ 2.8	42.0 $\pm$ 7.0	8.0 $\pm$ 0.2	1.53 $\pm$ 0.02	2.8 $\pm$ 0.13	0.4 $\pm$ 0.04	1	0.25 $\pm$ 0.03	1.16 $\pm$ 0.14
GST-GK(L165F) (<i>n</i> = 5)	14.0 $\pm$ 3.6	18.0 $\pm$ 4.0*	92.5 $\pm$ 9.5**	1.21 $\pm$ 0.03**	12.2 $\pm$ 1.5*	1.52 $\pm$ 0.08**	0.034 $\pm$ 0.009*	0.62 $\pm$ 0.06*	6.5 $\pm$ 0.17**
GST-GK(T206M) (<i>n</i> = 3)	13.0 $\pm$ 3.9	0.18 $\pm$ 0.14**	85.0 $\pm$ 27*	0.90 $\pm$ 0.03**	–	0.45 $\pm$ 0.13	0.002 $\pm$ 0.001*		
GST-GK(E265K) (<i>n</i> = 6)	3.5 $\pm$ 0.5 ^b	36.8 $\pm$ 7.9	15.5 $\pm$ 1.3*	1.43 $\pm$ 0.04	4.7 $\pm$ 0.6	0.63 $\pm$ 0.02*	0.4 $\pm$ 0.1*	0.20 $\pm$ 0.02	1.05 $\pm$ 0.05

Biochemical characterization of recombinant mutant GKs

In order to investigate the effect of the GK mutations Leu¹⁶⁵ $\rightarrow$ Phe, Thr²⁰⁶ $\rightarrow$ Met and Glu²⁶⁵ $\rightarrow$ Lys on GK activity, we bacterially expressed recombinant wild-type and mutant GKs as GST-fusion proteins [GST-GK, GST-GK(L165F), GST-GK(T206M) and GST-GK(E265K) respectively]. The strategy in which GK is expressed fused to GST allows the purification of the fusion protein in a single-step procedure [24], and has been used extensively by researchers to study GK mutations, also being recognized as advantageous for the analysis of labile mutants [15,16,25]. Six preparations of wild-type GK and at least three of each mutant derivative were purified, with yields ranging from 3.5 to 14 mg/l (Table 3), the lowest value being obtained for the

mutant GST-GK(E265K). The purity of each purified GST-GK protein was analysed by SDS/10 % PAGE and Coomassie Blue staining. In all cases, a single band of 75 kDa was detected (results not shown).

The kinetic behaviour of GK with its main substrate, glucose, is represented in Figure 2. The values for the kinetic variables, including the catalytic constant, *k*_{cat}, and substrate affinities (*S*_{0.5} for glucose and *K*_m for ATP) are shown in Table 3. The strongest effect on GK activity was observed for mutation Thr²⁰⁶ $\rightarrow$ Met, with an activity index corresponding to 0.2 % of the wild-type GK activity index. Data analysis for this mutant was somehow limited, since the specific activity was very low, despite good protein yields, and the kinetic results are therefore approximations. This mutant displayed a > 10-fold lower affinity for glucose than the

Figure 2 Glucose-dependent activity of the mutated forms of GK

Specific activity at each glucose concentration was calculated as described in the Experimental section. Results are means $\pm$ S.E.M. for n separate enzyme expressions.

wild-type protein and might have lost the co-operativity for this substrate, as shown by an approximate Hill coefficient close to 1. In contrast, the affinity for the second substrate ATP did not appear to be affected. The activity index of the mutant GK carrying mutation Leu¹⁶⁵ $\rightarrow$ Phe was approx. 4% of that of the wild-type GK. The catalytic constant of this mutant was reduced to one-half of the wild-type value, and the affinities for both substrates (glucose and ATP) were almost 12- and 4-fold lower respectively. Mutation Glu²⁶⁵ $\rightarrow$ Lys elicited the weakest kinetic effect and the activity index was only reduced to 40% of the wild-type GK activity index. This effect is mainly due to a decrease in the affinity for glucose and a slight decrease in affinity for ATP, since the k_{cat} remained almost unaffected.

Because all three mutations had an effect on the affinity for glucose, we tested the inhibitory effect of two well-known GK competitive inhibitors, namely GlcNAc and MH. In order to measure the affinity values for these effectors independently of the effect of the mutations on glucose affinity, the wild-type GST-GK and mutant derivatives were tested at a maximum substrate concentration corresponding to their respective $S_{0.5}$ values (see the Experimental section). As shown in Table 3, mutation Leu¹⁶⁵ $\rightarrow$ Phe conferred a significantly lower sensitivity to both the GK competitive inhibitors MH and GlcNAc. In contrast, mutation Glu²⁶⁵ $\rightarrow$ Lys had no effect on the affinity of GK for these compounds.

It has been described previously that certain MODY2 mutations affect GK protein stability and/or regulation by the GKR protein [16,25]. The effect of the Leu¹⁶⁵ $\rightarrow$ Phe, Thr²⁰⁶ $\rightarrow$ Met and Glu²⁶⁵ $\rightarrow$ Lys mutations on the protein–protein interaction between GK and GKR was tested in the two-hybrid system in yeast. This method has been extensively used to analyse mammalian protein–protein interactions [32]. All three mutations do not appear to affect the interaction of GK with GKR in two-hybrid assays (Figure 3). The protein stability of wild-type GST-GK and mutant derivatives was tested at different temperatures. As described previously [25], GK activity remained practically constant under a temperature increase of up to 50°C, but enzymatic activity fell abruptly at 52°C (Figure 4A). Both

Figure 3 Two-hybrid interaction between GK and mutant derivatives and the GKR

Two-hybrid interaction of Gal4-binding domain–GKR with Gal4-activating domain–GK and its mutant derivatives were tested in *S. cerevisiae* strain Y187. Filter lift assays were developed for 1 h. In each case, three independent transformants were tested, with similar results.

Figure 4 Effects of temperature on the stability of GST-GK variants

Stock enzyme solutions were diluted to 250 μ g/ml in storage buffer containing 30% (v/v) glycerol, 50 mM glucose, 10 mM glutathione, 5 mM DTT, 200 mM KCl and 50 mM Tris/HCl, pH 8.0. **(A)** The enzyme solutions were incubated for 30 min at different temperatures ranging from 30 to 55°C and then assayed at 30°C as described in the Experimental section. **(B)** The enzyme solutions were incubated for different periods of time from 5 to 60 min at 50°C. Means $\pm$ S.E.M. for three independent enzyme preparations are shown for each case.

mutations Leu¹⁶⁵ $\rightarrow$ Phe and Glu²⁶⁵ $\rightarrow$ Lys conferred thermal instability, since the enzymatic activity of the corresponding mutant proteins was decreased at 50 or 52°C, as compared with

the wild-type. The time-course analysis of thermal inactivation shown in Figure 4(B) indicates that mutation Glu²⁶⁵ → Lys produces the strongest effect on protein stability and that 80 % of GK activity is lost within the first 10 min of incubation at 50 °C. Interestingly, although mutation Leu¹⁶⁵ → Phe produced a 50 % decrease in GK activity after 10 min at 50 °C, the residual activity remained constant over the ensuing 30 min at the same temperature, but decreased to 20 % of the wild-type value after 60 min of incubation.

DISCUSSION

In the present study, we have identified two novel *GCK* mutations, resulting in GK mutations Leu¹⁶⁵ → Phe and Glu²⁶⁵ → Lys, in Spanish MODY families and have shown that these mutations co-segregate with diabetes in their respective families and are not found in over 100 chromosomes from non-diabetic control subjects. We completed this molecular diagnosis by a functional analysis of the molecular effects of these mutations on GK enzymatic activity, protein stability and interaction with the GKRP protein. Moreover, we report another missense mutation, Thr²⁰⁶ → Met, identified previously in other MODY families. The presence of this missense mutation in two of our families prompted us to include it in the functional study carried out for the novel mutations.

The functional characterization of GK mutations Leu¹⁶⁵ → Phe, Thr²⁰⁶ → Met and Glu²⁶⁵ → Lys, revealed that the corresponding residues are important for GK activity. As seen for most of the MODY2 mutations, these mutations are kinetically inactivating and affect one or more kinetic parameters and, in some cases, also protein stability. We found a good correlation between the structural localization of the mutated residue and the effect on enzymatic activity. The recently defined crystal structure of human GK indicates that this protein has a large and a small domain, separated by a deep cleft [14]. Glucose binds to the interdomain cleft, composed of residues of the large domain (Glu²⁵⁶ and Glu²⁹⁰), the small domain (Thr¹⁶⁸ and Lys¹⁶⁹) and the connecting region I (Asn²⁰⁴ and Asp²⁰⁵). Upon binding to substrates (glucose and ATP), GK undergoes a conformational change that brings the large and the small domains physically closer, resulting in a closed active conformation. Residue Thr²⁰⁶ is located in the α 5 helix, inside the hinge of the active site, corresponding to the glucose-binding site [13]. The importance of the α 5 helix for GK function has been demonstrated [14,33]. The effect of substitution of methionine for Thr²⁰⁶ is devastating since the enzyme was inactivated by more than 99 %. Although our results are only approximations due to the poor activity of this GK mutant, they suggest that this mutation has a strong effect on the affinity for glucose, but apparently not on the affinity for ATP. Although there are no 'hot spot' mutations in the *GCK* gene, nearly 50 mutations have been identified in more than one family [6]. In particular, mutation Thr²⁰⁶ → Met has been reported in five unrelated families, including the two described here, and a mutation in the same nucleotide position, which produces a Thr²⁰⁶ → Arg mutation, has also been identified in another MODY2 family [34]. Finally, a randomly generated Thr²⁰⁶ → Met mutation associated with hyperglycaemia has been obtained in a mouse model by using the chemical mutagen *N*-ethyl-*N*-nitrosourea [35].

The novel mutation Leu¹⁶⁵ → Phe is a substitution of phenylalanine, which is an aromatic and bulky residue, for a leucine residue that is located very close to the active site. This mutation affects the enzyme affinity for both substrates (glucose and ATP), although the strongest effect is observed on the $S_{0.5}$ for

glucose. It also affects enzyme co-operativity and decreases the affinity for the glucose competitive inhibitors MH and GlcNAc. In addition, we found that the mutant protein GST-GK(L165F) is thermally unstable *in vitro*. Since glucose has a dramatic effect on GK stability, the relative instability of GST-GK(L165F) could be a consequence of its poor affinity for glucose, mitigating the physiological protection of the enzyme by its substrate, as postulated for other MODY2 mutations [25]. This effect could explain why only a fraction of the enzyme appears to be unstable shortly after a shift to 50 °C, in our time-course experiments. Leu¹⁶⁵ is located on the β 7 strand in the GK structure. The predictable involvement of this region of the protein in the active site of the enzyme (Figure 1A) is supported further by the results of a site-directed mutagenesis of the neighbouring GK residue Asn¹⁶⁶ to an arginine residue. The Asn¹⁶⁶ → Arg mutation also affects the affinity of GK for glucose, although with the opposite effect, and decreases the enzyme co-operativity (a similar low Hill coefficient as for Leu¹⁶⁵ → Phe) [36].

In family P22 reported in the present paper, Glu²⁶⁵ is changed to a lysine residue to afford the novel mutation Glu²⁶⁵ → Lys. Glu²⁶⁵ is located in a region of the protein between the β 11 strand and the α 6 helix. From the structural model, it could not be anticipated whether this glutamic acid residue participates directly in the interaction of the active site with the substrates glucose and ATP [14,37]. Although the kinetic analysis indicates that this substitution has a small, but significant, effect on the affinity for both substrates, it does not appear to affect the sensitivity of GK to competitive inhibitors, suggesting that Glu²⁶⁵ would not be involved directly in the active site of the enzyme. In contrast, we found a strong effect of mutation Glu²⁶⁵ → Lys on protein stability. The change Glu²⁶⁵ → Lys corresponds to a substitution of a positively charged amino acid for a negatively charged one. This critical change in polarity might impair the folding of the enzyme during biosynthesis *in vivo*, as proposed for another MODY2 mutation that involves a similar amino acid substitution (Glu³⁰⁰ → Lys; [25,38]). Both the Glu³⁰⁰ → Lys and Glu²⁶⁵ → Lys mutant proteins are expressed at low levels in *E. coli*, which could be a result of their instability. The apparent thermal instability of these mutant proteins should be considered as a factor that might decrease the I_a of the enzyme further as compared with that of the wild-type. Alternatively, we cannot rule out a possible involvement of Glu²⁶⁵ in a specific function of GK, not related directly to the active site of the enzyme. Interestingly, this residue is only conserved among GKs, but not hexokinases. In addition, a MODY2 mutation that involves the contiguous amino acid (Gly²⁶⁴ → Ser) has no effect on enzyme kinetics [39]. We explored the possible involvement of Glu²⁶⁵ in the interaction with GKRP. However, none of the three missense mutations studied appeared to affect this interaction in the yeast two-hybrid system. We cannot discard the possibility that the Glu²⁶⁵ → Lys mutation might affect the association of GK with other known GK partners, such as the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [40,41], the dual-specific phosphatase [42] or the neuronal nitric oxide synthase [43].

Most of the patients from the different families studied had relatively mild basal levels of fasting hyperglycaemia even 10–30 years after clinical diagnosis, and were being treated with diet alone. Despite the differences in the effects of these mutations on the kinetic parameters of GK, fasting plasma glucose levels did not differ very much in the different families and all of them were within the average (6–8 mM) described for MODY patients carrying *GCK* mutations [6]. Based on the mathematical model proposed by Matschinsky's group [15], there is good evidence that any decrease in the I_a below 30 % of the wild-type value would have little further effect on the fasting plasma glucose

levels. Interestingly, the increment of plasma glucose levels after OGTT for probands P5 (−0.7 mM) and P7 (+5.4 mM) differed from each other, despite these probands carrying the same GK mutation Thr²⁰⁶ → Met. Such a difference can also be observed with patients carrying the same mutation, described in previous studies {(+0.2 mM) [20] and (+4.7 mM) [18]}. These results are in agreement with the fact that genetic background and/or life style of the individuals affect the phenotypic characteristics of the MODY2 mutations.

Despite the fact that a large number of *GCK* mutations associated with MODY2 have been reported previously, in most countries, including Spain, new cases are still emerging from molecular diagnosis [44,45]. The importance of performing a molecular diagnosis of MODY has already been discussed in depth and emphasized by the clinical and scientific community owing to the implications for clinical management. Furthermore, the functional analysis of novel GK mutations provides new clues to the mechanisms involved in the regulation of GK activity. The importance of these studies for the development of new diabetic therapies is emphasized further by the recent discovery of small drugs acting as allosteric activators of GK which are able to increase GK activity *in vitro* and in normal and diabetic animal models [46,47].

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