# Insights Into the Biochemical and Genetic Basis of Glucokinase Activation From Naturally Occurring Hypoglycemia Mutations

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**Glucokinase (GCK) is a key regulatory enzyme in the pancreatic β-cell and catalyzes the rate-limiting step for β-cell glucose metabolism. We report two novel *GCK* mutations (T65I and W99R) that have arisen de novo in two families with familial hypoglycemia. Insulin levels, although inappropriately high for the degree of hypoglycemia, remain regulated by fluctuations in glycemia, and pancreatic histology was normal. These mutations are within the recently identified heterotropic allosteric activator site in the theoretical model of human β-cell glucokinase. Functional analysis of the purified recombinant glutathionyl S-transferase fusion proteins of T65I and W99R GCK revealed that the kinetic changes result in a relative increased activity index (a measure of the enzyme's phosphorylating potential) of 9.81 and 6.36, respectively, compared with wild-type. The predicted thresholds for glucose-stimulated insulin release using mathematical modeling were 3.1 (T65I) and 2.8 (W99R) mmol/l, which were in line with the patients' fasting glucose. In conclusion, we have identified two novel spontaneous *GCK*-activating mutations whose clinical phenotype clearly differs from mutations in ATP-sensitive $K^+$ channel genes. In vitro studies confirm the validity of structural and functional models of GCK and the putative allosteric activator site, which is a potential drug target for the treatment of type 2 diabetes. *Diabetes* 52:2433–2440, 2003**

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GCK, glucokinase; GSIR, glucose-stimulated insulin release; GST, glutathionyl S-transferase; $K_{ATP}$, ATP-sensitive $K^+$; MODY, maturity-onset diabetes of the young.

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Glucokinase (GCK) is a key regulatory enzyme in the pancreatic β-cell. It plays a crucial role in the regulation of insulin secretion and has been termed the pancreatic β-cell sensor (1). Given its central role in the regulation of insulin release, it is understandable that mutations in the gene encoding glucokinase (*GCK*) can cause both hyper- and hypoglycemia. Heterozygous inactivating mutations in *GCK* cause maturity-onset diabetes of the young (MODY), in which mild hyperglycemia is present at birth but often only detected later in life during screening for other purposes (2,3). Homozygous inactivating *GCK* mutations result in a more severe phenotype presenting at birth as permanent neonatal diabetes (4). Two heterozygous activating *GCK* mutations have been reported (V455M and A456V) that cause hypoglycemia (5,6). These mutations are in close proximity to each other both genetically (neighboring codons in exon 10) and structurally; they are adjacent to each other in the structural model of GCK (6).

Studies that have investigated the overexpression of hepatic *GCK* in mice (7–11) have shown a lowering of plasma glucose concentrations, suggesting that enhancement of GCK activity could be a therapeutic strategy for the treatment of diabetes. However, the majority of these studies (9–11) have also shown that the decrease in plasma glucose concentration is accompanied by an undesirable increase in serum triglycerides.

The discovery of inactivating and activating *GCK* mutations and their functional characterization has led to important concepts that explain essential characteristics of glucose-stimulated insulin release (GSIR). In this study, we present two novel spontaneous activating *GCK* mutations, detailed functional characterization of these mutations, and the clinical characteristics of the families. These have led to important insights into the biochemical activation and regulation of GCK and the clinical phenotype of individuals with hypoglycemia due to GCK activation.

## RESEARCH DESIGN AND METHODS

### Subjects

***Family 1.*** The proband is a boy born at term (birth weight 3.13 kg) from a nonconsanguineous Dutch Caucasian family. He had neonatal hypoglycemia with blood glucose values between 2.0 and 2.6 mmol/l. He was asymptomatic and received no treatment. At age 15 years he presented with seizures and was found to have a fasting blood glucose of 2.3 mmol/l and an insulin level of 56

TABLE 1
Fasting lipid values for GCK-HI–affected family members

| | Total cholesterol (mmol/l) | HDL cholesterol (mmol/l) | LDL cholesterol (mmol/l) | Triglycerides (mmol/l) |
|---|---|---|---|---|
| T651 proband | 5 (4.7–6.5) | 1.26 (0.95–1.5) | 3.46 (<4.7) | 0.66 (0.8–2.0) |
| T651 proband's mother | 4.1 (4.7–6.5) | 1.02 (1.1–1.7) | 2.63 (<4.7) | 1.06 (0.8–2.0) |
| W99R proband* | 4.2 | NA | NA | 0.7 |
| W99R proband's father | 3.9 (3.3–6.5) | 1.08 (0.8–1.8) | 2.6 (1.0–4.2) | 0.5 (0.85–2.0) |

Data are patient's value (laboratory normal age range, when available). *Nonfasting sample, NA, variable not measured.

pmol/l. At this time he weighed 53 kg and had a BMI of 17.8 kg/m$^2$. Fasting blood glucose values before treatment ranged between 2.3 and 3.0 mmol/l. During normal feeds his blood glucose values ranged between 3.3 and 5.8 mmol/l, and his insulin levels were 230–323 pmol/l. He was treated with diazoxide 100 mg b.i.d., which resulted in an increase in blood glucose to 4.2–4.6 mmol/l. Frequent meals reduced his mild clinical symptoms of hypoglycemia.

The proband's mother had a history of seizures from age 15 years and was diagnosed with consistent fasting hypoglycemia at age 20 years. Her insulin level (93 pmol/l) was inappropriate for her hypoglycemia (2.2 mmol/l). When she was treated with a resection of the head of the pancreas, there was a slight increase in her blood glucose. At age 35 years a trial of treatment with diazoxide was commenced, which normalized her blood glucose levels but was discontinued due to the development of hypertrichosis. Histological examination of her pancreas showed normal histology with no hyperplasia, no hypertrophied insulin cells, and regular-sized islets of Langerhans. The proband's father, maternal grandparents, and mother's five siblings had normal fasting glucose levels.

***Family 2.*** The proband, a boy from a nonconsanguineous U.K. family, was born at 37 weeks' gestation by emergency section for fetal distress following maternal preeclampsia. His birth weight was 3.08 kg, head circumference 35 cm, and length 50 cm (all 50th percentile). On the first day of life he was admitted to the neonatal unit with hypoglycemia (1.8 mmol/l) and treated with intravenous glucose infusion. A dilutional exchange transfusion was performed on day 3 for polycythemia. He was initially treated with intravenous glucose infusion but was discharged at 28 days of age on standard formula milk. Investigations during this time showed normal plasma cortisol, growth hormone, lactate, amino acids, free fatty acids in response to glucagon, plasma and urinary amino acids, and organic acids. Plasma insulin levels (68–29 pmol/l) were inappropriately high for his glucose values (range 2.0–3.5 mmol/l). At 9 weeks of age he was started on a low dose of diazoxide and chlorothiazide, which had no effect on his blood glucose, but an increase in dose resulted in vomiting. At 16 weeks of age prefeed blood glucose levels were 2–2.6 mmol/l and postfeed levels were 2.6–3.4 mmol/l on 3-h feeds totaling 150 ml $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$. When his glucose was 2.9 mmol/l, insulin was 29 pmol/l and C-peptide 0.61 nmol/l. Plasma ammonia was normal. Diazoxide was increased to 20 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ with chlorothiazide, which resulted in some improvement in blood glucose levels. At 10 months of age he was admitted to the hospital again with hypoglycemia (blood glucose 1.2 mmol/l). On this occasion, insulin (<14 pmol/l) and C-peptide (<0.05 nmol/l) were suppressed, hydroxybutyrate was raised (1.10 mmol/l), and lactate was marginally elevated (2.4 mmol/l). Two days later blood glucose had returned to 2.4 mmol/l, insulin 31 pmol/l, C-peptide 0.8 nmol/l, hydroxybutyrate <0.5 mmol/l, and lactate 2.26 mmol/l. The proband was readmitted to the hospital at 16 months of age because of persistently low glucose levels on maximal diazoxide therapy (20 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$). In response to octreotide 20 μg (1.8 μg/kg) his blood glucose rose to >20 mmol/l for 8 h, fell to <3 mmol/l, and rose again to >20 mmol/l following a 5-μg dose for several hours before falling again to <3 mmol/l, but thereafter showed a modest rise in response to subsequent increasing doses on octreotide, which lasted for only 4 h, therefore treatment was not continued. Blood glucose levels improved on Nifedipine but did not result in an ability to reduce the dose of diazoxide. From the 2nd year of life the proband has been persistently neutropenic (0.24–1.26 $\times$ 10$^9$/l), which is most likely due to chlorothiazide, but the proband's blood glucose fell dramatically when this treatment was withdrawn. The proband remains on diazoxide 20 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$, chlorothiazide 10 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$, and nifedipine 0.45 mg $\cdot$ kg$^{-1}$ $\cdot$ day$^{-1}$ on which his blood glucose levels are variable, but mostly 3.0–3.9 mmol/l unless there is intercurrent illness.

The proband's father presented with symptoms suggestive of hypoglycemia following his son's diagnosis, and a random blood glucose was 2.8 mmol/l. Following this, a formal prolonged oral glucose tolerance test was performed that showed fasting, 30-, 60-, 90-, 120-, 150-, and 180-min glucose values of 3.1, 7.5, 3.3, 2.1, 2.1, 2.1, and 2.2 mmol/l, respectively. During the time when these lower blood glucose levels were found, the proband's father felt tired and hungry with occasional sweatiness but never lost consciousness. Six months after the oral glucose tolerance test he underwent a 48-h fast. He had several blood glucose values during this time, and they were all 2.7–3.0 mmol/l. During the fast his insulin levels were suppressed from 16 to <10 pmol/l, and C-peptide fell from 613 to 301 pmol/l. Outside of formal testing, the proband's father had no symptoms of hypoglycemia, and a magnetic resonance imaging scan of his pancreas was normal. The proband's father has been untreated for 18 months, and fasting plasma glucose value measured 3.1 mmol/l. At this time liver function tests were normal. The proband's mother, paternal uncle, and paternal grandparents had no symptoms of hypoglycemia. They all had normal fasting glucose values except for the paternal grandfather, who had a fasting glucose value of 6.5 mmol/l. Lipid measurements for all subjects are shown in Table 1.

**Identification of *GCK* gene mutations.** Genomic DNA was extracted from peripheral lymphocytes using a Wizard DNA extraction kit (Promega, Southampton, U.K.). The coding regions of the 10 exons and the intron-exon boundaries of *GCK* were amplified by PCR using published primer sequences. PCR products were purified using QIAquick PCR purification columns (Qiagen, Crawley, U.K.), and both strands were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Warrington, U.K.) according to the manufacturer's recommendations. Reactions were analyzed on an ABI 377 DNA sequencer (Applied Biosystems). The relationships between the samples in each family were confirmed using a panel of 10 microsatellite markers.

**Kinetic analysis.** Recombinant human islet T65I and W99R were generated using methods previously described (12). The enzyme was expressed in the form of a glutathionyl S-transferase (GST) fusion protein using the protocols developed during the study of GST-GCK V455M and other *GCK* mutations (13). The following modifications were made to the protocol. During the single-step affinity chromatography purification of the GST-GCK, the eluted fusion protein was collected in 1-ml fractions. Kinetic analysis was then performed on all protein-positive fractions with a concentration >0.50 mg/ml. Protocol A was carried out with 11 glucose dilutions between 0 and 30 mmol/l for T65I and W99R GST-GCK and 0 and 100 mmol/l for wild-type GST-GCK. Protocol B was carried out with glucose at $\times 10$ $S_{0.5}$ for all mutants and wild-type. The relative activity index, an expression of the proposed enzyme's in situ phosphorylation capacity based on expression at 5 mmol/l blood glucose, relative to wild-type GCK, was calculated as previously described (6). Statistical analysis to compare the kinetic parameters for the different protein fractions was performed by a Kruskal-Wallis test.

**Structural analysis.** Structural analysis of activating mutations in the GCK enzyme was performed using the structural model of Mahalingam et al. (14). The location of all described activating mutations in the heterotropic allosteric activating site is shown (Fig. 1).

**Mathematical modeling.** The kinetic data were used to calculate the glucose threshold for GSIR using a mathematical model previously described (1). This model predicts the threshold for GSIR by using the kinetic characteristics of the normal or mutated GCK enzyme and by accepting a few plausible assumptions. First, β-cell GCK serves as the glucose sensor that controls GSIR. Second, the physiological threshold for GSIR for wild-type GCK is 5 mmol/l, and therefore ~26% of total GCK phosphorylating capacity is needed to initiate insulin secretion. Third, the glucose dependency of β-cell glucose phosphorylation rate is defined by the Hill equation. The ATP dependency of β-cell glucose phosphorylation rate is defined by an expression based on Michaelis-Menton kinetics. The two equations can be combined to describe two-substrate kinetics of GCK. Finally, there is altered expression of both the mutant and wild-type GCK alleles due to adaptation to the blood glucose concentration (1,15,16). The following expression coefficient describes the relationship: $e = (S^h \times 2)/(S^h + S_{0.5}{}^h)$, where $S$ refers to the glucose level at threshold, $h$ is the Hill number for cooperativeness with glucose, the numerical value 2 indicates that half maximal induction is achieved at glucose $S_{0.5}$,

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TABLE 2
Kinetic characteristics of GCK T65I and W99R

| Kinetic parameter | Wild-type GCK | T65I GCK | W99R GCK |
|---|---|---|---|
| $K_{cat}$ ($S^{-1}$)* | 48.99 ± 4.90 | 20.10 ± 1.48 | 106.29 ± 2.15 |
| $K_{cat}$ ($S^{-1}$)† | 59.69 ± 0.44 | 22.09 ± 2.47 | 130.99 ± 3.96 |
| Glucose $S_{0.5}$ (mmol/l) | 8.45 ± 0.89 | 1.71 ± 0.04 | 4.90 ± 0.16 |
| nH (no units) | 1.44 ± 0.03 | 1.26 ± 0.01 | 1.44 ± 0.05 |
| ATP $K_m$ (mmol/l) | 0.50 ± 0.01 | 0.59 ± 0.01 | 1.10 ± 0.06 |
| Relative activity index (no units) | 1 | 9.81 ± 1.01 | 6.36 ± 0.46 |

Data are means ± SE. The results are the means of the kinetic analysis of three independent expressions of wild-type and mutant T65I GST-GCK and four independent expressions of mutant W99R GST-GCK. ATP $K_m$ was measured at a glucose concentration 10 times the enzyme's $S_{0.5}$. *Protocol A; †protocol B.

and $S_{0.5}$ refers to the concentration of glucose needed to achieve half maximal rate of phosphorylation (1).

## RESULTS

**Sequencing of *GCK*.** Direct sequencing of the entire coding region and exon-intron boundaries of *GCK* was performed in the probands from both families. In family 1, a heterozygous missense mutation substituting isoleucine for threonine at codon 65 (T65I) in exon 2 was identified. This mutation was subsequently identified in the proband's affected mother but not in the proband's normoglycemic father, maternal grandparents, and five maternal aunts and uncles. Analysis of the 10 microsatellite markers confirmed the family relationships, establishing that the mutation was a spontaneous mutation in the proband's mother.

In family 2, a heterozygous missense mutation substituting arginine for tryptophan at codon 99 (W99R) in exon 3 was identified in the proband. This mutation was present in the proband's affected father but not in the proband's normoglycemic mother, paternal grandparents, or paternal uncle. Microsatellite analysis confirmed family relationships and established that this was a spontaneous mutation in the father. Neither of these mutations has been found in 200 normal chromosomes.

**Biochemical characterization of T65I and W99R.** The mutant enzymes were expressed as GST fusion proteins, and the purified enzymes were subjected to kinetic analysis ($K_{cat}$ [maximal specific activity], glucose $S_{0.5}$, $h$, and ATP $K_m$ [concentration at half-maximal activation]) (Table 2). Two different $K_{cat}$ values are recorded, one calculated from data obtained with protocol A and the other with data from protocol B as previously described (13). At least three preparations of wild-type and each mutant GST-GCK were purified. We performed kinetic analysis on multiple protein fractions for each independent enzyme expression. All GCK protein fractions analyzed were found to be essentially pure as indicated by the presence of a single band at 75 kDa on phast gel (Amersham Pharmacia Biotech, Piscataway, NJ) electrophoresis (data not shown). There were no significant differences between the maximal specific activities ($K_{cat}$) of each fraction (data not shown). For this reason all fractions from each of the independent expressions were pooled. The functional data are shown in Table 2. Both mutant enzymes showed an increased affinity for glucose indicated by the decrease in glucose $S_{0.5}$ value. W99R had an approximate twofold decrease, whereas T65I was reduced approximately fourfold. The Hill number was decreased slightly for T65I but unchanged for W99R. The $K_{cat}$, or turnover number of the enzyme, was significantly increased for W99R (approximately twofold), but it was conversely reduced for T65I (approximately threefold). $K_m$ for ATP was in the normal range for T65I but increased for W99R (approximately twofold). The effective activation of the mutant GCKs are expressed by the increased relative activity index of

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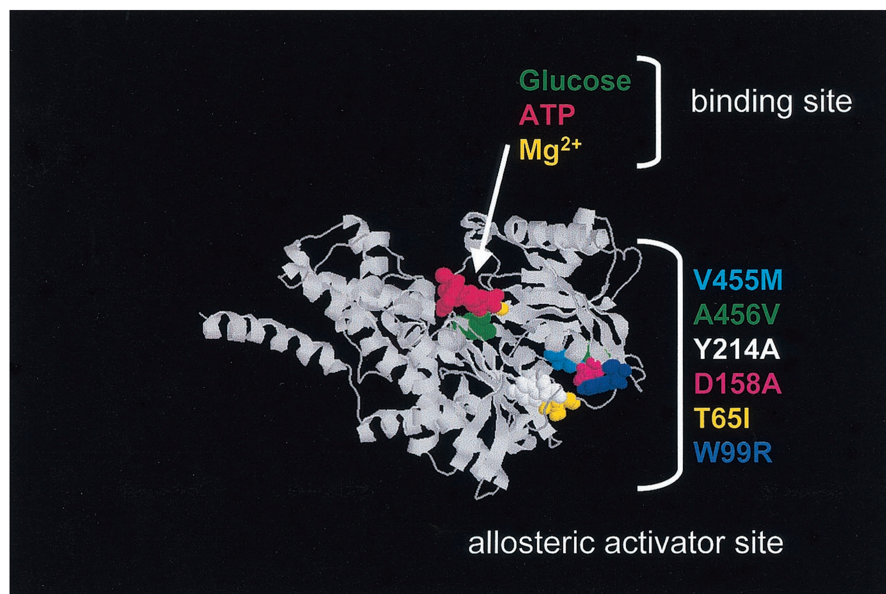
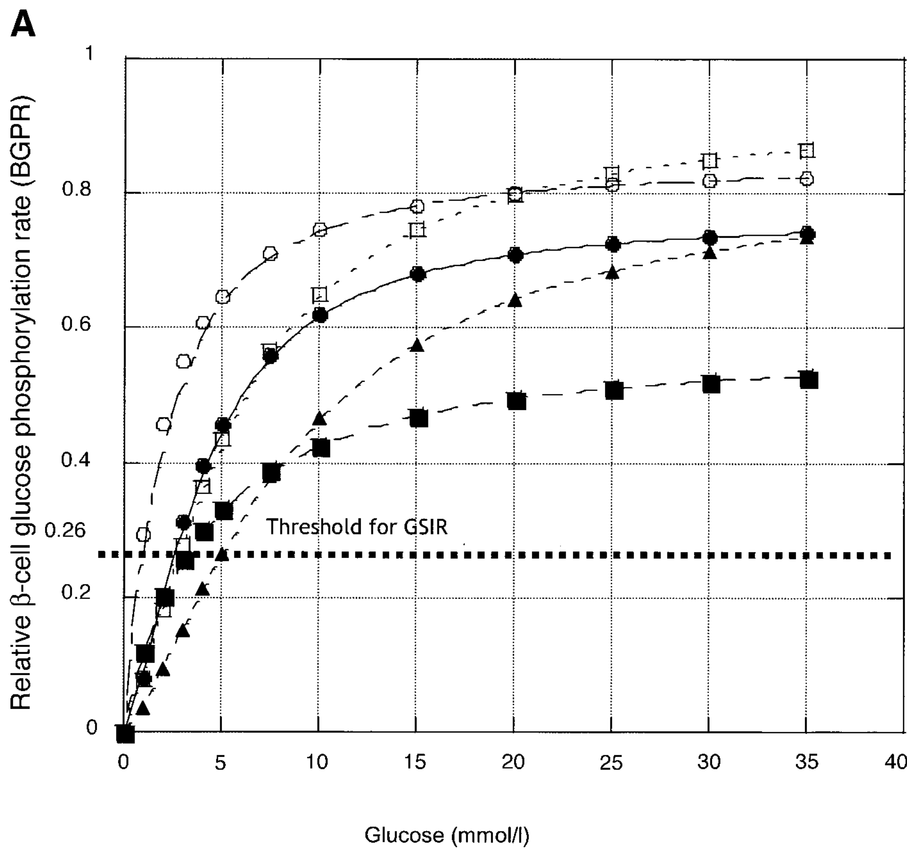



**FIG. 1. Heterotropic allosteric activating domain of GCK. The structural model shows the location of the naturally occurring (T65I, W99R, V455M, and A456V) and artificially created activating mutations (D158A and Y214A) in a circumscribed allosteric domain distinct from the binding cleft for glucose and MgATP.**



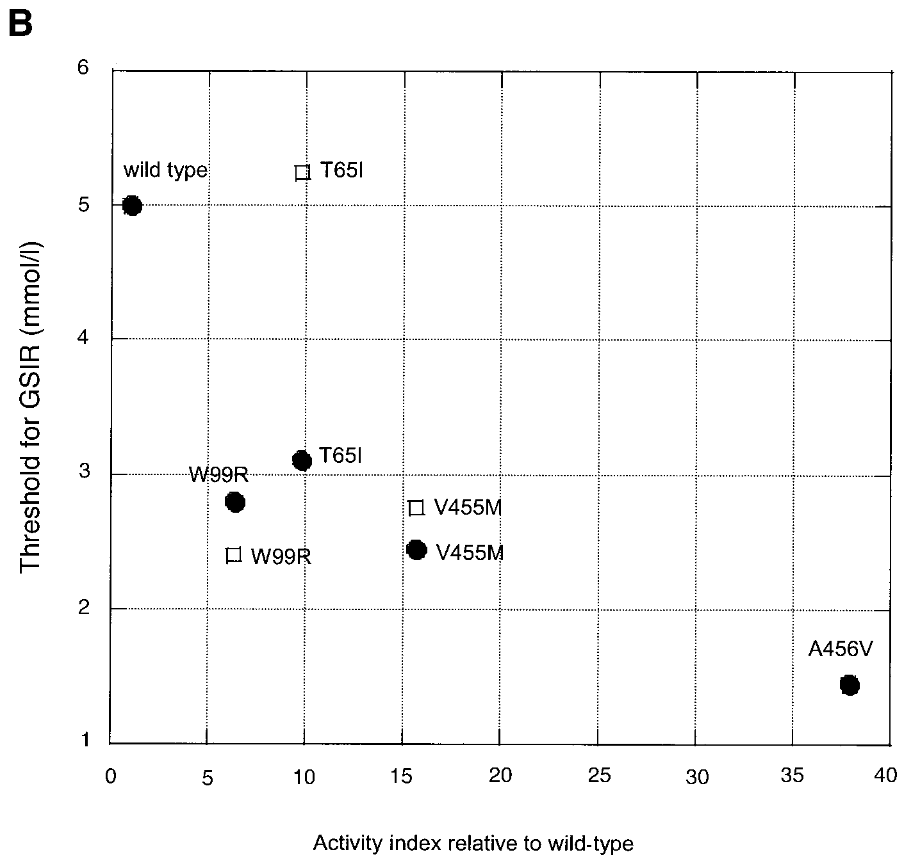




**FIG. 2.** ***A*****: Threshold shift of GSIR for naturally occurring activating mutations (T65I, W99R, V455M, and A456V) compared with wild-type GCK. ●, V455M; ○, A456V; ■, T65I; ▲, wild-type; □, W99R.** ***B*****: β-Cell thresholds for GSIR in wild-type homozygotes and heterozygotes with naturally occurring mutations (T65I, W99R, V455M, and A456V): a comparison of the threshold for GSIR in the adapted and nonadapted states (T65I, W99R, V455M, and A456V) compared with wild-type GCK. ●, adapted; □, nonadapted.**

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TABLE 3
Clinical characteristics of GCK-HI compared with SUR1/Kir6.2-HI

| Characteristic | GCK-HI | SUR1/Kir6.2-HI |
|---|---|---|
| Birth weight | Normal<br>Median birth weight SD score −0.22; range −2.32–1.43 | Increased<br>Median birth weight SD score +1.85; range −1.5 to 5.7 (20) |
| Response to diazoxide | 9 of 11 cases treated successfully with diazoxide | Poor and often totally refractory (heterozygous mutations respond better to diazoxide than homozygous) |
| Glucose levels | Low and consistent (Fig. 3) | Low and erratic<br>Median 1.8 ± 1.14 mmol/l; range 0.5–5 mmol/l ($n$ = 24) (20) |
| Insulin levels | Only high for level of glycemia and regulated (Fig. 3) | High and unregulated<br>Median 341.50 ± 325.03 pmol/l; range 78–1200 pmol/l ($n$ = 24) (20) |
| Counterregulatory response | Normal | Increased (raised glucagon, raised growth hormone, and raised cortisol) |
| Risk of type 2 diabetes later in life | 2 reported cases in GCK-HI families:<br>*1* dx 48 years (5)<br>*2* dx 72 years (6)<br>Mutation status unknown in both cases | Increased (19–21)<br>Slow progressive loss of β-cell function (21) |
| Inheritance | Autosomal dominant<br>Spontaneous mutations common | Autosomal recessive (most common)<br>Autosomal dominant<br>Loss of heterozygosity (somatic loss of maternal alleles on 11p15.1 + paternal SUR1 mutation in 30% of cases) |
| Histology | Normal | Irregular-sized islets of Langerhans with hypertrophied insulin cells<br>Can either be diffuse or focal (due to loss of heterozygosity) |

Birth weights for GCK-HI have been compared with a U.K. Caucasian population cohort.

~10-fold for T65I and ~6-fold for W99R, extrapolating to a marked enhancement of β-cell glucose usage.

**Structural analysis.** Structural analysis of T65I and W99R showed that they were in close proximity to the previously published, naturally occurring activating mutations (V455M and A456V) and the artificially created mutations (D158A and Y214A) that increase the glucose affinity and/or $K_{cat}$. All of these mutations are located in a putative allosteric activator site (Fig. 1). This domain is remote from the substrate binding cleft for glucose and MgATP. This region has been termed the heterotropic allosteric activator site.

**Mathematical modeling.** The physiological consequences of the kinetic characteristics of the T65I and W99R mutations on GSIR, and thus glucose homeostasis, were investigated using the published mathematical model, which takes into account the impact of blood glucose levels on GCK expression for both alleles (1). This model predicted thresholds for GSIR of 3.1 and 2.8 mmol/l, respectively. The previously published naturally occurring (V455M and A456V) activating mutations (5,6) were remodeled using this model. The predicted thresholds for GSIR were 2.45 and 1.40 mmol/l, respectively. The predicted threshold shifts of GSIR for all activating mutations are shown in Fig. 2*A*. A graphic illustration of the relationship between the threshold for GSIR and the relative activity index of each mutant enzyme is shown in Fig. 2*B*. Figure 2*B* also shows a comparison of the relationship between the threshold for GSIR and the relative activity index for each mutant in the adapted (adjusted for the effect of glucose on GCK expression) and nonadapted state. Adaptation has a large effect on the predicted threshold for T65I, a small effect for W99R and V455M, but no effect on A456V GST-GCK.

## DISCUSSION

The identification of mutations in the *GCK* gene has significantly increased our understanding of the GCK glucose-sensor paradigm for glucose homeostasis. The discovery and functional characterization of the two novel activating mutations reported in this study has given us the opportunity for further investigation and discussion of this model. In addition, it has increased the number of reported cases of hyperinsulinemia of infancy due to GCK mutations (GCK-HI) and hence our understanding of this disorder, thus allowing us to make comparisons between the different phenotypic presentations and clinical courses of GCK-HI and the cases with the most common cause, mutations in the $K_{ATP}$ channel genes (SUR1/*ABCC8* and Kir6.2/*KCNJ11*).

In the current study we have described two novel *GCK*-activating mutations and have shown that both are de novo in the second generation of the families tested. There are only two previous reports of a spontaneous mutation in the *GCK* gene (17,18). In addition to the lack of family history, the subclinical symptoms of this condition may hinder diagnosis.

Although GCK-HI is a subtype of hyperinsulinemia of infancy, it is important to note the differences in the clinical characteristics of these patients compared with those with mutations in the ATP-sensitive $K^+$ ($K_{ATP}$) channel genes. These differences have been summarized in Table 3.

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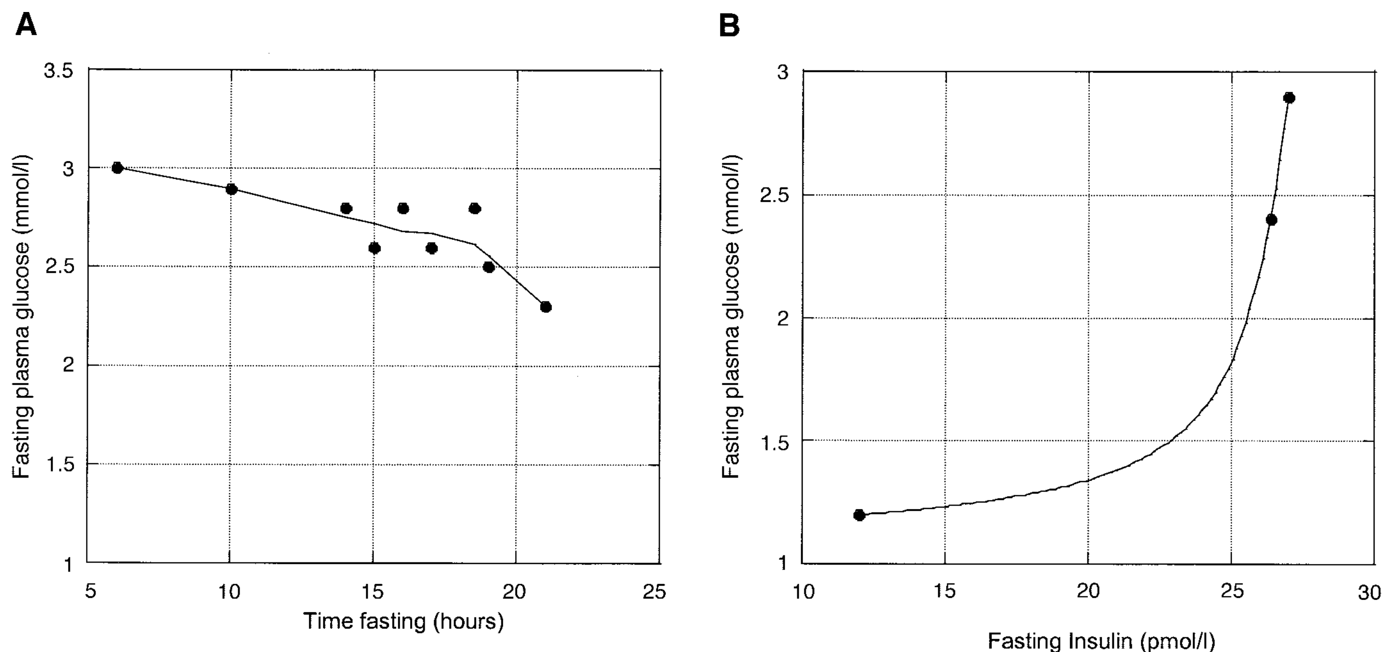


**FIG. 3.** ***A*****: Fasting plasma glucose levels during a 21-h fast for proband family 1 (T65I).** ***B*****: Fasting glucose versus fasting insulin values in proband family 2 (W99R).**

Mutations in the $K_{ATP}$ channel genes result in unregulated secretion of insulin due to a defective $K_{ATP}$ channel, but activating mutations in *GCK* result in a "resetting" of the GSIR threshold, while insulin secretion remains regulated. This is clearly shown in our patients whose insulin levels are suppressed and increased with fluctuations in glycemia around the normal fasting level and whose glucose values are consistently low (Fig. 3). In patients with GCK-HI the fasting insulin levels are in the normal range (21–114 pmol/l) and counterregulation is normal rather than increased. We predict that these patients, like those with inactivating mutations resulting in MODY2, will have a stable phenotype throughout life with a very gradual increase in fasting glucose in line with the normal population. A further clear phenotypic difference is that the head of the pancreas was histologically normal in the proband's mother in family 1, while in HI due to $K_{ATP}$ channel gene mutations there is diffuse hyperplasia with hypertrophied insulin cells and irregular-sized islets of Langerhans.

The differences in phenotype begin in utero, SUR1/Kir6.2-HI babies are born large for gestational age, but the GCK-HI children do not show increased birth weight. In some cases, e.g., family 1, this is because the mother was hypoglycemic during pregnancy [similar to the normal weight seen when the mother and fetus inherit an inactivating mutation, and the baby is normal birth weight (22)]. The treatment of these two forms of HI differs. Patients with SUR1/Kir6.2-HI respond poorly to potassium channel openers, such as diazoxide, whereas GCK-HI patients respond to or tolerate diazoxide. Because insulin secretion is regulated as long as blood glucose is not low enough to precipitate seizures and neuroglycopenia, no intervention may be required. This is seen in the father of family 2, who is asymptomatic despite having a lifetime of untreated fasting glucose values between 2.7 and 3.1 mmol/l. Activating mutations with kinetic changes that result in higher relative activities than those described in patients to date can be predicted from the artificial mutations with more dramatic increases in relative activity indexes (23). Such severe mutant enzymes, although still regulated by glucose, may result in more pronounced hypoglycemia, and patients might not respond sufficiently to diazoxide and have severe neuroglycopenic symptoms. Familial cases are less likely with these severe mutations, as the marked ill health is likely to greatly reduce lifespan and reproductive fitness.

There has been some debate as to whether patients with neonatal hyperinsulinemia are more likely to develop type 2 diabetes later in life. A recent study (21) carried out in an extended Finnish family has illustrated that mutations in the SUR1 (*ABCC8*) gene can present as hypoglycemia during infancy and diabetes in early adulthood. However, it is not known whether this is the case with GCK-activating mutations. We suggest that since the defect is in glucose sensing rather than excess insulin secretion, this is not likely to result in the "β-cell exhaustion" seen in $K_{ATP}$ channel mutations (21). None of the mutation carriers in the two families presented in this study have developed diabetes; however, the oldest is only 47 years of age. In the two previously reported cases, a family member of each family has developed diabetes later in life, but it was not known if these individuals had an activating mutation (5,6). This was likely in the V455M family, as the proband's father developed diabetes at age 48 years and had a history of hypoglycemia. Further longitudinal studies are required to investigate this.

The different clinical presentations of the two affected individuals from family 2 is also interesting. The proband presented at birth with severe recurrent hypoglycemia requiring multiple therapy, whereas his father was asymptomatic and presented only after his son's diagnosis. This has also been reported in the A456V family (6). It may be that there is a phenotypic modification by other environmental or genetic factors. However, it is also possible that

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some variation in the clinical presentation of father and son may represent a different approach of the physician's management of asymptomatic hypoglycemia in these cases.

The localization of the novel mutations in exons 2 and 3 in a structural model of GCK revealed that they were in close proximity to the previously described, naturally occurring mutations (V455M and A456V) in exon 10. Along with the clustering of artificially created mutations in exons 4 and 6 (D158A and Y214A) in this region (23,24), this finding adds further scientific evidence for the presence of a heterotropic allosteric activator site. The discovery of this site suggests that it is possible that an undiscovered endogenous activator molecule exists that acts through this site. The discovery of pharmacological agents that activate GCK in a similar way to these mutations suggests that they may be acting through this site (25). Since this site is remote from the substrate-binding cleft, it suggests that the "putative" activator is structurally different from the substrate.

The clinical characteristics of patients with *GCK*-activating mutations have important implications for any therapeutic agents targeted at this heterotropic allosteric activator site in GCK. Overexpression of hepatic *GCK* in mice has raised the issue of elevated serum triglycerides (9–11). None of the affected mutation carriers in this study had adverse lipid profiles. Apart from mildly raised serum triglyceride, the lipid profiles of the affected individuals with the A456V mutation were also in the normal range (6). These observations do not support a deleterious effect on a patient's lipid profile.

Activation of GCK catalysis by the mutations D158A, Y214A, V455M, and A456V is the result of lowering of glucose $S_{0.5}$, increasing $K_{cat}$, and reducing the Hill coefficient for glucose either separately or in combination. The kinetic changes result in a near hyperbolic enzyme, which contrasts with the catalytically less active sigmoidal wild-type GCK. Kinetic analysis of mutant T65I and W99R GCK showed that the mutations have different kinetic consequences. The decrease in maximal activity, $K_{cat}$, for T65I was the opposite of what had been predicted. With the previous activating mutations, the $K_{cat}$ increased or remained unchanged. However, the dramatic reduction in $S_{0.5}$ is more than sufficient to account for the increased activity of the enzyme. W99R is also unusual in that it is the first of the naturally occurring or artificially created activating mutations to have a significantly increased ATP $K_m$. This decrease in affinity for the substrate MgATP explains in part the relative activity of the enzyme, since the phosphorylation of glucose may be limited by the decreased affinity for the second substrate. The most active mutants are those with large increases in $K_{cat}$ and substantial decreases in $S_{0.5}$ (Y214A and A456V). These enzymes have much higher relative activity indexes (164 and 34, respectively) compared with those with less dramatic changes in both parameters (W99R, T65I, D158A, and V455M at 6.36, 9.81, 10.8, and 15.7, respectively).

We used a mathematical model to predict the threshold for GSIR in heterozygous carriers of mutant T65I and W99R GCK. This model takes into consideration the adaptation of both the mutant and wild-type alleles to blood glucose concentration. In MODY2, increased blood glucose levels favor increased expression of the wild-type allele that compensates for the decreased relative activity of the mutant allele. However, in HI the decreased blood glucose concentration favors adaptation of the mutant allele. If this model genuinely reflects what is happening in vivo, then it is possible that unlike MODY2 mutations, which have a predicted threshold for GSIR of ~7 mmol/l and patients have a very narrow range of fasting plasma glucose values (6–8 mmol/l), we will see greater heterogeneity with GCK-HI mutations. Although the mathematical model accurately predicts thresholds of GSIR for all mutants, it is important to stress that it is a minimal model and that there are other factors that affect blood glucose levels, particularly food intake. The fact that patients can control their symptoms by eating regular meals shows the influence of postprandial glucose levels on the threshold for GSIR. The comparison of the predicted thresholds for GSIR using the model with and without adaptation supports the minimal mathematical model used. Without adaptation the predicted threshold for T65I would be ~5 mmol/l, and the patient would therefore not have hypoglycemia. The relative contribution of the mutant allele's kinetics to expression levels of the enzyme results in a decreased threshold for GSIR and hypoglycemia.

In conclusion, we have identified two novel GCK-activating mutations that cause hyperinsulinemia of infancy. This study illustrates that activating GCK mutations may often be spontaneous. Our families and the two previously published families illustrate clear phenotypic differences between GCK-HI and SUR1/Kir6.2-HI. This study provides further support for the current structural model of GCK, as we have shown that both novel mutations are in close proximity to the previously identified, naturally occurring, and artificially created activating mutations in the proposed heterotropic allosteric activator site. Functional characterization of these mutations has shown that the changes to the kinetic properties of these enzymes will reduce the threshold for GSIR, thus leading to hypoglycemia. Finally, the different kinetic properties of these two mutant enzymes provide insight into the nature of the allosteric heterotropic activator site, which is a potential drug target for the treatment of type 2 diabetes.

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