Characterization of Glucokinase Mutations Associated With Maturity-Onset Diabetes of the Young Type 2 (MODY-2)

Different Glucokinase Defects Lead to a Common Phenotype

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Glucokinase (GK) is expressed in the pancreatic β-cells and liver, and plays a key role in the regulation of glucose homeostasis. The enzymatic activity and thermal stability of wild-type (WT) GK and several mutant forms associated with maturity-onset diabetes of the young type 2 (MODY-2) were determined by a steady-state kinetic analysis of the purified expressed proteins. The eight MODY-2 mutations studied were Ala53Ser, Val367Met, Gly80Ala, Thr168Pro, Arg36Trp, Thr209Met, Cys213Arg, and Val226Met. These missense mutations were shown to have variable effects on GK kinetic activity. The Gly80Ala and Thr168Pro mutations resulted in a large decrease in V_{max} and a complete loss of the cooperative behavior associated with glucose binding. In addition, the Gly80Ala mutation resulted in a sixfold increase in the half-saturating substrate concentration ($S_{0.5}$) for ATP, and Thr168Pro resulted in eight- and sixfold increases in the $S_{0.5}$ values for ATP and glucose, respectively. The Thr209Met and Val226Met mutations exhibited three- and fivefold increases, respectively, in the $S_{0.5}$ for ATP, whereas the Cys213Arg mutation resulted in a fivefold increase in the $S_{0.5}$ for glucose. These mutations also led to a small yet significant reduction in V_{max}. Of all the mutations studied, only the Cys213Arg mutation had reduced enzymatic activity and decreased thermal stability. Two mutants, Ala53Ser and Val367Met, showed kinetic and thermal stability properties similar to those of WT. These mutants had increased sensitivities to the known negative effectors of GK activity, palmitoyl-CoA, and GK regulatory protein. Taken together, these results illustrate that the MODY-2 phenotype may be linked not only to kinetic alterations but also to the regulation of GK activity. *Diabetes* 48:1645–1651, 1999

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GK, glucokinase; MODY-2, maturity-onset diabetes of the young type 2; n_H, Hill coefficient; psWT, pseudo–wild-type; RP, glucokinase regulatory protein; $S_{0.5}$, half-saturating substrate concentration; WT, wild-type.

Mutations in the glucokinase (GK) gene may be the most common cause of type 2 diabetes identified to date. More than 40 different mutations—including nonsense, missense, frameshifts, and splice-site mutations—have been linked to a subtype of type 2 diabetes called maturity-onset diabetes of the young type 2 (MODY-2) (1–8). This mild form of type 2 diabetes is characterized by its early onset, usually before the age of 25 years, and its autosomal dominant mode of inheritance. The mild hyperglycemia results primarily from a defect in glucose-induced insulin secretion rather than insulin action. Individuals with MODY-2 have an increased threshold for glucose-induced insulin secretion and decreased hepatic glycogen synthesis (9–11). The proposed role of GK as a "glucose sensor" in the insulin-secreting pancreatic β-cells (3,12,13) is consistent with the MODY-2 phenotype. In fact, Meglasson and Matschinsky (14) predicted that small reductions in β-cell GK activity might increase the threshold for glucose-induced insulin secretion, producing a type 2 diabetes phenotype.

Recently, the generation of hepatic and pancreatic β-cell specific gene knockouts in mice by Postic et al. (15) demonstrated a dual role for GK in glucose homeostasis. The mice that were globally deficient in GK or lacking GK only in the β-cells died within a few days of birth from severe diabetes, but the mice that were heterozygous for the β-cell expressed GK, survived, and were moderately hyperglycemic. Interestingly, hepatic GK knockout mice are also found to be mildly hyperglycemic and have impaired insulin secretion in response to glucose. The study indicated that alterations in both hepatic and β-cell GK likely contribute to the hyperglycemia of MODY-2.

GK (ATP:D-hexose 6-phosphotransferase [EC 2.7.1.1]) is a glycolytic enzyme expressed mainly in the insulin-secreting pancreatic β-cells and hepatocytes, where it plays a major role in the regulation of glucose metabolism (16–19). The enzyme is distinguished from other members of the mammalian hexokinase family by its lower affinity for glucose (K_m = 8 vs. 0.02–0.2 mmol/l for hexokinases), its molecular size (50 vs. 100 kDa), its lack of physiologically relevant product inhibition by glucose-6-phosphate, and its cooperative/sigmoidal kinetic behavior with respect to glucose (20). The cooperative behavior is unusual in that GK functions as a monomer

with a single active site. Several kinetic models have been proposed for its sigmoidal behavior.

The short-term regulation of GK activity depends on the substrate glucose causing activation of the enzyme through a "mnemonic mechanism" (21,22). Long-chain acyl-CoA esters in vitro and GK regulatory protein in vivo (in liver) are competitive inhibitors of the enzyme activity acting at a specific allosteric binding site (23,24).

The effects of many of the MODY-2 missense mutations on enzymatic activity have been examined and compared with that of wild-type (WT) β-cell GK (24–28). Molecular modeling of human β-cell GK (29) predicts that many of the missense mutations associated with the MODY-2 phenotype are located in the active site cleft or the surface leading into the cleft. Others are located in the region of the small domain predicted to undergo a glucose-induced conformational change that results in closure of the active site cleft. This conformational change is believed to be essential for catalysis (30). Consistent with this model, the GK mutations exhibit various reductions in the V_{max} for glucose phosphorylation, substrate affinity (ATP and glucose), cooperativity, and thermal stability. The diminished catalytic activity and stability of GK have been used to explain the MODY-2 phenotype by a gene dosage mechanism (25).

A set of GK mutations associated with the MODY-2 phenotypes was identified by Velho et al. (8) but not characterized kinetically. Here we examine the effect of six of the nine missense MODY-2 mutations on the enzymatic properties of GK. Two additional mutations identified by Hager et al. are included in the present kinetic characterization (7). WT and mutant forms of human pancreatic β-cell GK were expressed in *Escherichia coli*. The enzymatic properties of the purified expressed native proteins were compared in steady-state kinetic analyses.

RESEARCH DESIGN AND METHODS

Materials. Mono Q-Sepharose Fast Flow resin and a HiPrep Sephacryl S-100 High Resolution (26/60) column were obtained from Pharmacia Biotech (Piscataway, NJ). Glucose-6-phosphate dehydrogenase and leupeptin were from Boehringer Mannheim (Indianapolis, IN). SigmaUltra grades of glucose, ATP, and palmitoyl-CoA were from Sigma (St. Louis, MO). All other reagents obtained were of the highest grades available.

Site-directed mutagenesis of human pancreatic β-cell GK. Construction of the pET3a expression plasmid for human β-cell GK has been described previously (25). This construct, pEhgk-WT, contains the protein coding region and the 3′-untranslated region of the human β-cell GK cDNA clone phGK-20 (31). Mutations were generated by site-directed mutagenesis of the GK gene using the Altered Sites In Vitro Mutagenesis system from Promega (Madison, WI) or the QuikChange system from Stratagene (San Diego, CA). Restriction fragments containing the desired mutation were then subcloned into the β-cell GK expression plasmid by replacement of the appropriate restriction fragment. All of the mutations were confirmed by automated DNA sequencing (ABI 374 Sequencer; Perkin-Elmer, Foster City, CA). The human β-cell GK cDNA of pEhgk-WT (31) used to construct the expression plasmids in these studies contains a spurious Asp158Ala mutation and is termed pseudo–wild-type (psWT). Therefore, Asp158Ala mutation was corrected by site-directed mutagenesis as above in four of the constructs, reverting them to single MODY-2 mutants. It is presumed that the Asp158Ala mutation is an artifact that arose during cloning and is not a polymorphism (24).

Expression and purification of WT and mutant human pancreatic β-cell GK. WT and mutant GK genes were expressed in *E. coli* essentially as described previously (32). The pET expression plasmids were transformed into *E. coli* BL21(DE3)pLysS cells, which harbor the T7 RNA polymerase gene under the control of the inducible lacUV5 promoter. Cultures were grown at 37°C in Lauria broth medium containing ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) to an optical density at 600 nm of ~0.5. The cultures were then transferred to 22°C, and expression of the GK gene was induced with 0.4 mmol/l isopropyl-β-D-thiogalactopyranose (IPTG). After 24 h of induction at 22°C, the cells were harvested and stored at –80°C.

WT and mutant forms of GK were purified from *E. coli* extracts essentially as described previously (33). Briefly, GK was $(NH_4)_2SO_4$ precipitated (40–65%), chromatographed using Mono Q-Sepharose, and gel-filtered on a HiPrep Sephacryl S-100 High Resolution (26/60) column. The proteins were purified to homogeneity as judged by SDS-PAGE. The purified proteins were stored at –80°C in 30 mmol/l KPO_4 (pH 8.0), 30 mmol/l KCl, 0.7 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 6 mmol/l glucose, and 30% glycerol. After trichloroacetic acid precipitation, protein concentrations were determined by the method of Lowry et al. (34) using bovine serum albumin as the standard.

Kinetic analysis of WT and mutant GK. GK activity was measured at 30°C using a coupled spectrophotometric assay with glucose-6-phosphate dehydrogenase as the coupling enzyme (27). The reaction mixture for kinetic analysis (1.0 ml) contained 100 mmol/l Tris-HCl (pH 7.5), 100 mmol/l KCl, 1.0 mmol/l $MgCl_2$, 1.0 mmol/l dithiothreitol, 0.4 mmol/l $NADP^+$, 0.4 U/ml glucose-6-phosphate dehydrogenase, and various substrate concentrations. The total Mg^{2+} concentration in each reaction was 1.0 mmol/l in excess of the total ATP concentration. The stock $MgCl_2$-ATP solution was adjusted to pH 7.5 using 1.0 mol/l NaOH to obtain a final pH of 7.5 for the reaction assay mix. The reactions were initiated with ~0.018 U GK and followed by measuring the change in absorbance at 340 nm using the Kinetics/Time program on a Beckman DU650 spectrophotometer (Fullerton, CA). Initial rates were determined from a linear regression of the change in A_{340}. The glucose dehydrogenase activity of glucose-6-phosphate dehydrogenase observed in the absence of GK was measured at each substrate concentration, and the velocity was subtracted from that observed in the presence of GK. One unit of GK activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of product in 1 min at 30°C under the standard reaction conditions.

For the determination of the kinetic parameters (27,28), the experiments were carried out with either 12 glucose concentrations (100 μmol/l to 400 mmol/l) and saturating Mg•ATP (i.e., 10 × half-saturating substrate concentration [$S_{0.5}$] for ATP) or with 8 Mg•ATP concentrations (50 μmol/l to 40 mmol/l) and saturating glucose (i.e., 20 × $S_{0.5}$ for glucose). The range of substrate concentrations used depended on the $S_{0.5}$ of the enzyme. Velocities were obtained from triplicate measurements at each substrate concentration. The V_{max} was determined by a least-squares linear regression of the results of a Hanes-Woolf plot using Sigma Plot (Jandel Scientific, San Rafael, CA) and values obtained at high substrate concentrations (27,28). Calculation of the Hill coefficient (n_H) and $S_{0.5}$ were determined by linear regression of Hill plots using Sigma Plot (35). All assays were carried out in triplicate, and the values of the kinetic parameters shown represent an average ± SE from at least three independent experiments.

The thermal stability of the enzymes was analyzed by determining the GK activity after incubating the enzymes at 37°C. The purified enzymes were diluted to a final concentration of 0.001 U/μl in buffer containing 50 mmol/l KPO_4 (pH 8.0), 50 mmol/l KCl, 1.0 mmol/l EDTA, 0.2 mmol/l dithiothreitol, 1 mmol/l glucose, and 5% glycerol and then incubated at 37°C. After 0, 6, 12, 18, 24, 30, and 40 min of incubation, 10 μl was removed and the GK activity was measured under standard conditions with 400 mmol/l glucose and 25 mmol/l Mg•ATP at pH 7.5.

The affinity of WT and mutant GK for the effectors palmitoyl-CoA and rat liver GK regulatory protein (RP) was determined under conditions used previously (24,36,37). Briefly, the reactions were carried out at 30°C in buffer containing 25 mmol/l HEPES, pH 7.1, 1 mmol/l ATP, 1 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 200 μmol/l fructose-6-phosphate, 25 mmol/l KCl, 0.4 mmol/l $NADP^+$, and 0.4 U/ml glucose-6-phosphate dehydrogenase. The GK kinetics were assayed at pH 7.1 and in the presence of 25 mmol/l KCl because higher pH and Cl^- ions would antagonize the effect (24). Moreover, the presence of fructose-6-phosphate at 200 μmol/l is essential for GK binding with RP (24). After a 5-min preincubation, the reaction was initiated by the addition of 5 mmol/l glucose. The same amount of protein was used for the mutants and WT enzyme.

Molecular modeling of the human β-cell GK structure. A model for β-cell GK was developed based on the crystal structure of the related yeast hexokinase B (29). The model was examined using MOLSCRIPT software (38).

RESULTS

Expression and purification of WT and mutant forms of human pancreatic β-cell GK. Recently, 14 new mutations were identified in the GK gene of patients with MODY-2; the results of their clinical studies have been reported (8). Six of the nine MODY-2 missense mutations and three previously identified MODY-2 mutations (7) were examined here for their effect on GK activity. The location of these mutations within the human β-cell GK structural model is shown in Fig. 1. WT and mutant forms of human β-cell GK were expressed in a pET3a system (25), and the proteins were

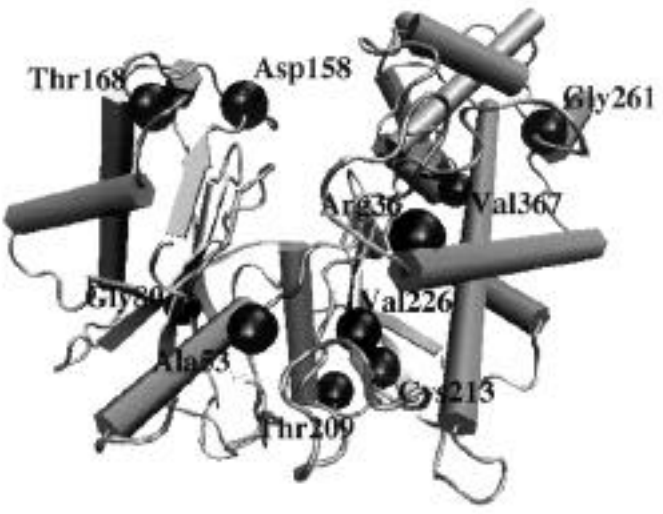

FIG. 1. Location of MODY-2 mutations in the human β-cell GK structural model. The human β-cell GK structure is represented by a ribbon drawing of the α-carbon backbone. This model is based on the crystal structure of the related yeast hexokinase B enzyme (29). The open conformation is shown. The missense mutations examined herein are shown as spheres and indicated by the affected amino acid residue. The model has been rotated compared with the view shown by Velho et al. (8) to clearly show the location of Asp158.

purified (33). One of the mutant forms of GK, Gly261Glu, failed to be induced by the pET3a system and could not be purified for further kinetic studies. Each of the purified enzymes was homogeneous and had an apparent molecular mass of ~50 kDa.

Kinetic properties of WT and mutant GK. In Table 1, the kinetic parameters of mutant forms of GK containing only the MODY-2 mutations are compared with WT GK. (As mentioned above, the spurious mutation of Asp158Ala was removed by site-directed mutagenesis.) The WT β-cell GK had a V_{max} of 93 μmol · min^{-1} · mg^{-1} and exhibited cooperative/sigmoidal glucose-dependent kinetics with an n_H of 1.5 (Table 1). The $S_{0.5}$ was 8.2 mmol/l for glucose. The enzyme displayed Michaelis-Menten kinetics with respect to Mg•ATP and an $S_{0.5}$ of 0.5 mmol/l Mg•ATP. These values are very similar to those previously reported (20,27,28,33,39).

Two of the MODY-2 mutations, Gly80Ala and Thr168Pro, exhibited dramatic effects on GK activity. The activities of the enzyme were severely impaired, with V_{max} reduced to <1% of that observed for WT GK. More interestingly, these mutations completely abolished the cooperative behavior associated with glucose, as indicated by the value approximately equal to 1.0 for n_H. These mutants also showed decreased affinity for the substrates. The $S_{0.5}$ for Mg•ATP was increased three- to fivefold for both mutants. Thr168Pro also caused a threefold increase in the $S_{0.5}$ for glucose. Surprisingly, two of the mutations, Ala53Ser and Val367Met, showed kinetic properties similar to that of WT GK, yet are associated with MODY-2 phenotype.

Kinetic properties of psWT and MODY-2 mutant GK in psWT background. The GK expression plasmid used in previous studies (25,26,33,39) and the present study codes for an Asp158Ala mutation (24). This mutation is also present in the original cDNA produced by Nishi et al. (31) and is called here psWT. In the present study, this mutation was reverted to WT by site-directed mutagenesis in all of the GK proteins analyzed in Table 1. However, all of the MODY-2 mutant GK proteins analyzed and presented in Table 2 carry this mutation of Asp158Ala (psWT) as background; thus, comparisons are made between MODY-2 mutants and psWT. The effect of this mutation on MODY-2 mutations has been questioned.

As shown previously (24) and here for comparison, the psWT GK exhibits a V_{max} (95.4 μmol · min^{-1} · mg^{-1}) similar to that of WT GK, as well as normal ATP-dependent kinetics with an $S_{0.5}$ of 0.5 mmol/l for ATP (Table 2). The enzyme also exhibited the characteristic cooperative kinetics for glucose, with an n_H of 1.5. However, the Asp158Ala mutation causes an approximately twofold decrease in the $S_{0.5}$ for glucose. The presence of an Asp158Ala mutation does not alter the effect of MODY-2 mutations on GK activity.

The MODY-2 mutations show a wide range of effects on enzymatic activity relative to the kinetic properties of the psWT, Asp158Ala mutant GK (Table 2). The enzymatic activities of Gly80Ala (psWT) and Thr168Pro (psWT) mutants are severely impaired compared with psWT GK (Table 2). These mutations drastically reduce the V_{max} and completely abolish the cooperative behavior associated with glucose and also show decreased affinity for the substrates. The $S_{0.5}$ for Mg•ATP is increased six- to sevenfold for both the mutants. The mutation Thr168Pro also causes a fivefold increase in the $S_{0.5}$ for glucose. These kinetic defects and their magnitude are similar to those observed by the same MODY-2 mutations in the absence of Asp158Ala mutation (Table 1). In addition, the Ala53Ser (psWT) and Arg36Trp (psWT) showed kinetic properties similar to those of psWT GK (Table 2).

Several other mutations associated with the MODY-2 phenotype have been examined as mutant forms of psWT GK (Table 2). The mutations Thr209Met, Cys213Arg, and Val226Met have less dramatic effects on GK activity. The pri-

TABLE 1
The kinetic properties of WT and mutant forms of human pancreatic β-cell glucokinase

Enzyme (background)	V_{max} (μmol · min^{-1} · mg^{-1})	Glucose		Mg•ATP	
		$S_{0.5}$ (mmol/l)	n_H	$S_{0.5}$ (mmol/l)	n_H
WT	93.4 ± 2.6	8.17 ± 0.5	1.57 ± 0.05	0.58 ± 0.05	1.01 ± 0.02
Ala53Ser (WT)	70.1 ± 1.8	6.80 ± 0.1	1.61 ± 0.06	0.37 ± 0.01	1.03 ± 0.005
Val367Met (WT)	85.6 ± 3.2	6.93 ± 0.3	1.55 ± 0.03	0.51 ± 0.03	0.97 ± 0.001
Gly80Ala (WT)	0.036 ± 0.003	13.87 ± 1.30	0.88 ± 0.02	2.92 ± 0.17	1.02 ± 0.024
Thr168Pro (WT)	0.726 ± 0.015	27.16 ± 1.65	1.03 ± 0.015	1.58 ± 0.21	0.96 ± 0.007

Data are means ± SE.

TABLE 2
The kinetic properties of psWT and mutant forms of human pancreatic β-cell glucokinase

Enzyme (background)	V_{max} (μmol · min^{-1} · mg^{-1})	Glucose		Mg•ATP	
		$S_{0.5}$ (mmol/l)	n_H	$S_{0.5}$ (mmol/l)	n_H
WT	93.4 ± 2.6	8.17 ± 0.5	1.57 ± 0.05	0.58 ± 0.05	1.01 ± 0.02
psWT*	95.4 ± 6.9	3.52 ± 0.2	1.54 ± 0.02	0.53 ± 0.01	1.04 ± 0.02
Ala53Ser (psWT)	79.7 ± 2.5	4.17 ± 0.3	1.52 ± 0.04	0.39 ± 0.04	1.04 ± 0.04
Arg36Trp (psWT)	100.0 ± 1.6	6.02 ± 0.5	1.74 ± 0.04	0.40 ± 0.005	1.02 ± 0.01
Gly80Ala (psWT)	0.043 ± 0.001	5.78 ± 0.5	1.03 ± 0.03	3.04 ± 0.1	0.98 ± 0.003
Thr168Pro (psWT)	1.45 ± 0.08	19.3 ± 2.7	1.02 ± 0.02	3.31 ± 0.2	1.00 ± 0.02
Thr209Met (psWT)	47.4 ± 1.5	5.75 ± 0.4	1.30 ± 0.04	1.47 ± 0.03	1.00 ± 0.03
Cys213Arg (psWT)	63.9 ± 2.8	16.5 ± 2.3	1.50 ± 0.02	0.79 ± 0.02	1.01 ± 0.01
Val226Met (psWT)	68.5 ± 4.3	5.07 ± 0.3	1.35 ± 0.04	2.29 ± 0.12	1.02 ± 0.006

Data are means ± SE. *psWT contains the Asp158Ala mutation.

mary effects of the Thr209Met and Val226Met mutations are three- and fourfold increases in the $S_{0.5}$ for Mg•ATP, respectively. In contrast, the Cys213Arg mutation causes a fivefold increase in the $S_{0.5}$ for glucose without significantly affecting Mg•ATP affinity. All of these mutations are also associated with a slight to modest reduction in V_{max}. Interestingly, although the mutation Ala53Ser is associated with MODY-2 phenotype, it has no significant effect on psWT GK activity or kinetic properties. These observations should be interpreted with the caveat that the effect of the Asp158Ala mutation cannot be predicted, even though it had no effect on the MODY-2 mutations that were directly compared on WT and psWT backgrounds.

Thermal stability of WT and mutant GK. Changes in protein stability rather than activity have been reported for some of the MODY-2 mutations (27,28). Thus, the thermal stability of WT and mutant forms of GK were examined as a function of time at 37°C. The mutations that had no effect on catalysis—Ala53Ser and Val367Met in WT and Arg36Trp in psWT—were found not to affect thermal stability (data not shown). These mutant proteins exhibited a thermal stability profile similar to that of the WT (and psWT) GK, retaining 85–90% of their activity after a 30-min incubation at 37°C. In contrast, only the Cys213Arg mutant protein in psWT showed reduced thermal stability compared with the others. After a 30-min incubation at 37°C, its activity was reduced to ~50% of that observed without this treatment. Thus, in addition to the kinetic effects, mutation of Cys213 to Arg also destabilizes the enzyme.

Inhibitory effects of palmitoyl-CoA and RP on GK activity. As shown in Table 1, two of the single mutant forms of GK, Ala53Ser and Val367Met, exhibit kinetic properties and thermal stability similar to those of WT GK. The fact that these mutations are associated with the MODY-2 phenotype suggests that the proteins may be defective in some other property of GK that is important for its biological role or function. Therefore, we studied the sensitivity of these GK mutants and WT to inhibition by palmitoyl-CoA and GK RP.

The inhibition of these MODY-2 mutants and WT GK activity by 1 and 2 μmol/l palmitoyl-CoA is shown in Fig. 2. Identical amounts of protein were assayed in these experiments. In the absence of palmitoyl-CoA, the GK activity of WT and mutants were similar. However, the mutant GK activities were inhibited to a much greater extent compared with WT GK. At 1 μmol/l palmitoyl-CoA, their approximate K_i value (24), the inhibition of Val367Met and Ala53Ser GK activity was 4- and 10-fold greater, respectively, than that of WT GK. A similar effect was observed at 2 μmol/l palmitoyl-CoA. These results suggest that the Val367Met and Ala53Ser mutations cause an increase in the affinity for the inhibitor palmitoyl-CoA.

The inhibition of the WT and mutant GK activity by the RP is compared in Fig. 3. Again, identical amounts of protein were assayed and similar GK activities were observed for WT and mutants in these experiments in the absence of RP. The mutant GK activities were almost twice as sensitive to inhibition by RP compared with WT GK activities at 2.5 U/ml of RP. However, this difference in sensitivity to the inhibitor is lost at a higher concentration of 5.0 U/ml of RP. These results suggest that these two mutant GK proteins also have an increased affinity for RP at the lower concentration.

DISCUSSION

A model for the three-dimensional structure of human β-cell GK has been developed based on the known crystal structure

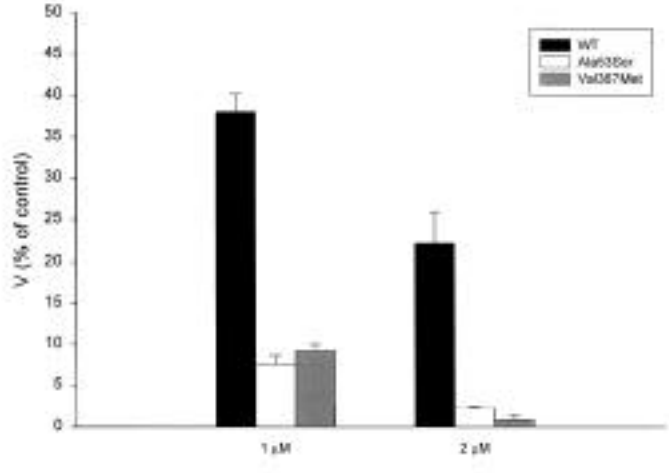

FIG. 2. Inhibitory effect of palmitoyl-CoA on the activity of WT human β-cell GK and two mutants (Ala53Ser and Val367Met) showing WT kinetic and thermostable characteristics. Assays were carried out after palmitoyl-CoA and GK were preincubated at 30°C for 5 min in 25 mmol/l HEPES (pH 7.1), 1 mmol/l ATP, 1 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 200 μmol/l fructose-6-phosphate, 25 mmol/l KCl, 0.4 mmol/l $NADP^+$, and 0.4 U/ml glucose-6-phosphate dehydrogenase. After a 5-min incubation, the reaction was initiated by the addition of 5 mmol/l glucose. The same amount of protein (150 μg) was used for both the mutants and the WT enzyme.

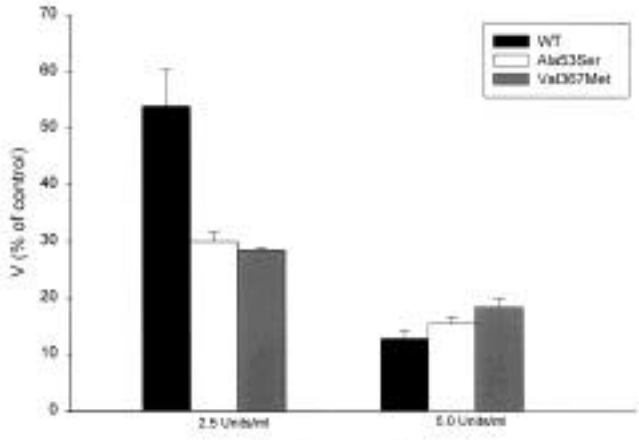

FIG. 3. Inhibitory effect of rat liver GK regulatory protein on the activity of WT human β-cell GK and two mutants (Ala53Ser and Val367Met) showing WT kinetic and thermostable characteristics. Assays were carried out after rat liver RP and GK were preincubated at 30°C for 5 min in 25 mmol/l HEPES (pH 7.1), 1 mmol/l ATP, 1 mmol/l $MgCl_2$, 1 mmol/l dithiothreitol, 200 µmol/l fructose-6-phosphate, 25 mmol/l KCl, 0.4 mmol/l $NADP^+$, and 0.4 U/ml glucose-6-phosphate dehydrogenase. After a 5-min incubation, the reaction was initiated by the addition of 5 mmol/l glucose. The same amount of protein (150 µg) was used for both the mutants and the WT enzyme.

of yeast hexokinase B (29). GK is predicted to fold into two domains separated by a deep active site cleft. Glucose binds at the bottom of this cleft. By analogy with yeast hexokinase B, a paradigm for induced-fit catalysis, the small domain of GK is thought to undergo a conformational change upon substrate binding, resulting in the closure of the cleft. This substrate-induced conformational change is believed to be essential for catalysis (30). The locations of the currently reported missense mutations relative to the catalytic cleft are indicated in the α-carbon ribbon drawing of the open conformation of GK shown in Fig. 1.

Based on predictions from the GK model, one of the MODY-2 mutations, Thr168Pro, involves a residue that forms the glucose-binding site in yeast hexokinase. This conserved residue is also predicted to be near the expected site for ATP binding (40) and be involved in the substrate-induced conformational change required for catalysis. Consistent with these predictions, the Thr168Pro mutation decreases the enzyme affinity for both glucose and ATP, resulting in complete loss of cooperative behavior (17,33,39).

Two of the MODY-2 mutations, Gly80Ala and Val226Met, involve conserved residues that are located within predicted ATP-binding site sequence motifs (40). Gly80 occurs in a conserved region on the surface of the active site cleft within the PHOSPHATE1 sequence motif (amino acid residues 74–99 of human β-cell GK [29,40]). This residue is predicted to be part of the β-turn structure of one of the ATP-binding hairpins. The decreased ATP affinity associated with the Gly80Ala mutation may result from steric disruption of the ATP-binding hairpin. It is worth noting that this mutation and other putative ATP-binding mutations (41) that abolish cooperativity are located on the small domain surface of the cleft in regions predicted to form substrate binding sites. This region is predicted to participate in the substrate-induced conformation change that results in cleft closure, and destabilization of this induced fit is probably responsible for the dramatic changes in activity. This mutation also demonstrates that loss of the cooperative behavior need not involve active site interactions with glucose, as had been previously predicted (17,39).

Val226 of GK is conserved among mammalian hexokinases (for example, rat brain hexokinase I and rat liver GK [42]), but not yeast hexokinase B, and lies within the PHOSPHATE2 sequence motif (amino acid residues 223–242 of human β-cell GK [29,40]). This residue is thought to be involved in ATP binding adjacent to the β-turn structure. The Val226Met mutation primarily affects ATP affinity, supporting the prediction that this region is important for ATP binding.

In the model for GK, two of the mutations, Thr209Met and Cys213Arg, involve residues that are predicted to be near the active site. Thr209 is a conserved residue located in α-helix 5 within the CONNECT1 ATP sequence motif (amino acid residues 197–216 of human β-cell GK [29,40]). This region is part of the putative interdomain hinge for ATP binding (40). The primary effect of the Thr209Met mutation is a decrease in ATP affinity, albeit only threefold, but consistent with this prediction. Thr209 also lies near the predicted glucose binding site (39,43) and is thought to interact with the catalytic Asp205 residue (32). This may explain the slight decrease in glucose affinity and V_{max} seen in this mutant. Cys213 is an internal residue located in the same region described above. Mutation to Arg introduces a large, positively charged side chain that is predicted to disrupt the structure of GK. This distortion could be transmitted along the helix to the active site Asp205 (32) and thereby affect catalysis.

To date, nearly all of the GK missense mutations associated with the MODY-2 phenotype appear to cluster within or near the active site cleft. Moreover, these mutant enzymes exhibit some level of reduced activity or stability. The reduced level of GK activity is thought to provide a biochemical explanation for the development of the MODY-2 phenotype. Decreased GK activity in pancreatic β-cells is predicted to alter glucose sensing and thus increase the threshold for glucose-induced insulin secretion by these cells (14). Since all of the MODY-2 mutations occur in regions common to both the pancreatic and hepatic isoforms of GK, the mutant isoforms expressed in hepatocytes are likely to exhibit similar kinetic defects as those observed in the β-cell isoform. In hepatocytes, the decreased activity of GK would reduce glucose phosphorylation and thereby decrease glycogen synthesis (11). The inhibition of endogenous glucose production by hyperglycemia is also decreased in MODY-2 patients, possibly as a result of decreased hepatic GK activity (44). This results in excessive liver glucose output with respect to glycemia levels in MODY-2 patients.

Interestingly, however, three of the mutations described here, Ala53Ser and Val367Met in WT and Arg36Trp in psWT backgrounds, were found to have no effect on GK kinetic properties. Additionally, these mutations do not affect the enzymatic stability of GK. The residues Ala53 and Val367 (or Arg36 in psWT) are predicted to lie on the protein surface far from the active site (see Fig. 1), suggesting a possible role in protein–protein interactions (45). These mutations could affect some noncatalytic functions important for the biological role of GK as the glucose sensor in pancreatic β-cells. These mutations could affect glucose sensing at the level of the glucose transporter (1). It has been suggested but not experimentally shown that glucose sensing requires the facil-

itative glucose transporter isoform, GLUT2, acting in concert with GK (14,46,47). These proteins may be coexpressed and form a tissue-specific multisubunit complex required for channeling/coordinating glucose transport and phosphorylation (48). The residues Arg36, Ala53, and Val367 could be important for this interaction. Disruption of this complex could raise the threshold for glucose sensing and lead to the MODY-2 phenotype. However, since GLUT-2 is minimally expressed in β-cells, this possibility would be restricted to the liver.

Second, these residues may participate in allosteric sites that bind long-chain acyl-CoAs, known to be inhibitors of GK (49). Thus, increased affinity of palmitoyl-CoA for GK mutants Ala53Ser and Val367Met reported herein (Fig. 2) would lead to a greater inhibition of GK activity. At the same concentration of palmitoyl-CoA, a more inhibited GK could be sufficiently compromised to bring about the MODY phenotype. However, despite the inhibitory kinetic effect shown here in vitro, palmitoyl-CoA might not be acting as a physiologic effector for GK in vivo, since it is located in the mitochondria of liver or bound to a cytosolic acyl-CoA binding protein (50). Furthermore, palmitate decreases glycolytic flux, but this effect is likely to be mediated by a decrease in concentration of fructose 2,6-bisphosphate, an important stimulator of 6-phosphofructo-1-kinase (51).

A similar reasoning could be applied to the observed increased affinity of the Ala53Ser and Val367Met GK mutants (Fig. 3) for the RP. Binding of RP is known to inhibit GK activity (23). The results of the present study indicate that there could be a higher threshold for the release of GK from the nuclear GK-RP complex in these MODY-2 mutants compared with WT at physiologic concentrations of RP. At 2.5 U/ml of RP treatment, the mutant GK activities were almost twice as inhibited as WT GK activities. This could lead to the expression of MODY-2 phenotypes in these mutants. However, at 5.0 U/ml of RP, this difference in the mutants and WT GK activities may be lost due to saturation by RP.

Several reports suggest that both the long-chain palmitoyl-CoA and RP show similar effects by binding to an allosteric site on GK (24,45). Both inhibited the GK activity competitively with respect to glucose (23,24). RP has been shown to transduce the effect of fructose-6-phosphate for binding and fructose-1-phosphate for the release of GK both in vitro and in vivo (45,52). These allosteric sites are considered to be overlapping, since palmitoyl-CoA and RP behave as mutually exclusive inhibitors (24). The similar responses of the two mutants Ala53Ser and Val367Met to the two effectors also support the contention that palmitoyl-CoA and RP interact at a common or overlapping site on the GK protein. This interpretation, however, is not consistent with the distant locations of residues A53 and V367 in the GK structural model. Resolution of this discrepancy must await the determination of crystal structure.

The interaction of GK with RP, which is affected by the MODY-2 mutations, may be necessary for the subcellular localization/distribution of GK, thought to play an important role in glucose metabolism in pancreatic β-cells and hepatocytes. RP and GK are localized in the nucleus of hepatocytes, but only GK is translocated from the nucleus to the cytoplasm in response to high glucose concentrations (53). This correlation between glucose metabolism and translocation of GK from nucleus to cytosol is consistent with the known kinetic properties of GK in association with RP (54). Therefore, the increased affinity of the GK mutants for RP not only may increase GK inhibition but also may prevent translocation out of the nucleus, and thus impair normal GK metabolic and physiologic function. This defect could lead to a shift toward sequestration of GK in the nucleus, which would lead to an increase in intracellular glucose concentration and a decrease in glucose-6-phosphate concentration (45,54–57). Clearly, the interactions of the proteins in the nucleus, GK, RP, and a possible anchoring protein, as well as the control by fructose-6-phosphate and fructose-1-phosphate, are indeed complex and require further study.

In conclusion, the findings described here show that the causes of the MODY-2 phenotype may be more complex and varied than simply a loss of GK activity as shown in the Ala53Ser and Val367Met mutants. Regulation of GK activity by translocation is a good candidate for the defect leading to MODY-2 phenotypes. Recently, the role of RP in shuttling of GK between nucleus and the cytoplasm in primary cultures of rat hepatocytes in glucose metabolism has been suggested by using immunohistologic and cellular fractionation techniques (54–59). These observations warrant direct testing of the MODY-2 mutants Ala53Ser and Val367Met for translocation defects. These studies are underway using mutant-GK adenoviral vectors for immunohistologic localization.

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