Insights into the Structure and Regulation of Glucokinase from a Novel Mutation (V62M), Which Causes Maturity-onset Diabetes of the Young*

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Glucokinase (GCK) serves as the pancreatic glucose sensor. Heterozygous inactivating *GCK* mutations cause hyperglycemia, whereas activating mutations cause hypoglycemia. We studied the *GCK* V62M mutation identified in two families and co-segregating with hyperglycemia to understand how this mutation resulted in reduced function. Structural modeling locates the mutation close to five naturally occurring activating mutations in the allosteric activator site of the enzyme. Recombinant glutathionyl *S*-transferase-V62M GCK is paradoxically activated rather than inactivated due to a decreased $S_{0.5}$ for glucose compared with wild type (4.88 *versus* 7.55 mM). The recently described pharmacological activator (RO0281675) interacts with GCK at this site. V62M GCK does not respond to RO0281675, nor does it respond to the hepatic glucokinase regulatory protein (GKRP). The enzyme is also thermally unstable, but this lability is apparently less pronounced than in the proven instability mutant E300K. Functional and structural analysis of seven amino acid substitutions at residue Val[62] has identified a non-linear relationship between activation by the pharmacological activator and the van der Waals interactions energies. Smaller energies allow a hydrophobic interaction between the activator and glucokinase, whereas larger energies prohibit the ligand from fitting into the binding pocket. We conclude that V62M may cause hyperglycemia by a complex defect of GCK regulation involving instability in combination with loss of control by a putative endogenous activator and/or GKRP. This study illustrates that mutations that cause hyperglycemia are not necessarily kinetically inactivating but may exert their effects by other complex mechanisms. Elucidating such mechanisms leads to a deeper understanding of the GCK glucose sensor and the biochemistry of β-cells and hepatocytes.

Glucokinase (GCK)[1] plays a critical role in the regulation of insulin secretion and has been termed the pancreatic β-cell glucose sensor on account of its kinetics, which allow the β-cells to change glucose phosphorylation rate over a range of physiological glucose concentrations. These kinetic characteristics are the enzyme's low affinity for glucose ($S_{0.5} \sim 7.5$ mM), cooperativity with glucose (Hill number of ~1.7), and lack of inhibition by its product glucose 6-phosphate. Glucokinase plays an important role in glucose sensing not only in the pancreatic β-cell but also in the liver and a variety of neural/neuroendocrine cells. These include the pancreatic α-cell, L- and K-type gut enterocytes, and certain rare neurons in the central nervous system, mainly in the hypothalamus (1–3). It is the sum of its actions in these multiple sites that ultimately determines the blood glucose concentration. In the liver glucokinase is regulated by glucokinase regulatory protein (GKRP), which acts as a competitive inhibitor with respect to glucose (4, 5). In addition to this role GKRP also determines the subcellular location of glucokinase within the liver cell (6). Glucokinase translocates between the nucleus and the cytoplasm depending on the metabolic state of the cells. When glucokinase is not bound to hepatic GKRP and therefore present in the cytoplasm, the enzyme facilitates hepatic glucose utilization and glycogen synthesis thereby helping to lower the blood glucose concentra-

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[1] The abbreviations used are: GCK, glucokinase; GKRP, glucokinase regulatory protein; MODY, maturity-onset diabetes of the young; fpg, fasting plasma glucose; GST, glutathionyl *S*-transferase; S6P, sorbitol 6-phosphate; WT, wild type.

tion. Consistent with this role in the liver, mice that totally lack hepatic glucokinase have impaired glucose tolerance (7). There is controversy in the literature as to whether GKRP is expressed in the β-cell (8–10) and whether glucokinase translocates from the nucleus to the cytoplasm during glucose stimulation (11, 12).

Inactivating mutations in the gene encoding this enzyme (*GCK*) cause a subtype of maturity-onset diabetes of the young (MODY2/GCK-MODY) (13, 14). To date over 190 mutations have been reported in the literature (15). These mutations include non-sense, frameshift, deletions, splice-site, and missense mutations, and they are distributed throughout the ten exons (1a–10) of the pancreatic isoform of the gene.

The functional characterization of over 35 missense *GCK* mutations that cause MODY, has shown that a number of these kinetic parameters are altered and that usually more than one parameter is changed (16–18). These changes usually include an increase in the glucose $S_{0.5}$ and/or a decrease in the turnover of the enzyme (k_{cat}). This exploration has also uncovered mutants that are thermally labile (19). These mutations may have additional defects or appear kinetically close to normal or entirely normal (19). The stability of the mutant glutathionyl *S*-transferase (GST)-E300K-GCK has been studied extensively in a cell biological assessment of enzyme expression and stability in addition to *in vitro* thermolability assays providing evidence that instability is indeed pathogenic in this case (20).

Mutations in *GCK* not only cause hyperglycemia (MODY), they can also result in hypoglycemia, hyperinsulinemia of infancy (21–24). Five missense mutations have been reported (T65I, W99R, Y214C, V455M, and A456V), and functional characterization of these mutations has shown that they are activating mutations, because there is a decrease in the glucose $S_{0.5}$ and/or an increase in the k_{cat} (21–24). In a structural model of GCK (25) these mutations have been shown to cluster in a region remote from the substrate binding site termed the allosteric activator site (21). Recently, a novel class of small molecular activators of GCK has been described (26–28); these have been shown to lower blood glucose levels in rodent models of type 2 diabetes (26). These molecules act at the allosteric activator site and have a similar effect to the naturally occurring activating *GCK* mutations (26, 27). Given the existence of this allosteric activator site and the fact that GCK responds to this novel class of pharmacological activators, an endogenous allosteric activator of GCK has been proposed (19, 21). Recently the crystal structure of glucokinase has been solved in its free and liganded forms (22, 23), which has confirmed the presence of an allosteric activator site and demonstrated that there are global conformation changes, which could be the structural corollary of the mnemonical mechanism that is invoked to explain the cooperative kinetics of the enzyme with glucose (27).

In this study we present a novel *GCK* mutation (V62M), which has been identified in two families and shown to cosegregate with MODY. When functionally characterized, recombinant GST-V62M GCK is paradoxically mildly activating rather than inactivating. This study provides the most detailed functional assessment of a *GCK* mutation to date and includes extensive mutagenesis studies at residue Val[62], which increase our structural insights and enhance our understanding of the allosteric activator site.

MATERIALS AND METHODS

Human Subjects Studied

Family 1 (Exeter, UK)—The female proband (III:4) presented with gestational diabetes during her first pregnancy. This diagnosis was made on the basis of an oral glucose tolerance test performed at 26-week gestation, which showed a fasting value of 5.5 and a 2-h value of 9.2 mM. She was managed on diet alone. Her male infant was born at 41 weeks (birth weight, 3.27 kg; length, 50 cm; head circumference, 35 cm;

percentiles were 16th, 17th, and 27th, respectively). An oral glucose tolerance test performed on the proband at 8 weeks post partum showed a fasting level of 6.5 and a 2-h value of 7.1 mM. The proband's father (II:6) was diagnosed with abnormal glucose tolerance at the age of 57 years during routine screening and had been treated by diet. The deceased paternal grandmother had diabetes. Anthropometrical measurements (height, weight, body mass index), fasting blood glucose (fpg), lipids, and HbA1$_c$ were measured in all family members available for testing; including one brother, two paternal aunts, one paternal uncle, and four paternal first cousins (see Fig. 1*a*).

Family 2 (Italy)—The male proband (IV:1) and his identical twin brother (IV:2) were diagnosed with MODY on the basis of elevated fpg values at the age of 6 years. Both boys were born at 39 weeks (birth weight, 3.3 kg). Details of this family are shown in Fig. 1*b*.

Identification of Glucokinase Gene Mutations by Direct Sequencing

Genomic DNA was extracted from peripheral lymphocytes using a Wizard DNA extraction kit (Promega, Southampton, UK). The coding regions of exons 1a–10 and the intron-exon boundaries of the glucokinase (*GCK*) gene were amplified by PCR using published primer sequences (29). PCR products were purified using QIAquick PCR purification columns (Qiagen), and both strands were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Warrington, UK) according to the manufacturer's recommendations. Reactions were analyzed on an ABI 3100 DNA sequencer (Applied Biosystems).

Kinetic Analysis

Recombinant human islet wild type enzyme and the mutants V62A, V62E, V62F, V62K, V62L, V62M, V62Q, V62T, and E300K were generated using methods previously described (30). The enzymes were expressed in the form of GST fusion proteins using the protocols developed during the study of GST-GCK V455M and other *GCK* mutations (17). The following modifications to the protocol were made. Protocol A was carried out with 11 glucose dilutions between 0 and 100 mM for each mutant and WT GST-GCK. Protocol B was carried out with glucose at ×10 $S_{0.5}$ for wild type, each Val[62] mutant and E300K GCK. Further kinetic analysis was performed in the presence of the newly discovered compounds (RO028165, RO0274375, and RO0283946; Fig. 2) that allosterically activate wild type GCK (26). Experiments were performed in the presence of 0.3 μM, 1 μM, 3 μM, 9 μM, 27 μM, and 60 μM RO0281675, RO0274375, and RO0283946. The results were compared with those obtained with wild type GST-GCK preparations freshly made for the present investigation. The activity index, an expression of the proposed *in situ* phosphorylation capacity of the enzyme, was calculated as previously described (24). Kinetic analysis was also performed with human and rat recombinant glucokinase regulatory protein (GKRP). GKRP is a competitive inhibitor of glucose (31), and consequently the kinetic analysis was carried out as previously described with glucose at 3 mM for wild type, V62F, and V62Q and to account for the decreased or increased glucose $S_{0.5}$ values at 1 mM for V62L, 1.5 mM for V62M, 2.5 mM for V62K, 4.5 mM for V62E, 7.5 mM for V62T, and 8.5 mM for V62A (17).

Thermal stability of the mutant V62M-GST GCK enzyme, the known instability mutant E300K-GST GCK, and wild type GST-GCK was tested using protocols previously described (32). The following modifications were made: enzyme stock solutions were diluted in storage buffer containing either 0 or 50 mM glucose. The enzymes were incubated in a water bath at 30, 32.5, 35, 37.5, 40, 42.5, 45, 47.5, 50, and 52.5 °C for 30 min. Glucokinase activity was then determined spectrophotometrically as described above. Glucose has been shown to stabilize glucokinase (33). The stabilization of mutant V62M, E300K, and wild type GCK by the addition of glucose was therefore investigated. Briefly, enzyme stock solutions lacking glucose were diluted in buffer with increasing concentrations of glucose ranging from 0 to ~100 mM and to achieve comparable protein concentrations of ~75 μg/ml. The enzymes were incubated in a water bath at 42.5 °C for 30 min. Glucokinase activity was then determined spectrophotometrically as described above.

Structural Analysis

Structural analysis of V62M and published activating mutations in the glucokinase enzyme was performed using a homology model based on the WT-GCK crystal structure, co-cystallized with α-D-glucose and the allosteric activator (RO0283946), as a template (42). In addition, homology models for each of the mutations at amino acid position 62 were built. Interaction energies between the amino acids comprising
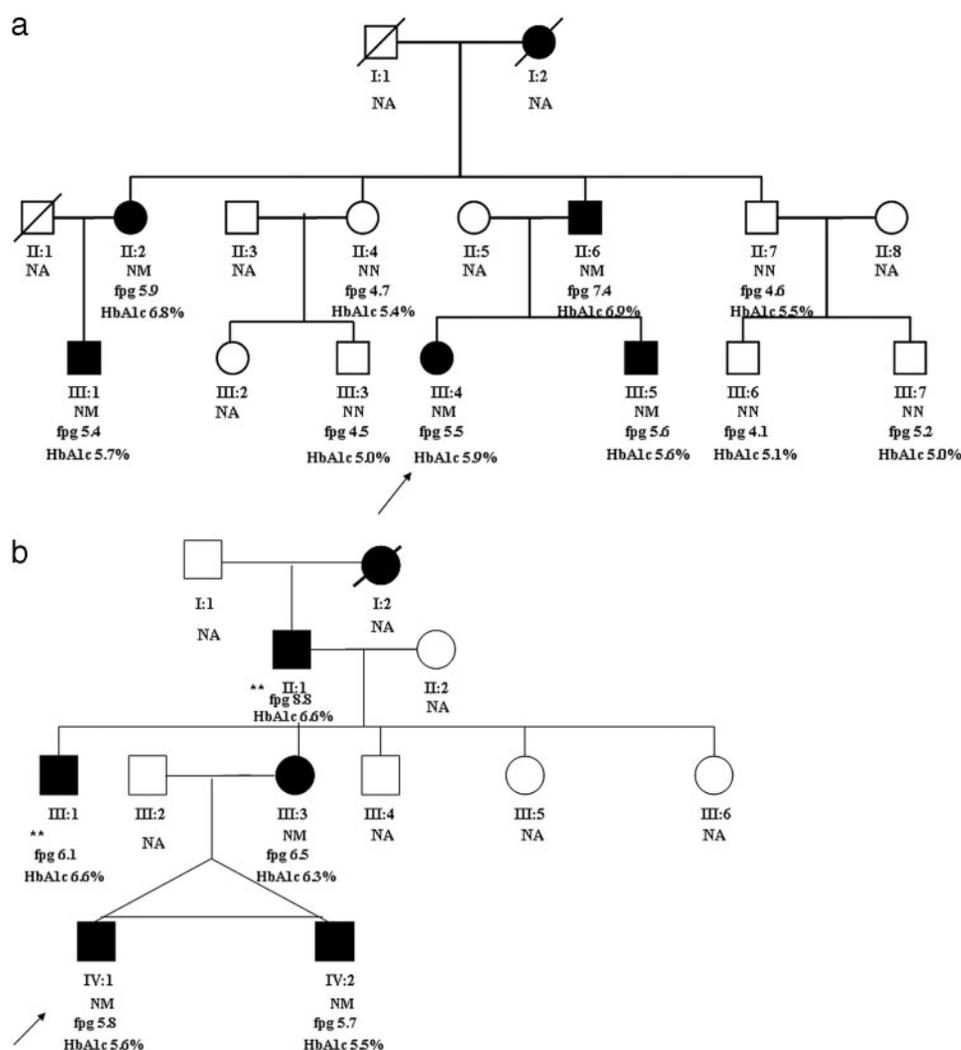
FIG. 1. **Pedigrees for MODY families showing co-segregation of the V62M GCK mutation with elevated fasting plasma glucose values.** *a*, UK family. *b*, Italian family. *Circles* represent females, *squares* males. *Shaded shapes* represent affected individuals. The *number* under each member represents the sample identifier. *NN*, no mutation; *NM*, heterozygous for V62M mutation. Fasting plasma glucose (fpg) values and HbA1c values are shown where available. *NA*, not available for testing; **, sample not available for genetic testing.

the activator binding site and each of the pharmacological activators (RO028165, RO0274375, and RO0283946) in its x-ray position were calculated using MOE[2] software and MMFF94X force fields.

RESULTS

Sequencing of Glucokinase Gene—Direct sequencing of the entire coding region and exon-intron boundaries of the glucokinase gene was performed in the probands from both families. In both families a heterozygous missense mutation substituting methionine for valine at codon 62 (V62M; $\underline{G}TG>\underline{A}TG$) in exon 2 was identified. In family 1 (UK), this mutation was also identified in the proband's father, brother, paternal aunt, and cousin (Fig. 1*a*) and co-segregated with hyperglycemia as shown by either a raised fasting glucose of >5.5 mM or an elevated HbA1c above the normal range. In family 2 (Italy), the mutation was identified in the proband, the proband's identical twin brother, and the proband's mother. The other affected family members were not available for testing (Fig. 1*b*). This mutation was not found in over 100 normal chromosomes.

Biochemical Characterization of V62M—The mutant enzyme was expressed as a GST fusion protein, and the purified enzyme was subjected to kinetic analysis (k_{cat}, glucose $S_{0.5}$, n_H, and ATP K_m) (Table I). Four preparations of wild type, mutant

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[2] Chemical Computing Group Inc., Quebec, Canada.

V62M, and four of E300K GST-GCK were purified. All GCK proteins analyzed were found to be essentially pure as indicated by the presence of a single band at 75 kDa on phast gel (Amersham Biosciences) electrophoresis (data not shown). The functional data are shown in Table I (except those for E300K, which have been published already (12, 13)). The mutant enzyme showed a significant increase in affinity for glucose indicated by the decrease in glucose $S_{0.5}$ value (4.88 ± 0.25 *versus* 7.55 ± 0.23 V62M and WT, respectively). Paradoxically, the change to the kinetic parameters of this mutant result in an increase in the activity of this enzyme compared with wild type of ~4-fold inconsistent with this mutation causing MODY.

To explain how an enzyme that is kinetically more active than wild type causes MODY we investigated whether the mutation might result in an enzyme that is thermally labile. Analysis of the thermal stability of GST-GCK showed that the wild type enzyme was slightly activated after 30-min incubation as the temperature was raised from 30 to 47.5 °C but that its k_{cat} decreased at higher temperatures falling to about 10 s^{-1} (by 80%) at 50 °C (Fig. 3*a*). The activity of V62M GST-GCK increased similarly as that of the wild type as the temperature rose but decreased abruptly at temperatures above 42.5 °C. In contrast, the previously reported instability mutant

FIG. 2. **Flexible overlap of compounds RO0274375 and RO0281675 with compound RO0283946 at its x-ray conformation.**
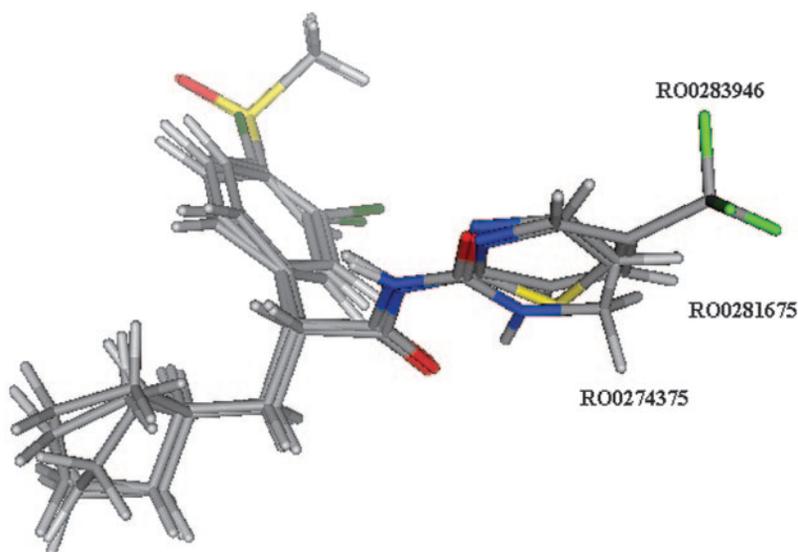
TABLE I
Mutational Analysis of the V62 GKA binding site

Data are means $\pm$ S.E. for wild type and V62M GCK. The results are the means of the kinetic analysis of four independent expressions of wild type and mutant V62M GST-GCK. For the remaining mutants two independent expressions were prepared and the mean and individual data points are shown.

Mutants	Parameters							
	Yield	Protein concentration	k_{cat}	Glucose $S_{0.5}$	ATP K_m	n_H	GI[a]	EC$_{50}$ for RO0274377
	mg/liter	*mg/ml*	*s^{-1}*	*mM*				*μM*
WT	41.3 ± 3.33	3.03 ± 0.16	62.3 ± 4.75	7.55 ± 0.23	0.41 ± 0.33	1.74 ± 0.04	1.45 ± 0.11	6.85 ± 0.43
V62A	23.1 (20.6, 25.6)	1.56 (1.37, 1.75)	43.1 (48.8, 37.3)	27.4 (24.6, 30.2)	0.20 (0.20, 0.20)	1.51 (1.50, 1.51)	0.34 (0.38, 0.29)	13.2 (10.0, 16.3)
V62E	5.64 (4.61, 6.66)	0.50 (0.48, 0.52)	4.92 (5.22, 4.62)	19.5 (23.8, 15.1)	0.07 (0.08, 0.05)	1.15 (1.31, 0.98)	0.16 (0.16, 0.15)	NA[b]
V62F	17.4 (21.2, 13.5)	1.08 (1.22, 0.94)	33.7 (39.4, 28.0)	8.93 (9.48, 8.37)	0.71 (0.70, 0.71)	1.43 (1.45, 1.40)	1.09 (1.37, 0.81)	NA
V62K	6.08 (5.32, 6.84)	0.48 (0.38, 0.57)	6.42 (7.44, 5.40)	10.0 (11.4, 8.60)	0.16 (0.18, 0.14)	1.18 (1.13, 1.23)	0.37 (0.44, 0.30)	NA
V62L	4.25 (1.46, 7.04)	0.59 (0.26, 0.91)	59.9 (65.3, 54.4)	3.26 (3.58, 2.94)	0.61 (0.59, 0.63)	1.39 (1.43, 1.35)	9.27 (10.2, 8.34)	NA
V62M	29.7 ± 6.29	1.99 ± 0.38	53.6 ± 2.51	4.88 ± 0.25	0.46 ± 0.03	1.49 ± 0.12	4.68 ± 0.48	NA
V62Q	6.69 (7.04, 6.34)	0.61 (0.55, 0.66)	14.9 (14.9, 14.8)	12.7 (11.5, 13.9)	0.11 (0.11, 0.10)	1.14 (1.16, 1.12)	0.75 (0.75, 0.74)	NA
V62T	14.3 (15.4, 13.2)	1.23 (1.13, 1.32)	25.7 (26.6, 24.8)	26.3 (30.5, 22.1)	0.22 (0.31, 0.13)	1.36 (1.47, 1.25)	0.26 (0.27, 0.24)	4.07 (5.85, 2.29)

[a] GI, the activity index for the enzyme was calculated as previously described (24).
[b] NA, not applicable.

E300K GST-GCK (16) showed much greater thermolability than the wild type enzyme and was also more unstable than V62M. The stabilizing effect of glucose on each enzyme, WT, E300K, and V62M GST-GCK was also investigated. The instability of the mutants was tested at 42.5 °C, because this was the highest temperature at which point the least variation between the three enzymes was visible during temperature titration. Decreasing or eliminating of glucose in the heat step had no effect on the activity of wild type GST-GCK, however this treatment markedly lowered the activity (k_{cat}) of the instability mutant E300K from about ~41 to ~8 s^{-1}. Glucose removal caused a far less pronounced decrease in the activity of V62M GST-GCK from ~58 to ~36 s^{-1} (Fig. 3b). In fact, glucose titration at 42.5 °C and using physiological glucose levels showed that V62M is virtually as stable as wild type.

Inhibition with human and rat GKRP was also determined. WT-GST-GCK showed an expected reduction in activity with increasing concentrations of both human (Fig. 4a) and rat (data not shown) GKRP with and without sorbitol-6-phosphate (S6P). However, there was no reduction in enzyme activity for V62M-GST-GCK with either human (Fig. 4, *a* and *b*) or rat GKRP (data not shown). Further kinetic analyses, of V62M GST-GCK in response to a newly discovered class of glucokinase-activating drugs (RO028165, RO0274375, and RO0283946), were performed. The potency and efficacy of the three compounds was similar (Table II). Wild type GST-GCK responded to the drug as demonstrated by increasing maximal-specific activities (k_{cat}) of the enzyme with increasing concentrations of the drug (as previously described (26)). There was also a marked decrease in the $S_{0.5}$ for glucose with increasing concentrations of the drug. In contrast V62M GST-GCK did not respond to the drug, there was no increase in k_{cat}, no decrease in $S_{0.5}$, and consequently no increase in the relative activity index. The kinetic parameters for wild type and mutant V62M-GST-GCK have been plotted against increasing concentrations of the drug in Fig. 5 (*a* and *b*).

Biochemical Analysis of Additional Mutants at Residue 62—To understand the relationship between structure and function at residue 62 seven additional mutants (V62A, V62E, V62F, V62K, V62L, V62Q, and V62T) were generated and functionally characterized. Two preparations of each mutant were purified (Table I). The mutations that introduced polar residues at position 62 (Glu, Lys, and Gln) had a marked effect on k_{cat}, whereas the less polar residue (Thr) had a less pronounced effect. The replacement with the smaller alanine (Ala) residue resulted in an increased $S_{0.5}$, whereas the opposite effect was observed with the leucine (Leu) and methionine (Met) mutations, which introduce slightly bulkier residues. The response to human GKRP and three different GCK activators of the propionic acid amide series (RO0274375, RO0283946, and RO281675) was also investigated (Fig. 4 and Table II). V62A, V62T, and V62L responded to the activator as seen by an increase in the activity index, although this increase was variable and depended on the nature of the compounds. V62Q, V62E, V62F, and V62K were resistant to the activators. The

(a) Effect of temperature on enzyme activity

(b) Effect on enzyme activity of glucose concentration

Mutant	n	Mean Protein concentration (μg/ml)	Range of Protein concentration (μg/ml)
WT	3	84.7 2 ± 2.96	79.0-89.0
V62M	3	61.7 ± 4.21	53.5-67.5
E300K	3	71.0 ± 11.6	50.0-90.0
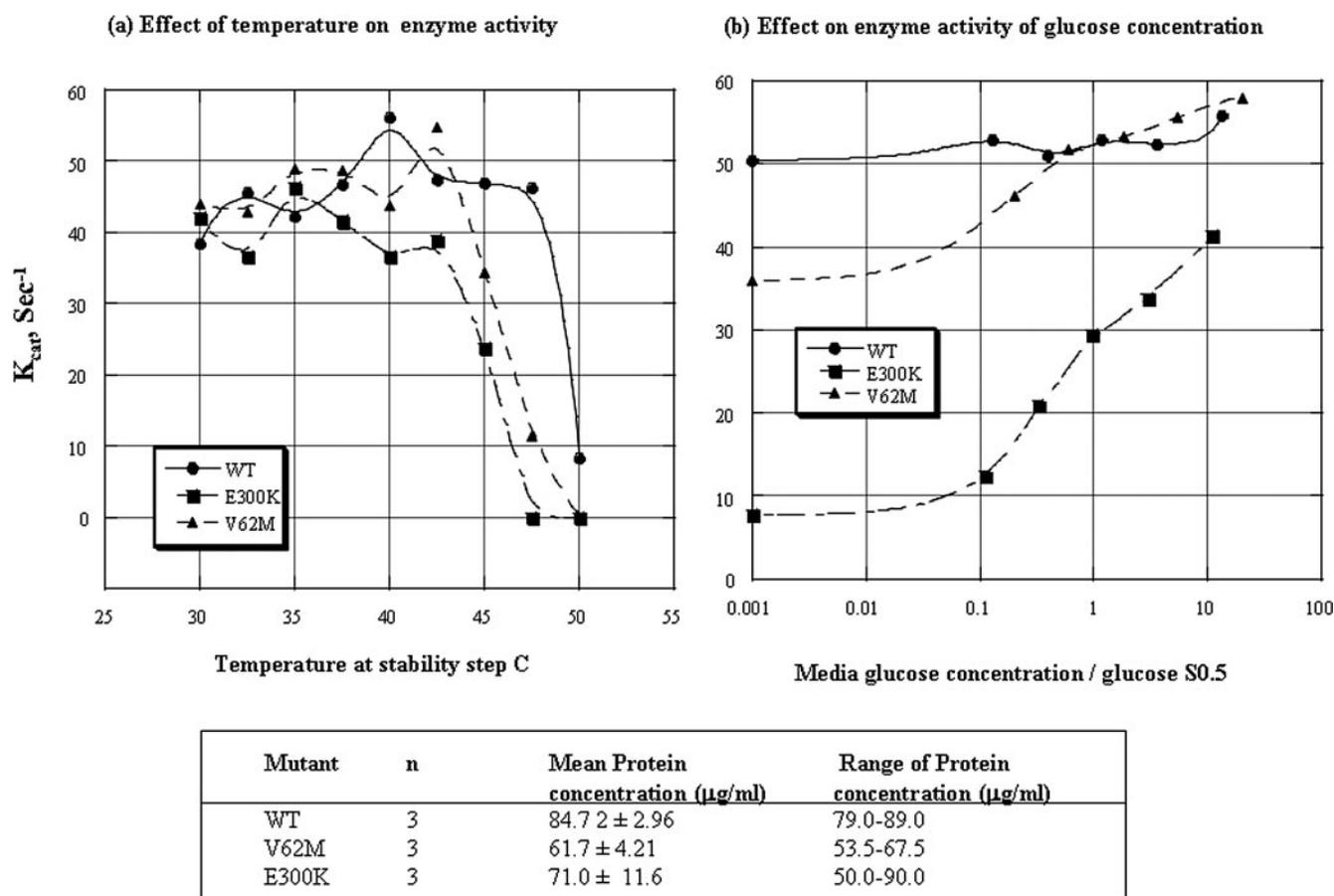
FIG. 3. **Assessment of thermal instability and effect of glucose concentration on stability of wild type GCK, V62M-GCK, and E300K-GCK.** *a*, effect of temperature on enzyme activity. Enzymes were diluted in buffer containing 0.1 M HEPES buffer, 150 mM KCl, 6 mM MgCl$_2$, 30% glycerol, and 8 mM glucose (*WT*), 5 mM glucose (*V62M*), and 9.2 mM glucose (*E300K*). Enzymes were incubated for 30 min at each temperature prior to kinetic analysis. This graph shows the mean results of three independent expressions of WT, E300K, and V62M-GST-GCK. For clarity S.E. are not shown. Similar results were seen for enzymes stored in 50 mM glucose and heat treated at 50 mM (data not shown). Protein concentrations at the heat step are listed. *b*, stock enzymes were stored in buffer containing 0 mM glucose. Enzymes were diluted in buffer containing 0.1 M HEPES buffer, 150 mM KCl, 6 mM MgCl$_2$, 30% glycerol, glucose levels were adjusted to range from zero to ~100, and the enzymes were then incubated for 30 min at 42.5 °C followed by kinetic analysis. The ratio of the glucose concentration to the glucose $S_{0.5}$ was used to plot the data to account for differences in glucose affinities of the enzymes. This graph shows the mean results of three independent expressions of WT, E300K, and V62M-GST-GCK. For clarity S.E. are not given. Similar results were seen for enzymes stored in 50 mM glucose (data not shown). Protein concentrations at the heat step are shown *beneath the graphs*.

kinetic analysis of the mutant and wild type enzymes in the presence of human GKRP showed that only wild type and V62A, V62T, and V62E were significantly inhibited (<80% enzyme activity remaining).

Structural Analysis—Structural analysis of V62M showed that it is in close proximity to the previously published naturally occurring activating mutations (T65I, W99R, Y214C, V455M, and A456V) and the artificially created (D158A) mutation that increase the glucose affinity and/or k_{cat}. All these mutations are located in a domain of the enzyme ~20 Å from the substrate binding cleft for glucose and MgATP suggesting the existence of a specific allosteric activator site (Fig. 6, *a* and *b*). The crystallisation of GCK has revealed significant differences between the structures of hexokinase I and GCK in the region between Ser 64 and Gly 72 (27). The allosteric activator site is located near this glucokinase-specific region (27). Therefore, a homology model for the V62M mutation was built using the WT GCK structure, co-crystallized with RO0283946, as a template and used to investigate why the substitution of a valine for a methionine residue at codon 62 prevents the allosteric activator from having any effect. The methionine residue blocks the ligand structure core from taking the preferred binding position (Fig. 6*c*). This is consistent with the fact that residue 62 is one of the residues involved in the binding of

activators to glucokinase and forming a hydrophobic interaction with the drug.

A homology model for each of the mutations at amino acid position 62 was built using the WT GCK structure, co-crystallized with RO0283946, as a template. RO0274375 and RO0281675 were docked into the binding pocket through flexible alignment with the observed crystal structure of RO0283946 (Fig. 6*d*). Interaction energies between the amino acids in the activator binding site and glucokinase activators in its x-ray position were calculated using software MOE[2] and MMFF94X force fields. The results are shown in Table II and indicate a strong non-linear correlation between glucokinase activator activation and the van der Waals interaction energies. This relationship suggests that the ability to activate is related to favorable hydrophobic interactions between activator and binding pocket. Smaller van der Waals interaction values translate into better hydrophobic interaction, whereas a large positive value indicates the ligand is unlikely to fit in the binding pocket.

DISCUSSION

We have demonstrated that a novel *GCK* mutation (V62M) that co-segregates with MODY in two families results in hyperglycemia but does not show reduced kinetic function. The
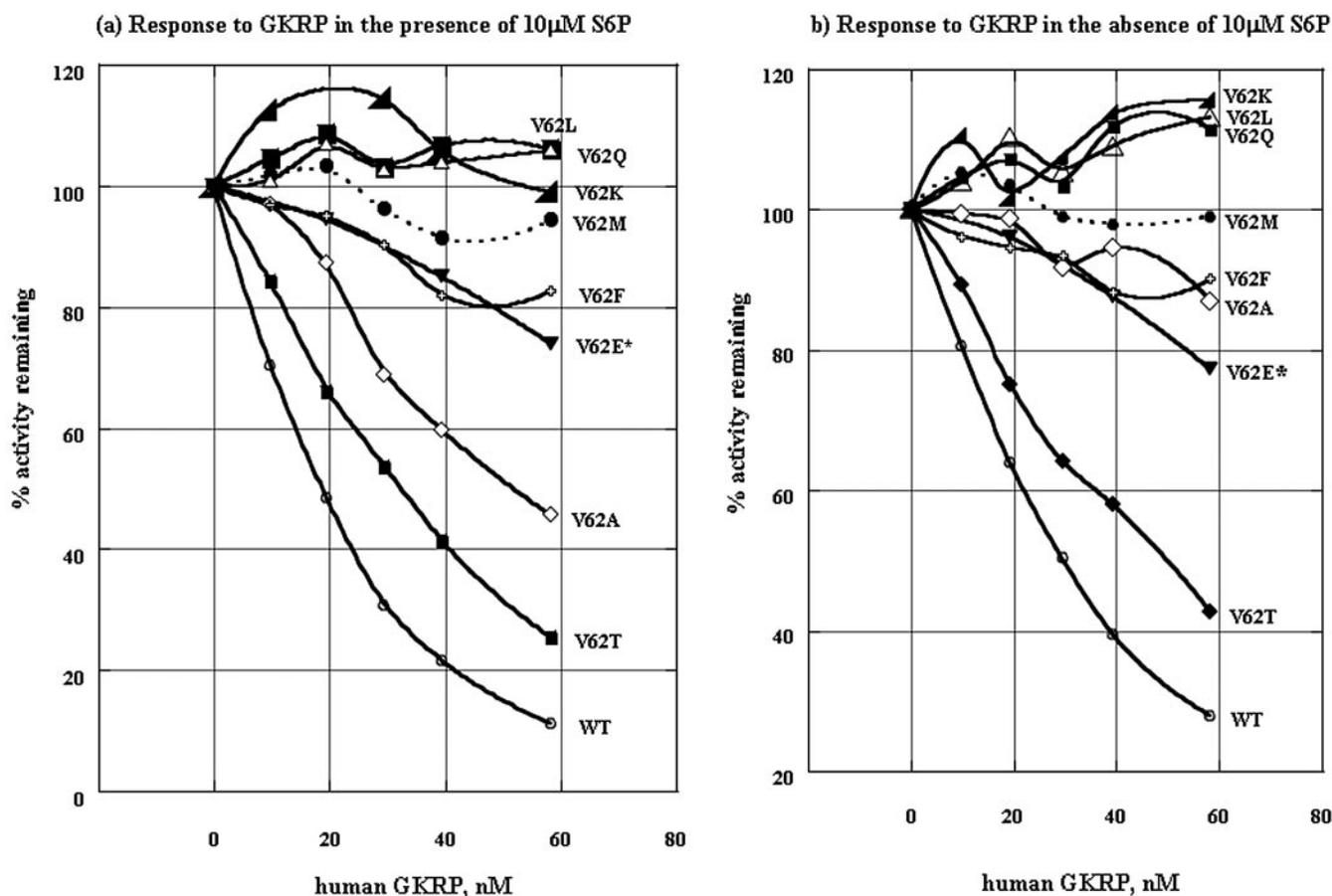
FIG. 4. **Effect of human GKRP on wild type and 8 different Val[62] GST-GCK mutants.** *a*, effect of human GKRP on wild type and mutant Val[62] GST-GCK in the presence of sorbitol 6-phosphate (*S6P*). Results represent the outcome of studies with single preparations of Val[62] mutant GST fusion enzymes. Experiments were performed in the presence of sorbitol 6-phosphate (*S6P*, 10 μM). The enzyme concentrations were 22.2 nM. *, note, however, that the concentration of the low activity mutant GST-GCK V62E was 44.5 nM resulting in the false impression of relatively low responsiveness. *b*, effect of human GKRP on wild type and mutant Val[62] GST-GCK in the absence of S6P. Results represent the outcome of studies with single preparations of Val[62] mutant GST fusion enzymes. Experiments were performed as indicated in *a* but without S6P. *, see note to *a*.

TABLE II

Effect of glucokinase activators (RO0274375, RO0281675, RO0283946) on glucokinase activity and its Van der Waals interaction energies with amino acids in the activator binding site for wild type glucokinase and mutant glucokinase forms

Data are means ± S.E. for wild type and V62M GCK for RO0274375. The results are the means of the kinetic analysis of four independent expressions of wild type and mutant V62M GST-GCK. For the remaining mutants two independent expressions were prepared. For RO0274375 two preparations were studied and the means and individual data points are given. For the other two compounds only one preparation was analyzed in each case. The GKA efficacies were expressed in terms of the -fold increase of the activity index. The Van der Waals interaction between GKAs with WT and all other mutants at the binding site was calculated in kilocalories. A statistically significant non-linear relationship between GKA activation and Van der Waals interaction energies for all three compounds was confirmed by Spearman's ρ method at a confidence level of 95%.

| | GCK mutation | | | | | |
| | RO0274375 | | RO0281675 | | RO0283946 | |
Mutants	GKA activation	Van der Waals interaction energy	GKA activation	Van der Waals interaction energy	GKA activation	Van der Waals interaction energy
	-fold	*kcal*	*-fold*	*kcal*	*-fold*	*kcal*
Wild	15.8 ± 0.56	−3.26	33.6	−10.59	20.5	−24.88
V62A	4.72 (5.07, 4.37)	−1.65	63.5	−9.40	1.69	−23.67
V62T	3.57 (3.67, 3.46)	−2.07	39.0	−9.90	2.00	−24.26
V62L	1.46 (1.55, 1.37)	1,766.52	13.3	415.24	2.14	200.95
V62M	1.24 ± 0.18	130.28	3.73	153.11	0.76	82.94
V62Q	1.16 (1.22, 1.09)	2353	0.68	3,571.66	0.60	3,547.22
V62E	1.16 (1.19, 1.13)	1,017.58	0.80	499.34	0.57	177.47
V62F	1.10 (1.25, 0.94)	1,693.55	1.23	653.33	1.14	299.11
V62K	0.94 (0.95, 0.93)	6,495.40	0.89	11,517.02	0.95	12,943.95

clinical observations in these two families fit exactly with the phenotype seen in GCK MODY, with life-long, mild, fasting hyperglycemia (fpg > 5.5 mM) and a small increment between the fasting and 2-h values on an oral glucose tolerance test (mean value 2.1 mM) (34). Complications are rare and patients are usually managed on diet alone.

The initial functional characterization of V62M resulted in a paradox: an enzyme with an increased affinity for glucose ($S_{0.5}$ for glucose decreased from 7.40 in wild type to 4.88 mM in the mutant), but resulting in hyperglycemia. Because it is known from *in vitro* (H137R, V367M, M298K, and S263P) (17) and cell biological *in vivo* studies (E300K) (20) that certain GCK-MODY

(a) Response of wild type GCK to RO0274375

(b) Response of mutant V62M GCK to RO0274375
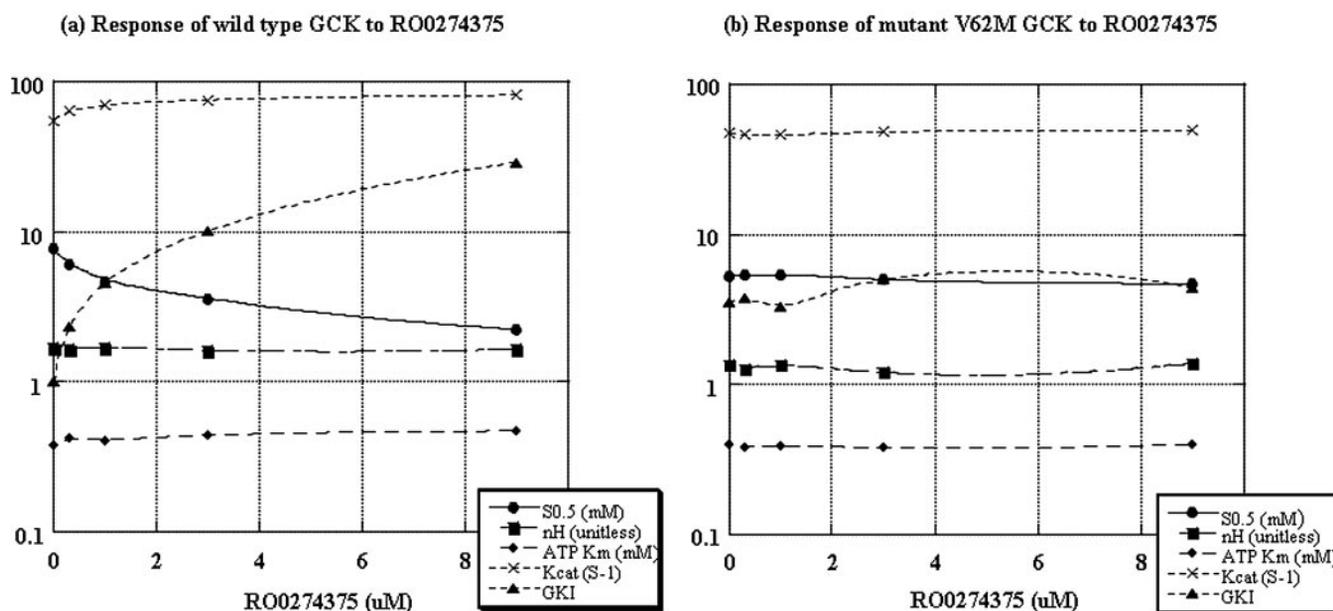
FIG. 5. **Response of wild type and mutant V62M-GCK and RO0274375.** *a*, effect of RO0274375 on all kinetic parameters for wild type GST. Results are the means of the kinetic analysis of three independent expressions of WT-GST GCK. For clarity, S.E. are not shown. *b*, effect of R00274375 on all kinetic parameters for mutant V62M-GST. Results are the means of the kinetic analysis of three independent expressions of V62M-GST GCK. For clarity S.E. are not shown.

mutations are unstable and that this instability is glucose-dependent, we have assessed the thermal stability of V62M in the presence and absence of glucose. V62M is thermally labile, but this thermal lability is less pronounced than the proven instability mutant E300K. Using a simple mathematical model to predict the threshold for GSIR (16, 19) we determined that the instability factor required to result in GCK-V62M resulting in a threshold for GSIR of ~7.0 mM could be <0.01 (data not shown) compared with 0.1 for E300K and 1.0 for stable mutations (16). This equates to a severe defect in thermal instability not seen in our experiments. It is not surprising that kinetically activating mutations would require a large degree of structural instability to result in thresholds for GSIR of ~7 mM. Indeed, mathematical modeling of the reported activating mutations (T65I, W99R, Y214C, V455M, and A456V) also predicts instability factors of <0.01 (data not shown). This suggests that thermal instability on its own is probably not sufficient to account for the phenotype seen in these patients. However, further biological studies, including perhaps the generation of an animal model, are required to fully address this issue.

Structural modeling of the mutation using a homology model based on the crystal structure of WT-GCK co-crystallized with RO0283946 revealed that the mutation is located in the recently described allosteric activator site (21). Evidence suggests that this site is where the allosteric activator RO0281675 interacts with GCK (26). We addressed the question that defective regulation of GCK could account for the elevated fpg values seen in our families. Indeed, V62M-GCK was totally refractory to the allosteric activator, and structural modeling in a homology model of GCK based on the crystal structure of GCK revealed that the reason for this was that a methionine residue at position 62 blocks the preferred ligand binding position (Fig. 6*c*). Interestingly, our data also show that V62M-GCK does not respond to GKRP. The crystal structure of GKRP has not been solved, and little is know about how it interacts with GCK. Site-directed mutagenesis studies have shown that residues Glu[51]-Leu[52] and His[141]-Leu[144] may play a role in the interaction between GCK and GKRP (35). This suggests that GKRP binds to a broad region on glucokinase encompassing the smaller domain and the region of the larger domain next to the

hinge. It has been proposed that GKRP binds to the super-open form of GCK (27). However, if GKRP does indeed bind to the super-open form of glucokinase it would result in an increase in the Hill number that is the opposite to that observed in kinetic studies (36). However, the V62M mutation prevents GKRP from inhibiting GCK, and it is unlikely that it is due to a conformational change that prevents GCK from existing in the open or super open forms, because this would alter the catalytic cycle responsible for the sigmoidal glucose dependence (27) and therefore alter the Hill coefficient. Our findings may suggest that GKRP interacts with residues located in the narrow crevice between the larger domain and the smaller domain, thereby freezing GCK in its open conformation. A direct interaction with Val[62] is unlikely, but there could be an interaction with the glucokinase-specific Tyr[61] residue. A full understanding of how this mutation effects the interaction between GCK and GKRP will have to await the crystal structure of the glucokinase-GKRP complex.

It has been known for sometime that glucokinase is regulated in the liver by GKRP (4, 5). However, there is controversy in the literature as to whether GKRP plays a role in the pancreatic β-cell (8–10). A recent report has shown that an alternatively spliced variant of the GKRP gene is expressed in the β-cell and is the major isoform in this tissue (10). This could be an attractive candidate for regulation of glucokinase in the pancreatic β-cell. In this study V62M-GCK was not inhibited by human or rat GKRP, which may have implications for glucose homeostasis in both the liver and β-cell. Homozygous GKRP null mice have been shown to have a 40% reduction in liver GCK content, while heterozygous mice have a less severe reduction of ~16% (8), suggesting that this mutation could result in a reduction of β-cell GCK in the liver and β-cell. However, GKRP null mice do not have elevated fasting plasma glucose levels (8) nor do patients with heterozygous nonsense or missense mutations in the human GKRP gene (37) indicating that haploinsufficiency or complete loss of GKRP is not sufficient to cause the elevated fpg levels observed in both families. GKRP is not the sole candidate for regulation of GCK in the β-cell, and other candidates include long chain fatty acyl-CoA (31, 38), the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2–6-
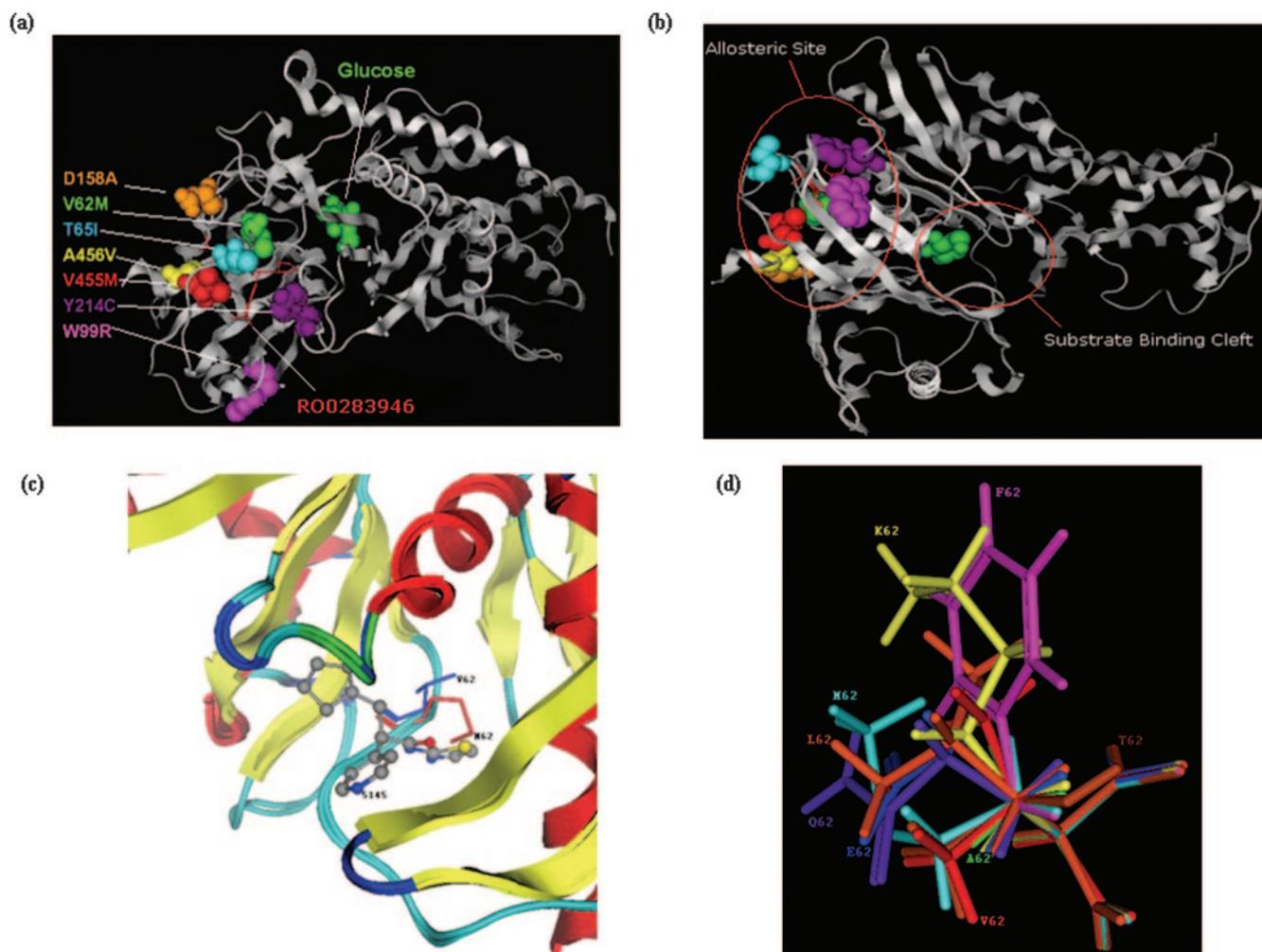
FIG. 6. **Structural modeling of GCK.** *a*, structural model of glucokinase using the WT-GCK structure co-crystallized with RO0283946 as a template, showing the location of the naturally occurring (T65I, W99R, Y214C, V455M, and A456V) artificially created (D158A) activating mutations, the MODY mutation V62M and the substrate binding cleft. *b*, the figure illustrates the distinct substrate binding site and allosteric activator site. *c*, homology model of glucokinase built using the WT-GCK structure co-crystallized with RO0283946, as a template. The model shows the location of the V62M mutation and effect on binding of the allosteric activator. *d*, homology model of glucokinase built using the WT-GCK structure co-crystallized with RO0283946, as a template. Overlay of all the mutations at position 62 with wild type- GCK (V62).

bisphosphate (39), dual specific phosphatase (DUSP12) (40), and as yet unidentified compounds. Further studies will be needed to clarify the roles of these putative regulators in the β-cell and to search for other potential candidates.

Additional mutagenesis of residue 62 has demonstrated that this residue is essential for the correct conformation of the allosteric activator binding site and that amino acid substitutions that effect the charge of the residue (V62E, V62K, and V62Q) or the size (V62E, V62F, V62L, V62M, and V62Q) will interfere with the hydrophobic interaction of this residue with R00281675 and prevent the drug from binding. The V62A mutation has been reported in the literature and causes GCK-MODY (41). Our study would suggest that the mechanism is a decrease in the affinity for the substrate glucose as indicated by the increased $S_{0.5}$ for glucose. Interestingly, our data suggest that the amino acid substitutions that prevent the pharmacological activator from binding, with the exception of V62E, also appear to prevent GKRP from functioning, implying that the correct conformation of the allosteric activator site is also important for the optimal binding and function of GKRP. The mutagenesis studies at residue 62 show that the polar residues (Table II) result in a marked decrease in k_{cat}. It is interesting to speculate that the V62M mutation could exert its effect due to oxidation of the methionine to methionine sulfoxide, which is a

polar residue. This change could take place *in vivo* with an unpredictable stoichiometry and result in a marked decrease in k_{cat} and therefore a kinetically inactivating mutation.

The possibility that a MODY phenotype may result from a mutation that affects the regulation of GCK by an endogenous regulator suggests that there might exist other mutations in *GCK* that could cause glycemic disease by affecting regulation of GCK by either an endogenous activator or inhibitor. The careful kinetic analysis of 40 mutant recombinant enzymes has uncovered five mutants that show practically normal kinetic characteristics; A53S, H137R, G264S, R275C, and V367M (16, 19). It is possible that these are functionally silent polymorphisms rather than pathogenic mutants, but the fact that these variants have not been detected in over 100 normal chromosomes would argue against this. These mutations do not map to the allosteric activator site, but they could be important for regulation by other compounds. Additional studies are needed to investigate this further and to identify such endogenous regulators.

We propose that V62M may cause MODY by a complex mechanism that involves thermal instability and the inability of the enzyme to respond to GKRP and/or an endogenous activator that acts at the same binding site as the allosteric activator drugs. This observation coupled with our detailed func-

tional studies provides evidence for the existence of an as yet unknown naturally occurring endogenous allosteric activator of glucokinase. The facts that GCK is vulnerable to allosteric activation by single point mutations in a circumscribed cluster of amino acids (21) and responds to GCK-activator drugs (26) clearly argue in favor of the existence of endogenous GCK activators. The present results and considerations encourage the search for such molecules.

In conclusion we have identified a novel *GCK* mutation (V62M), which co-segregates with MODY in two families. Functional studies have shown that this mutation results in an enzyme that is mildly activating, mildly thermally labile, and totally refractory to allosteric activators of GCK and GKRP. We propose that this mutation may cause MODY by a complex mechanism, which involves thermal instability and loss of regulation by GKRP and an endogenous allosteric activator of GCK. It is likely that the precise mechanism for this intriguing mutation will only be fully elucidated by the study of a mouse model. The present study provides the most comprehensive functional assessment of a *GCK* mutation to date and illustrates that *GCK* mutations that cause hyperglycemia are not necessarily kinetically inactivating and that they may exert their effects by other complex mechanisms.

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Insights into the Structure and Regulation of Glucokinase from a Novel Mutation (V62M), Which Causes Maturity-onset Diabetes of the Young

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