Original Article

From Clinicogenetic Studies of Maturity-Onset Diabetes of the Young to Unraveling Complex Mechanisms of Glucokinase Regulation

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Glucokinase functions as a glucose sensor in pancreatic β-cells and regulates hepatic glucose metabolism. A total of 83 probands were referred for a diagnostic screening of mutations in the glucokinase (*GCK*) gene. We found 11 different mutations (V62A, G72R, L146R, A208T, M210K, Y215X, S263P, E339G, R377C, S453L, and IVS5 + 1G>C) in 14 probands. Functional characterization of recombinant glutathionyl *S*-transferase–G72R glucokinase showed slightly increased activity, whereas S263P and G264S had near-normal activity. The other point mutations were inactivating. S263P showed marked thermal instability, whereas the stability of G72R and G264S differed only slightly from that of wild type. G72R and M210K did not respond to an allosteric glucokinase activator (GKA) or the hepatic glucokinase regulatory protein (GKRP). Mutation analysis of the role of glycine at position 72 by substituting E, F, K, M, S, or Q showed that G is unique since all these mutants had very low or no activity and were refractory to GKRP and GKA. Structural analysis provided plausible explanations for the drug resistance of G72R and M210K. Our study provides further evidence that protein instability in combination with loss of control by a putative endogenous activator and GKRP could be involved in the development of hyperglycemia in maturity-onset diabetes of the young, type 2. Furthermore, based on data obtained on G264S, we propose that other and still unknown mechanisms participate in the regulation of glucokinase. *Diabetes* 55:1713–1722, 2006

Glucokinase phosphorylates glucose to glucose-6-phosphate in the first step of glycolysis. Owing to its kinetic characteristics, glucokinase is capable of phosphorylating glucose over the physiological range of 3–8 mmol/l. Unique kinetic characteristics are low affinity for the substrate glucose ($S_{0.5}$ ~7.5 mmol/l) at pH 7.4, physiological saturation with MgATP, cooperativity with its substrate glucose (Hill coefficient n_H ~1.7), and lack of inhibition by intermediates and products of the glycolytic pathway. These are important features that allow glucokinase to serve a special function in the glucose sensing of several tissues including pancreas (β- and α-cells), hepatoportal, L- and K-type enteroendocrine cells, and certain neurons mainly in the hypothalamus (1–3). In the β-cell, glucokinase plays a critical role in glucose-stimulated insulin release (GSIR), encapsulated by the term "glucokinase glucose sensor" (4). Total glucokinase deficiency in patients with homozygous or compound heterozygous glucokinase (*GCK*) mutations leads to severely reduced insulin secretion (5–7). In the liver, glucokinase lowers blood glucose levels by facilitating hepatic glucose utilization and glycogen synthesis, demonstrated by the fact that mice lacking hepatic glucokinase have impaired glucose tolerance (8).

In the hepatocyte, glucokinase is regulated by glucokinase regulatory protein (GKRP), a competitive inhibitor with respect to glucose (9). Reversible nuclear sequestration and inhibition of glucokinase is accomplished by binding to GKRP, whereas free active glucokinase is shuttled back to the cytosol in response to increased glucose levels in blood and in hepatocytes (10). It is still questionable whether GKRP is expressed and functional in pancreatic β-cells (11–13). Furthermore, glucokinase has been found in insulin secretory granules, but it is unclear whether this particulate fraction of the enzyme translocates to the cytosol during glucose stimulation (14–16).

More than 190 different *GCK* mutations have been identified (17), of which the majority are associated with maturity-onset diabetes of the young, type 2 (MODY2) (18), a condition with a biochemical phenotype character-

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GKA, glucokinase activator; GKRP, glucokinase regulatory protein; GSIR, glucose-stimulated insulin release; GST, glutathionyl *S*-transferase; MODY2, maturity-onset diabetes of the young, type 2; PEOE, partial equalization of orbital electronegativities; PNDM, permanent neonatal diabetes mellitus.

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ized by mild fasting hyperglycemia (6–8 mmol/l) and a 2-h increment during an oral glucose tolerance test <3.5 mmol/l (19). Antidiabetes treatment is rarely necessary, and long-term diabetes complications are uncommon (20–22).

Functional evaluation of about 40 *GCK* point mutations using recombinant glutathionyl *S*-transferase (GST)-glucokinase has in most instances revealed greatly reduced enzymatic capacity due to changes in one or several kinetic parameters (4,23,24). These kinetic parameters together with other characteristics of the enzyme were used to predict the β-cell threshold for GSIR by mathematical modeling for wild type as well as the majority of mutants. However, based on the same model, several mutants associated with MODY2 are characterized by near-normal or even increased enzyme activity, indicating that mechanisms other than kinetic inactivation are involved in the development of hyperglycemia (4,24). For instance, there is strong evidence of thermal instability in the mutant (GST)-E300K-glucokinase, providing a sufficient explanation for the development of hyperglycemia related to this *GCK* mutation (25). Moreover, functional characterization of the mutant (GST)-V62M-glucokinase suggests that loss of regulation by GKRP and an endogenous allosteric activator are additional mechanisms contributing to the deteriorated glucose homeostasis (26). Therefore, a thorough genetic and biochemical investigation of a particular mutant in glucokinase is of unique interest in terms of what it might tell us about the enzyme, as illustrated in this article as well as others (25,26).

Five heterozygous *GCK* mutations (T65I, W99R, Y214C, V455M, and A456V) identified thus far result in hyperinsulinemia in infancy and hypoglycemia (27–30). These mutants cause an activation of the enzyme due to decreased glucose $S_{0.5}$ and/or increased k_{cat}. Furthermore, the mutated residues are localized to a region in the protein's structure remote from the substrate binding site according to a homology model (31). Recently, a new class of glucokinase activators (GKAs) has been discovered (32–34). One of these, RO0281675, was shown to increase the V_{max} and decrease the glucose $S_{0.5}$ of the wild-type enzyme and was able to lower blood glucose levels in rodent models of type 2 diabetes (32). Moreover, these molecules act as an allosteric activator with a similar effect on enzyme kinetics as the naturally occurring activating point mutations of glucokinase (32,33). Based on this information, the existence of a putative endogenous allosteric activator has been proposed (4,29) but remains to be identified. The crystal structure of glucokinase has recently been solved, confirming the presence of an allosteric activation site (32,33,35). These studies also revealed substantial conformational changes from a super-open inactive to a closed active enzyme when bound to substrate, and this offers a plausible explanation for the cooperative kinetics with glucose.

By functional characterization of *GCK* mutations identified in subjects with mild hyperglycemia or permanent neonatal diabetes mellitus (PNDM), we show that glucokinase is regulated by complex mechanisms, including differences in protein stability and loss of regulation by an allosteric activator and by GKRP. Moreover, data from at least one mutant with near-normal activity indicate that additional and yet unknown processes for regulation of glucokinase exist.

RESEARCH DESIGN AND METHODS

A total of 83 probands were referred to the Department of Pediatrics, Haukeland University Hospital, for a diagnostic screening for mutations in *GCK*, of which some have previously been reported (5,36). For comparison of clinical characteristics with MODY2 patients, 13 healthy subjects constituted the control group. Missense mutations identified in this diagnostic screening as well as one recently reported mutation (G264S) (6) were functionally studied.

Mutation screening. Genomic DNA was extracted from peripheral lymphocytes and exons 1a and 2–10, and flanking intron boundaries of *GCK* were sequenced on a capillary sequencer and subsequently analyzed by standard methods.

Generation of recombinant wild-type and mutant glucokinase. Recombinant wild-type and mutant human β-cell glucokinase were generated and expressed as GST fusion proteins in *Escherichia coli* as previously described (23,37). Point mutations were introduced into the pGEX-3X vector using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutants were transformed into *E. coli* cells and verified by DNA sequencing. The mutant enzymes made for this project included the following missense mutations: V62A, G72R, L146R, A208T, M210K, S263P, G264S, E339G, R377C, and S453L. The mutant A378V was not further investigated, since this was a severely inactivating mutation according to a previous study (6). We also generated additional mutants at residue 72 (G72E, G72F, G72K, G72M, G72Q, and G72S). All mutant glucokinase proteins except G72S were expressed in significant amounts and were found to be essentially pure, as indicated by the presence of a single band at 75 kDa on phast gel electrophoresis. Purified protein was stored at −80°C, either with 50 mmol/l glucose or in absence of glucose (23,37).

Kinetic analysis of glucokinase. Proteins were purified and kinetic properties determined as previously described (23,37). Glucokinase activity was measured spectrophotometrically using an $NADP^+$-coupled assay with glucose-6-phosphate dehydrogenase, and at least two serial experiments were performed. The following modifications to the protocol were made. In protocol A, kinetic studies were carried out in 11 glucose dilutions between 0 and 100 mmol/l in the presence of 5 mmol/l ATP. In protocol B, a glucose concentration at $10 \times S_{0.5}$ was used but with varying ATP concentrations.

We also performed kinetic analysis as influenced by a GKA (32). Measurements were made in the presence of 0.3, 1, 3, 9, 27, and 60 μmol/l GKA; the kinetic constants were determined; and the results were compared with those obtained with wild-type GST-glucokinase. In these studies, 1% DMSO was included in the medium, a concentration that did not affect the kinetic constants of wild-type or mutant enzymes. An activity index was calculated, based on the proposed enzyme's in situ phosphorylation capacity (23,24,28). Moreover, we calculated the maximal fold drug effect on k_{cat} (A), glucose affinity (B), and the activity index (C). To calculate the maximal values for A, B, and C, KaleidaGraph software (Abelbeck Software, Malvern, PA) was used. The half-maximal drug concentrations (EC_{50}) for these parameters were computed for the majority of mutant enzymes.

Guided by the kinetic properties that were initially found, we performed kinetic analyses with human recombinant GKRP in practically all mutants except those with a very low activity index because of technical difficulties. The analysis was carried out as previously described, with glucose and ATP concentrations adjusted to account for differences in the kinetic constants (23,38).

Thermolability tests. Thermal stability of the mutants G72R, S263P, and G264S GST-glucokinase as well as wild type was tested using protocols previously described (23,39), with some modifications. Enzyme stock solutions were stored in either absence of (0 mmol/l) or in 50 mmol/l glucose. For temperature titration studies, enzymes were diluted in buffer containing 0.1 mol/l HEPES buffer (pH 7.4), 150 mmol/l KCl, 6 mmol/l $MgCl_2$, 30% glycerol, and glucose at the $S_{0.5}$ concentrations. The enzymes were incubated in a water bath at 30, 32.5, 35, 37.5, 40, 42.5, 47.5, 50, and 52.5°C for 30 min. Glucokinase activity was then immediately determined by spectrophotometry as described. Since glucose has been shown to stabilize glucokinase (40), we also studied stabilization of wild type and the three mutants with increasing glucose concentrations. For these glucose titration studies, the enzyme stock solutions devoid of glucose were diluted in buffer (composition see above), and increasing glucose concentrations were then added from 0 to 100 mmol/l and to achieve comparable protein concentrations of 50–100 μg/ml. The enzymes were incubated in a water bath at 42.5°C for 30 min, and the glucokinase activity was quickly determined using spectrophotometry as described and the k_{cat} recorded as an index of stability.

Homology modeling. To help understand the structural basis of the interactions observed between GKA and mutants such as M210K and G72X, homology models were built using MOE package (version 2004.3; Chemical Computing Group, Montreal, Canada). The wild-type glucokinase structure

TABLE 1
Clinical characteristics of *GCK* mutation carriers and healthy control subjects

	GCK mutation carriers	Healthy control subjects	*P* value
n	42	13	
Fasting serum glucose (mmol/l)	7.4 ± 1.8	5.4 ± 0.7	<0.001
2-h serum glucose during an OGTT (mmol/l)	9.5 ± 3.4	5.1 ± 1.2	<0.001
2-h increment during an OGTT (mmol/l)	2.5 ± 2.9	−0.3 ± 1.4	0.002
A1C (%)	6.7 ± 0.8	5.6 ± 0.4	<0.001

Data are means ± SD. OGTT, oral glucose tolerance test.

with the cocrystallized GKA and glucose were removed and used as templates. The side chains of the mutated amino acids were allowed to move to their optimal positions through energy minimization under MMFF94 force field (41–45).

Statistical analysis. Statistical analysis was performed with independent samples *t* test using an SPSS 13.0 package.

RESULTS

***GCK* mutation screening and clinical characterization.** From the diagnostic mutation screening of 83 probands, we found five novel (L146R, A208T, E339G, R377C, and IVS5 + 1 G>C) and six previously reported (V62A, G72R, M210K, Y215X, S263P, and S453L) *GCK* mutations in 14 probands and 28 family members. The mutations cosegregated with diabetes, and the novel mutations were not identified in 50 anonymous blood donors. The clinical and biochemical phenotype of the probands and family members are presented in Table 1. In general, there was mild hyperglycemia with mean fasting blood glucose of 7.4 mmol/l (range 5.6–14.9) and a mean 2-h serum glucose increment after an oral glucose load <3.0 mmol/l (−2.2 to 11.2). Furthermore, the mean HbA_{1c} (A1C) was 6.7% (5.6–9.4). Overall, the clinical phenotype in respect of fasting blood glucose, 2-h serum glucose increment during an oral glucose tolerance test, and A1C values was similar in most glucokinase mutation carriers.

Enzyme kinetics of wild-type and spontaneous mutant GST-glucokinase. In Table 2, the enzyme kinetic data of mutants generated on the basis of the *GCK* mutation screening in the MODY2 patients in addition to mutations identified in subjects with PNDM (5,6) are shown. The mutants V62A, L146R, A208T, M210K, E339G, R377C, and S453L demonstrated reduced enzyme activity due to decreased k_{cat} and reduced affinity for glucose (elevated $S_{0.5}$). The ATP K_m of E339G was remarkably high (i.e., five times the control value). In contrast, the mutant G72R showed increased glucose affinity ($S_{0.5}$ of 5.32 mmol/l), a low k_{cat}, and smaller n_H compared with wild type extrapolating to an increased activity index, which was unexpected for this case. Two other mutants (S263P and G264S) had normal k_{cat} and slightly increased glucose $S_{0.5}$ compared with wild type, and their activity index was near normal. Interestingly, E339G showed different k_{cat} values in protocols A and B. In protocol A, glucose was the variable substrate, whereas MgATP was kept constant at 5 mmol/l. In protocol B, however, glucose was held constant at 10 × $S_{0.5}$ and MgATP was varied. The reason for this observation could be enzyme instability due to the mutation E339G (23).

Thermolability tests. Even with a slightly increased or near-normal enzyme activity index for the mutants G72R, S263P, and G264S, the clinical phenotype of the mutation carriers was similar to those carrying severely inactivating *GCK* mutations, suggesting other mechanisms being involved in the development of hyperglycemia in MODY2. The three mutants were therefore subjected to additional functional studies. Analysis of thermal stability showed that wild-type glucokinase was slightly activated after 30-min incubation as the temperature rose from 30°C but with an abrupt decrease at 47.5°C (Fig. 1*A*). The mutant G72R, which showed an increased activity index according to our kinetic analysis, was not activated when the temperature rose, whereas its activity began to fall at 45°C, a small but significant difference compared with wild type. The activity of the mutant G264S began to decline at 45°C, very similar to the findings with G72R. In contrast, the activity of the mutant S263P declined abruptly at 40°C in the thermolability test, a marked 7.5° left shift in the profile compared with the control enzyme. Glucose has a stabilizing effect on the enzyme, and we therefore explored this aspect with the three mutants at 42.5°C (Fig. 1*B*), the highest temperature at which k_{cat} varied the least between the wild-type and mutant enzymes. With decreas-

TABLE 2
Kinetic characteristics of recombinant human wild-type and mutant glucokinase

Enzyme	*n*	Yield (mg/l)	k_{cat} (s^{-1})*	k_{cat} (s^{-1})†	$S_{0.5}$ (mmol/l)	n_H	ATP K_m (mmol/l)	Relative activity index
Wild type	5	41.3 ± 3.33	61.6 ± 7.07	63.0 ± 8.75	7.55 ± 0.23	1.74 ± 0.04	0.41 ± 0.03	1.00‡
V62A	2	25.6, 20.6	49.6, 47.9	39.0, 35.6	24.6, 30.2	1.50, 1.51	0.20, 0.20	0.23‡
G72R	5	23.7 ± 3.70	29.9 ± 2.78	33.5 ± 2.99	5.32 ± 0.26	1.45 ± 0.03	0.76 ± 0.04	1.71
L146R	3	17.6 ± 2.67	0.23 ± 0.09	0.21 ± 0.01	123 ± 1.87	0.95 ± 0.03	0.10 ± 0.01	0.002
A208T	3	14.6 ± 0.58	2.86 ± 0.12	4.41 ± 0.20	10.1 ± 0.24	1.23 ± 0.03	2.61 ± 0.04	0.12
M210K	2	11.4, 4.16	23.3, 13.4	26.7, 18.8	52.8, 31.8	1.64, 1.37	1.58, 0.42	0.05§
S263P	2	44.0, 37.1	66.5, 55.7	64.7, 59.5	12.2, 12.3	1.59, 1.51	0.51, 0.63	0.76
G264S	4	38.6 ± 7.22	60.7 ± 3.04	65.3 ± 2.36	9.76 ± 0.74	1.57 ± 0.05	0.48 ± 0.05	0.89§
E339G	2	29.1, 18.2	34.9, 41.7	60.2, 66.9	32.1, 20.5	1.34, 1.53	1.87, 2.47	0.06
R377C	2	0.57, 0.91	5.87, 9.11	5.92, 10.3	52.3, 33.2	1.64, 1.85	0.28, 0.45	0.01
S453L	3	27.2 ± 0.95	8.17 ± 0.70	8.89 ± 0.55	16.0 ± 1.62	1.39 ± 0.07	0.69 ± 0.03	0.22

Data are means ± SE for wild type, G72R, L146R, A208T, G264S, and S453L-glucokinase with the number of independent experiments shown by *n*. For the remaining mutants, two independent expressions were prepared and the mean or individual data are shown. Kinetics of glucokinase were obtained with preparations stored in the presence of 50 mmol/l glucose. *Protocol A using glucose as a variable; †protocol B using ATP as a variable. ‡The original data are also included in another published report (26) and are reproduced here. §Data represent repeat analyses of preparations used before (5,24).

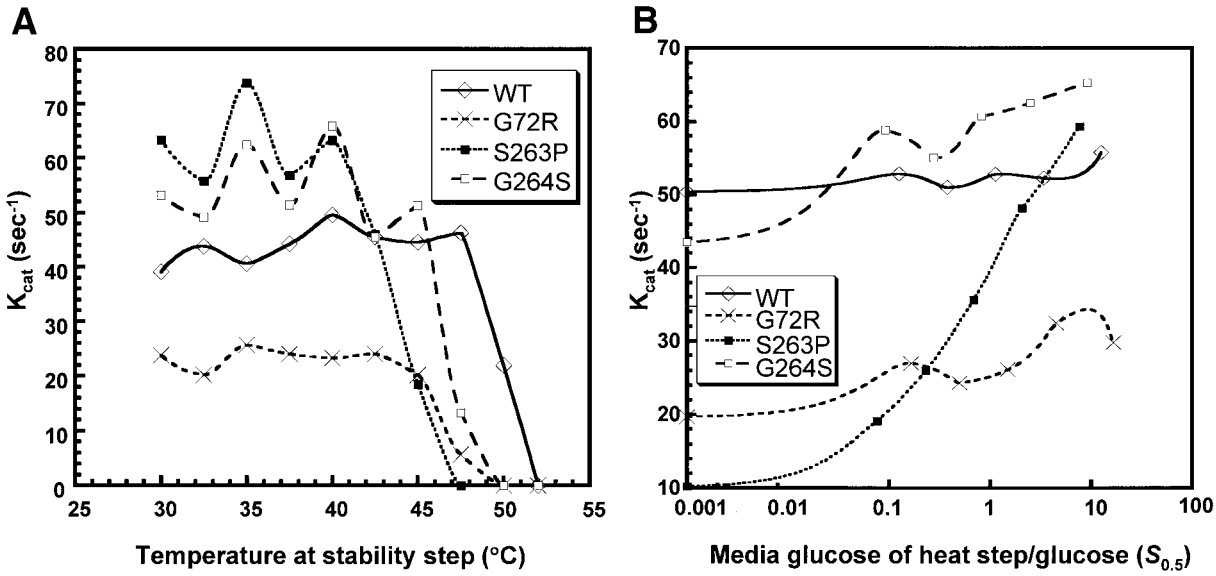

FIG. 1. Assessment of thermal instability and effect of glucose concentration on stability of wild-type (WT), G72R, S263P, and G264S-glucokinase. *A*: The effect of temperature on enzyme activity was investigated. The stock enzymes were stored at 0 mmol/l glucose. Enzymes were diluted in a buffer containing glucose at the $S_{0.5}$ of the particular enzyme (see RESEARCH DESIGN AND METHODS) and incubated for 30 min at different temperatures before kinetic analysis. Each graph shows mean results of three independent protein expressions and thermolability experiments of each recombinant enzyme. *B*: The effect of glucose concentration on enzyme activity was studied. Stock enzymes were stored at 0 mmol/l glucose and then diluted in a buffer (see RESEARCH DESIGN AND METHODS) and incubated at 42.5°C for 30 min at different glucose concentrations followed by kinetic analysis. Due to differences in glucose affinities between enzymes, the ratio of the glucose concentration to the glucose $S_{0.5}$ was used to plot the data. Each graph represents the mean value from three independent protein expressions and thermolability experiments for each enzyme.

ing glucose concentrations in the heat step, the activity of the wild-type GST-glucokinase remained unchanged. The k_{cat} value for the mutant G72R changed by ~30% from ~30 s^{-1} to ~20 s^{-1}, and the absence of glucose lowered the k_{cat} value from ~65 s^{-1} to ~44 s^{-1} for G264S (also by ~30%). In contrast, the k_{cat} was lowered drastically from ~59 s^{-1} to ~10 s^{-1} for the mutant S263P. These model experiments indicate that the mutants G72R and G264S are somewhat less stable than the wild type, whereas S263P is a highly unstable protein.

Interaction with GKRP. We then determined the inhibition of wild-type GST-glucokinase by human GKRP both with sorbitol-6-phosphate present and absent (Fig. 2 and Table 3). Wild-type GST-glucokinase and S263P, G264S, E339G, and S453L demonstrated reduced activity with increasing concentrations of GKRP. In contrast, the activity of G72R-GST-glucokinase was not inhibited by GKRP (Fig. 2). The mutant M210K-GST-glucokinase also showed reduced responsiveness to increasing GKRP concentrations, although not as pronounced as G72R.

Effect of an allosteric GKA. All point mutations and wild-type enzymes were subjected to kinetic analyses using a GKA (Table 4). Using KaleidaGraph, we calculated the maximal fold effect of GKA on k_{cat} (A), glucose affinity

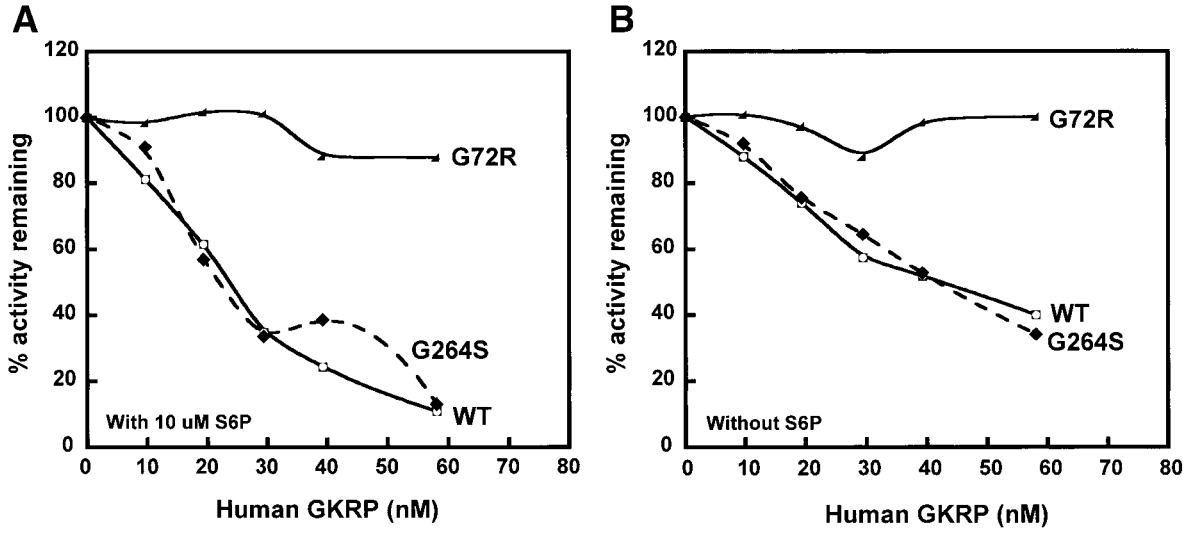

FIG. 2. Effect of human GKRP on wild-type (WT), G72R, and G264S-glucokinase. *A*: Effect of GKRP on wild-type, G72R, and G264S-glucokinase in the presence of sorbitol-6-phosphate (S6P; 10 μmol/l). Enzyme concentration was 22 nmol/l. Two investigations were performed for each enzyme. *B*: Effect of GKRP on wild-type, G72R, and G264S-glucokinase in the absence of sorbitol-6-phosphate. Two investigations were performed for each enzyme.

TABLE 3
Inhibition of wild-type and mutant glucokinase by human GKRP

	Substrate in assay (mmol/l)			Maximal % inhibition	
	n	Glucose	ATP	Without S6P	With 10 μmol/l S6P
Wild type	2	3	1	62.3	89.1
V62A	1	9	1	13.0	54.1
G72E	1	4	1	0.0	21.5
G72F	1	3.5	3.25	5.1	3.9
G72K	1	2.5	1	13.9	15.2
G72Q	1	3	1.5	10.7	19.8
G72R	2	2	1	0.0	12.1
M210K	1	13.5	2.5	16.9	24.9
S263P	1	4	1	47.5	75.2
G264S	2	3.5	1	63.8	87.1
E339G	1	8	5	76.7	89.0
S453L	1	4.5	2	24.1	70.6

Inhibition of recombinant wild-type and mutant glucokinase expressed as GST-glucokinase fusion proteins was investigated in the presence and absence of sorbitol-6-phosphate (S6P). Glucokinase concentration was 22 nmol/l. GKRP concentration was 58 nmol/l. The number of independent investigations is presented by *n*. L146R, A208T, and R377C not studied (activity too low).

(B), and the activity index (C). From this, we also determined the half-maximal GKA concentrations for the effect on all or selected kinetic parameters. Wild-type GST-glucokinase responded to the increasing concentrations of GKA with increased k_{cat} values and simultaneously reduced glucose $S_{0.5}$. There was an almost 20-fold maximal increase of the activity index for the wild-type enzyme. The effect on the activation of GKA on the mutants GST-glucokinase S263P, G264S, and E339G were in the same order of magnitude as wild type, whereas clearly smaller increases in activity (about three- to sevenfold) were observed for V62A, G72R, and S453L. M210K did not respond at all to the GKA. In general, the GKAs did not alter the n_H or ATP K_m. The effect of increasing concentrations of GKA on the kinetic parameters of wild-type and G72R GST-glucokinase is presented in Fig. 3*A* and *B*. As we investigated the effect of the compound on k_{cat}, glucose affinity, and activity index, we found that the half-maximal GKA concentrations (EC_{50}) differed significantly between wild-type and several mutants of GST-glucokinase (Table 4).

Functional characterization of designer mutants at residue 72 of glucokinase. We generated additional mutants at residue 72 and performed a functional characterization of these (G72E, G72F, G72K, G72M, G72R, G72Q, and G72S) (Table 5). G72S GST-glucokinase could not be expressed for unknown reasons, whereas G72M was expressed with a good yield but, on examination, turned out to be totally inactive. At least two preparations of each mutant were purified (Table 5). The replacement of G72 by polar (E, K, R, and Q) and also by nonpolar amino acids (F) resulted in decreased k_{cat}, and this reduction was least pronounced for the mutants G72R and G72Q. G72R was the only mutant with clearly demonstrable increased glucose affinity, whereas all the others showed lowered affinity for this substrate. Effects on the ATP K_m were absent or small. For G72R, there was a slightly increased enzyme activity index, whereas G72Q showed a normal activity index compared with wild type. The most severely affected mutants were G72M and G72F. The mutants in this series were activated less than threefold by GKA compared with wild type, S263P, G264S, and E339G, showing a 15- to 20-fold increase at 60 μmol/l GKA (Table 5 and Fig. 3*C*). All mutants had greatly reduced responsiveness to GKRP (Table 3).

Structural analysis. The structural analysis focused on mutants of G72 and M210 because the loss of regulation by GKA and GKRP was most pronounced in these cases. A

TABLE 4
Effect of GKA on recombinant human wild-type and mutant glucokinase

Enzyme	*n*	A*	EC_{50} (μmol/l) of A†	B	EC_{50} (μmol/l) of B†	C	EC_{50} (μmol/l) of C†	n_H^{ctr}/n_H^{act}	$^{ctr}ATPK_m/^{act}ATPK_m$
Wild type	5	1.53 ± 0.05	0.73 ± 0.12	4.34 ± 0.06	0.87 ± 0.05	19.6 ± 1.32	6.73 ± 0.44	1.13 ± 0.02	0.91 ± 0.04
V62A	2	2.04, 2.03	7.32, 4.48	2.04, 2.03	2.44, 8.49	7.16, 5.96	16.2, 9.98	1.16, 1.10	0.63, 0.62
G72R	5	1.59 ± 0.04	4.59 ± 1.02	1.86 ± 0.03	3.02 ± 0.56	2.92 ± 0.08	10.3 ± 1.29	1.03 ± 0.04	1.16 ± 0.04
M210K	2	NR	NR	NR	NR	NR	NR	1.02, 1.01	0.95, 0.90
S263P	2	1.52, 1.48	0.83, 0.50	5.57, 5.45	0.60, 0.52	18.2, 20.5	7.08, 9.54	1.09, 1.12	1.04, 1.18
G264S	2	1.55, 1.52	0.68, 0.55	5.14, 5.30	0.77, 0.60	20.0, 19.8	6.74, 6.48	1.13, 1.08	1.05, 0.95
E339G	2	1.43, 1.53	1.07, 0.56	6.55, 5.51	0.51, 0.56	7.50, 21.7	20.8, 8.42	0.94, 0.97	0.98, 0.91
S453L	3	2.31 ± 0.12	4.19 ± 0.50	3.11, 1.33	0.90, 6.06	3.67 ± 0.47	12.3 ± 3.61	0.97 ± 0.04	1.86 ± 0.08

Data are means ± SE for wild-type, G72R, and S453L glucokinase. The results are means from independent analyses of which the number of expression is represented by *n*. Note that in case of two expressions only, the individual data are shown. Kinetics of wild-type and mutant glucokinase are obtained with preparations stored in the presence of 50 mmol/l glucose. *"A" denotes the maximal fold drug effect on k_{cat}, "B" on the glucose affinity, and "C" on the activity index. †EC_{50} values are the half-maximal drug concentrations for the effect on the k_{cat}, the glucose affinity, and the activity index. NR, not responsive to the GKA.

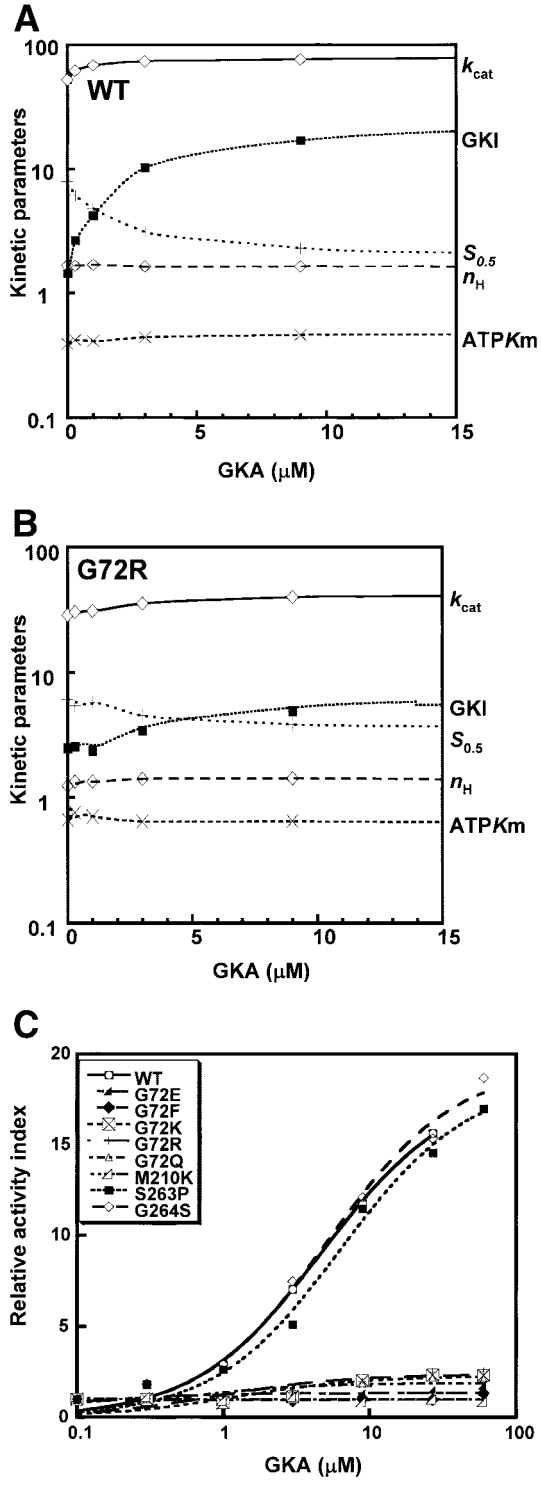

FIG. 3. Response of wild-type and G72R-glucokinase to the allosteric GKA RO02704375. *A*: Effect of RO02704375 on kinetic parameters for wild-type glucokinase. Results are presented as mean from three independent experiments. *B*: Effect of RO02704375 on kinetic parameters of G72R-glucokinase. Results are presented as mean from three to five independent experiments. *C*: Effect of RO02704375 on the activity index of wild-type and mutant glucokinase. The results for wild type, S263P, and G264S are comparable. M210K and five different amino acid substitutions at position 72 from G to E, F, K, R, and Q reduce the responsiveness to the activator markedly. Relative effects are shown by normalizing the baseline to unity for all enzymes. GKI, glucokinase activity index.

homology model was built using the wild-type glucokinase crystal structure as a template in the MOE software package. The M210K side chain was allowed to go through energy minimization under MMFF94 force field to find a low-energy conformation in the absence of any GKA. Next, RO0283946 was put into the activator binding pocket according to the position observed in the X-ray structure (Fig. 4*A*). Potential energy for the interaction between RO0283946 and the M210K clearly indicates that the mutation had a significant impact on the allosteric binding pocket environment. The Van der Waals interaction, which describes the hydrophobic interaction between ligand and protein, changed from very favorable in wild type (−26 kcal/mol) to completely repulsive in M210K (737 kcal/mol). This observation, very similar to the Van der Waals interaction trend we observed for V62 mutants (26), likely explains why M210K is completely unresponsive to activation by GKAs. The unfavorable Van der Waals interaction is shown in Fig. 4*B*.

To better understand why G72 mutants are refractory to GKAs, modeling efforts were used to calculate atomic partial charges using the partial equalization of orbital electronegativities (PEOE) method (46). The energetically minimized side chain conformations in the corresponding homology models were used in the calculation (Fig. 4*C* and *D*). The total negative Van der Waals surface area (PEOE_VSA_NEG) was found to be significantly nonlinearly correlated with the degree of the mutants' activation in response to treatment with GKA. The red surface areas shown in Fig. 4*E* and *F* are examples used to demonstrate the PEOE_VSA_NEG values for G and F. A regression analysis using fold activation as the y variable and $(\text{PEOE\_VSA\_NEG})^{-1}$ as the descriptor yielded a statistically significant fit with $R^2 = 0.93$.

DISCUSSION

Some 200 *GCK* mutations have been reported thus far (17). The majority are associated with MODY2, whereas a few result in PNDM (5–7,47) or congenital hyperinsulinemia of infancy (27–30). A minority of the mutations have been studied biochemically (4,23–26). These studies as well as the study here show that a thorough genetic and biochemical characterization of a particular mutant provides important information about glucokinase. Our diagnostic screening of *GCK* in 83 probands with mild hyperglycemia revealed 11 different mutations comprised of nine missense (V62A, G72R, L146R, A208T, M210K, S263P, E339G, R377C, and S453L), one nonsense (Y215X), and one splice-site (IVS5 + 1G>C) mutation in 14 families. We believe the mutations are pathogenic, as they cosegregated with disease, and the novel mutations were not identified in 50 blood donors. Notably, G72R has been independently reported in three MODY2 pedigrees, thus supporting that this mutant is pathogenic. Moreover, a previous mutation screening of subjects with PNDM revealed four homozygous or compound heterozygous mutations in *GCK* (M210K, G264S, A378V, and IVS8 + 2T>G) (5,6).

Our MODY2 subjects had typical signs of this disease (20–22), including moderately increased fasting blood glucose (>5.5 mmol/l), a 2-h increment <3.0 mmol/l after an oral glucose load, and mildly elevated A1C values. Moreover, the clinical phenotype was similar irrespective of mutation in *GCK*.

At least 40 different missense *GCK* mutations have been

TABLE 5
Mutational analysis of the G72 glucokinase binding site for the GKA

Mutants	n	Yield (mg/l)	Protein concentration (mg/ml)	$k$cat (s^{-1})	Glucose $S_{0.5}$ (mmol/l)	n_H	ATP K_m (mmol/l)	GI	GKA activation (fold)
Wild type	5	41.3 ± 3.33	3.03 ± 0.16	62.3 ± 4.75	7.55 ± 0.23	1.74 ± 0.04	0.41 ± 0.03	1.45 ± 0.11	15.8 ± 0.56
G72E	2	16.0, 3.36	0.95, 0.28	13.5, 12.5	17.6, 15.5	1.25, 1.19	0.33, 0.30	0.54, 0.49	1.70, 1.84
G72F	2	14.6, 8.45	0.85, 0.48	12.8, 11.3	16.0, 12.0	1.41, 1.15	1.28, 1.21	0.48, 0.41	1.46, 1.19
G72K	2	7.07, 6.66	0.52, 0.37	18.2, 17.9	10.1, 8.47	1.33, 1.17	0.49, 0.47	1.20, 1.14	2.46, 2.23
G72M	2	27.3, 24.1	1.28, 1.24	NA	NA	NA	NA	NA	NA
G72R	5	23.7 ± 3.70	1.12 ± 0.17	31.7 ± 2.02	5.32 ± 0.26	1.45 ± 0.03	0.76 ± 0.04	2.47 ± 0.23	2.60 ± 0.09
G72Q	2	14.4, 9.65	0.82, 0.67	34.7, 30.5	11.1, 11.0	1.26, 1.22	0.66, 0.65	1.49, 1.32	2.19, 2.08

Data are means ± SE for wild-type and G72R glucokinase. The results are means from independent analyses of which the number of expression is represented by n. Note that in case of two expressions only, the individual data are shown. G72S could not be expressed. GI, the activity index for the enzyme was calculated as previously described (28); NA, not applicable.

functionally studied. Most of these were found to be kinetically inactivating with alterations of one or more kinetic parameters (4,23,24). A few mutations are associated with hyperinsulinemia in infancy compatible with increased enzyme activity (27–30). The clinical phenotype of the glucokinase mutation carriers identified in our study could in most instances be explained by the inactivating nature of the mutations, as demonstrated by the kinetic analyses. However, one mutant had an increased enzyme activity index (G72R), whereas two others (S263P and G264S) were near normal compared with wild-type GST-glucokinase. These three mutations were therefore subjected to further functional characterization to uncover possible alternative mechanisms that could explain the development of hyperglycemia. Earlier studies have shown that some *GCK* mutations result in an unstable protein (23,25,39) and that this instability is inversely related to the ambient glucose concentration. We therefore assessed the thermal stability of G72R, S263P, and G264S in the presence and absence of glucose. A strong case of thermal instability as cause for the disease can be made for S263P, whereas the two other mutants are much less susceptible to inactivation by heat. According to a recent study, mathematical modeling of activating mutants predicts very low instability factors (26). From this and other considerations, it was suggested that thermal instability on its own is not sufficient to account for the hyperglycemia seen in patients with mutants who show only marginal thermal lability as revealed by our assays. It must be appreciated, however, that it remains difficult to define precisely what might be tolerable biological variation as contrasted with a severe pathogenic defect of this biophysical property. This critical issue will have to be resolved by cell-based approaches.

Regulation and nuclear localization of glucokinase in the hepatocytes is achieved by GKRP, a competitive inhibitor with respect to glucose (9,10). We therefore investigated the regulation of the mutants G72R, S263P, and G264S GST-glucokinase by human GKRP. Our data show that G72R was absolutely refractory to GKRP inhibition. Several explanations of the lack of regulation are possible. First, there may be a direct interaction between GKRP and residue G72 on glucokinase. An earlier study using site-directed mutagenesis suggested that GKRP might interact directly with the residues Glu-51, Glu-52, and also with a sequence from His-141 to Leu-144 or nearby residues (48). From our case, we cannot reject the possibility of direct interaction between GKRP with residue G72, and further investigations are needed. Second, it has been suggested that GKRP binds to the super-open form of glucokinase (33). This could then indicate that the mutation G72R inhibits the interaction between glucokinase and GKRP by preventing glucokinase from existing in its super-open form or changing the equilibrium between the open and closed forms. In principle, this could change the catalytic cycle, which is thought to be responsible for the cooperativity of the enzyme, and subsequently alter the n_H. This was, however, not observed for G72R. Recently, a lack of regulation of glucokinase by GKRP was reported for the mutation V62M (26), and it was concluded that the absence of inhibition by GKRP on V62M was not likely due to a conformational change of the enzyme. It was proposed that GKRP interacts with residues in a narrow region between the larger and smaller domain, thereby freezing glucokinase in its open conformation and interfering with glucose binding. The crystal structure of the glucokinase-GKRP complex is currently under investigation (33). The results from these studies promise to provide important data concerning the interactions between the two proteins.

All of our mutants were investigated with increasing concentrations of the newly discovered allosteric GKA RO0274375 (49). Most mutants responded to the GKA in a manner similar to that of the wild type with an increased k_{cat} and increased glucose affinity. M210K was, however, totally refractory to the allosteric GKA, and responsiveness of the mutants V62A, G72R, and S453L was significantly reduced. The existence of a putative endogenous allosteric activator has been proposed (4,29), though it remains to be identified. Our results with G72R illustrate that hyperglycemia in MODY2 may indeed be due to loss of an endogenous allosteric GKA and thereby support a similar finding for the mutation V62M by Gloyn et al. (26). Previously, a cocrystal structure between glucokinase and another GKA (RO0275145) revealed that the allosteric activator could interact with glucokinase at many residues, including a hydrophobic surface formed by the side chains of residues V62, I159, M210, I211, M235, and V452, covering the floor of the allosteric binding site. Our finding of total loss of effect of GKA on the mutant M210K is in line with a direct interaction between the allosteric activator and residue M210.

We extended our studies by performing additional mutagenesis of residue 72 and investigated the kinetic characteristics as well as the response to the allosteric activator RO0274375 of the different mutants. This study showed an increased activity index for G72R (as already noted), whereas it was normal for G72Q. The other mu-

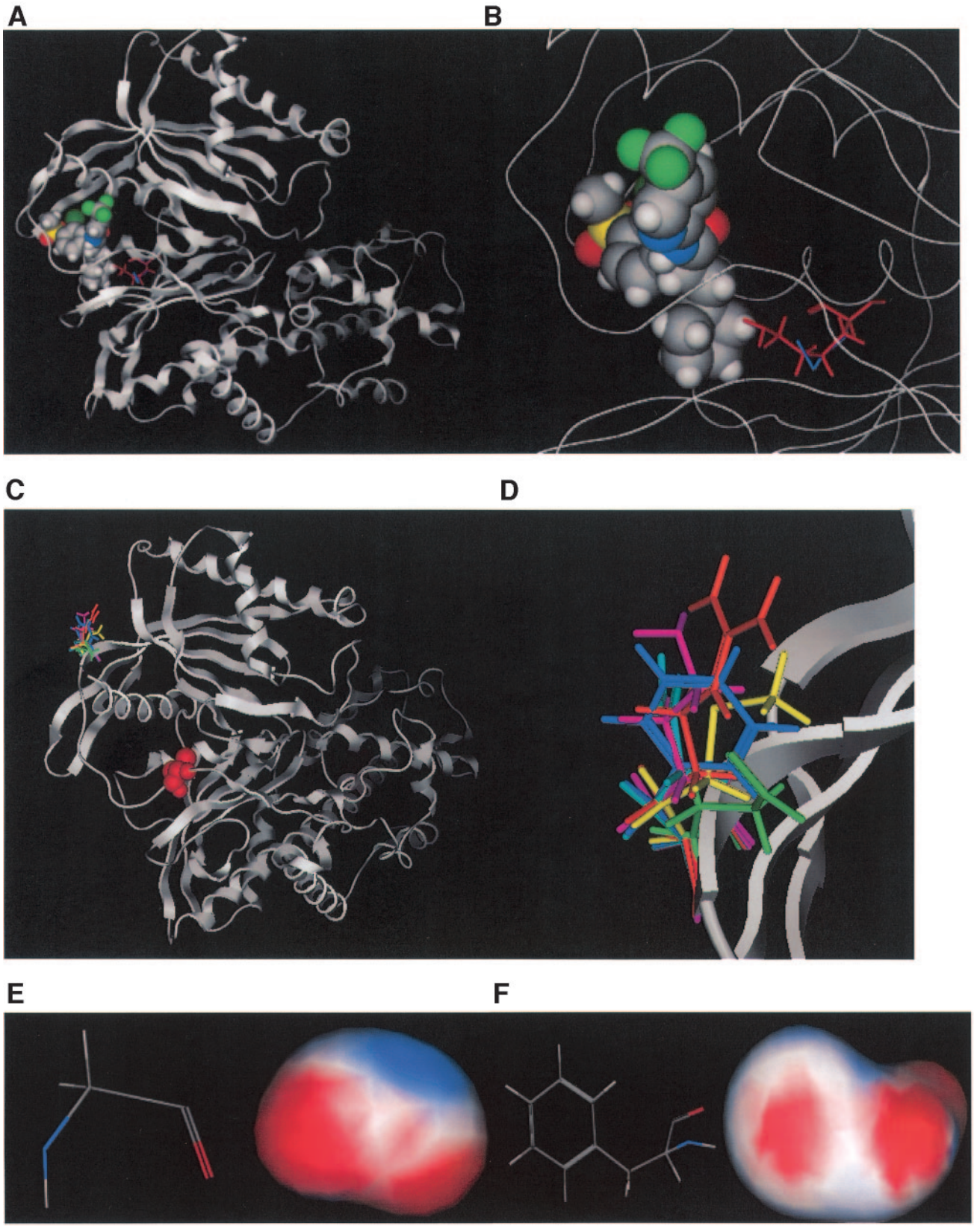

FIG. 4. Structural models of glucokinase. ***A*****: X-ray structure of glucokinase protein with RO0283946 in the allosteric binding pocket. Residue M210 in wild type is shown in blue and K210 in M210K mutation is shown in red.** ***B*****: Same as** ***A*****, but with a close up view of the allosteric pocket with M210 in blue and K210 in red. Global view (*** ***C*** **) and close up view of side chains (*** ***D*** **) of G72 mutations outlined in Table 5. V62 on the other end of the loop is colored in red (*** ***C*** **). The different amino acids in position 72 are as follows: E (green), F (blue), K (pink), M (yellow), Q (turquoise), and R (orange).** ***E*** **and** ***F*****: Examples of 3D amino acid structures and their Van der Waals surface areas colored by partial atomic charges. Red color is for the negatively charged atoms, white is for the neutral atoms, and blue is for the positively charged atoms.** ***E*****: Glycine.** ***F*****: Phenylalanine.**

tants showed reduced enzyme activity indexes. There was a less than threefold increase of the activity index resulting from maximal treatment with GKA for all mutants compared with the near 20-fold enhancement of the wild type. This lack of activation was observed irrespective of the nature of the substitute amino acid, suggesting that G plays a very critical role in this position. Glycine at the position 72 is apparently also critical for inhibition of GKRP.

A previous study of different amino acids in residue 62 (26) showed varying effects of several activators depending on the mutant, and it was concluded that this was due to differences in strength of interactions between the binding site and the compound. However, G72 is apparently not a direct contact point for RO0281675.

Modeling suggests that the negatively charged Van der Waal's surface area of the side chains for these point mutations at position 72 play an important role in modulating glucokinase responsiveness to activation by GKAs. As the side chains' negative surface area increases, glucokinase responsiveness sharply declines. Because these side chains are pointed toward the solvent environment and are not directly in the allosteric binding pocket, it is possible that the residues at position 72 may lie on a critical path needed for GKA to access the binding pocket. Since all GKA compounds have very polar core structure, a reasonable hypothesis can be proposed that the electrostatic interaction between GKAs and the side chain of the amino acid at position 72 can affect the glucokinase activation via its effect on how GKAs approach the binding site.

Our functional characterization of different *GCK* mutations illustrates that the mechanisms leading to hyperglycemia in MODY2 may be very complex. Even though for most mutations the phenotype can be explained simply by reduced glucose phosphorylation due to altered enzyme kinetic parameters, some mutants show near-normal or even increased enzyme activity. The study of the mutant G72R provides further evidence that loss of regulation by GKRP and an endogenous allosteric activator might be involved in altered glucose homoeostasis, perhaps aggravated by small reduction of protein stability, which may be of less consequence when GKRP and GKA responsiveness are preserved. Still, severe thermal instability may be the single sufficient cause of disease, as seen with the mutant S263P. In contrast, the mutant G264S showed near-normal enzyme activity, responded to GKRP as well as GKA, and was remarkably stable in the glucose titration test at 42.5°C. One could argue that this is a nonpathogenic mutation, but this is very unlikely because severe PNDM was diagnosed in a mutation carrier with the compound heterozygous *GCK* mutation G264S/IVS8 + 2 T>G (6). G264S is not mapped to any site known to interact with either GKA or GKRP, but it could be involved in the regulation of glucokinase by other cellular components. Recently, a provocative hypothesis was proposed based on studies of glucokinase induction in isolated pancreatic islets using glucose in the presence of the competitive inhibitor mannoheptulose (50). From these studies, the idea of glucokinase functioning as a metabolic messenger in pancreatic islet cells and hepatocytes (the "glucokinase switch") independent of glucose metabolism was advanced, implying the existence of intracellular targets for this metabolism-independent glucokinase switch. It would follow from this hypothesis that defects in such interactions and processes could be involved in the development of MODY2. Studies using mutations such as G264S may be helpful to further elucidate the regulation of glucokinase, which seems to be more complex than originally thought. The majority of nearly 200 *GCK* mutations remain unstudied, providing a great challenge and untapped resource for further study of the regulation of glucokinase.

In summary, our study provides evidence that *GCK* mutations can cause MODY2 by a complex mechanism, including thermal instability and loss of regulation by GKRP as well as a postulated endogenous allosteric activator. The impact of mutations in different residues on the effect of the GKA provides evidence of a binding site for such an endogenous allosteric activator. We also discovered mutants causing MODY2 that cannot be explained by any known or other plausible mechanism, suggesting additional and yet unrecognized ways of glucokinase regulation. Further comprehensive studies on such mutants may teach us more about an intricate regulatory network critical for glucokinase function.

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