Glucokinase Thermolability and Hepatic Regulatory Protein Binding Are Essential Factors for Predicting the Blood Glucose Phenotype of Missense Mutations*

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To better understand how glucokinase (GK) missense mutations associated with human glycemic diseases perturb glucose homeostasis, we generated and characterized mice with either an activating (A456V) or inactivating (K414E) mutation in the gk gene. Animals with these mutations exhibited alterations in their blood glucose concentration that were inversely related to the relative activity index of GK. Moreover, the threshold for glucose-stimulated insulin secretion from islets with either the activating or inactivating mutation were left- or right-shifted, respectively. However, we were surprised to find that mice with the activating mutation had markedly reduced amounts of hepatic GK activity. Further studies of bacterially expressed mutant enzymes revealed that GK^{A456V} is as stable as the wild type enzyme, whereas GK^{K414E} is thermolabile. However, the ability of GK regulatory protein to inhibit GK^{A456V} was found to be less than that of the wild type enzyme, a finding consistent with impaired hepatic nuclear localization. Taken together, this study indicates that it is necessary to have knowledge of both thermolability and the interactions of mutant GK enzymes with GK regulatory protein when attempting to predict in vivo glycemic phenotypes based on the measurement of enzyme kinetics.

Studies over the past 2 decades have firmly established that glucokinase (GK), plays a key role in determining the blood glucose concentration in mammals. In humans, there is a reciprocal but nonlinear relationship between GK activity and the blood glucose concentration. First, heterozygous gene mutations that diminish enzyme expression or otherwise lower catalytic flux cause maturity onset diabetes of the young type (MODY)-GK (1–4), a disease characterized by mild hyperglycemia. Second, inactivating mutations in both alleles of the human GK gene lead to persistent neonatal diabetes-GK, a severe but rare form of hereditary hyperglycemia that, if untreated, is fatal (5). Third, heterozygous gene mutations that increase the activity of GK cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI)-GK, a disease that is characterized by clinically significant hypoglycemia (6–8).

The analysis of genetically engineered rodent models has unequivocally established that GK gene expression in both pancreatic β-cells and hepatocytes independently contributes to the maintenance of blood glucose homeostasis (9–14). Increased GK in either cell type leads to more glucose uptake and glucose phosphorylation, whereas diminished GK expression has the opposite effect. In the β cell, these changes impact insulin secretion by altering the threshold for glucose-stimulated insulin secretion. Indeed, in mice with a β cell-specific knock-out of GK, glucose-stimulated insulin secretion is so markedly impaired that death occurs shortly after birth due to severe hyperglycemia. In the liver, alterations in GK activity directly affect rates of glucose uptake and glycogen synthesis. However, the total absence of GK in hepatocytes, unlike β cells, results only in mild hyperglycemia. In contrast to the pancreatic β cell and hepatocyte, our understanding of the role of GK in other sites of expression, such as pancreatic α-cells, gut L- and K-type enterocytes, the pituitary, and certain hypothalamic nuclei of the brain, is less extensive (15–18). However, it is likely that GK also functions as a determinant of glycolytic flux in each of these cell types.

In hepatocytes, GK activity is regulated by both transcriptional and post-translational mechanisms (19–23), the latter of which is dictated by interactions between GK and the GK regulatory protein (GKRP). When blood glucose concentrations are low, GKRP inhibits GK activity by decreasing the affinity for glucose (24). The interaction between GKRP and GK is stimulated by fructose 6-phosphate and inhibited by fructose 1-phosphate (25–29). Moreover, glucose itself directly modulates the interaction between GKRP and GK by competing with fructose 6-phosphate. The binding of GK to GKRP leads to the nuclear compartmentalization of the enzyme (30–34). In addition to sequestering the enzyme away from other glycolytic enzymes, the binding of GKRP to GK may also protect it from degradation (35, 36). This latter notion is supported by studies of GKRP...
null mice, which have a phenotype of diminished hepatic GK activity (35, 36).

The biochemical analysis of missense mutations identified in pedigrees with GK-associated glycemic disorders has provided new insights into the function of this enzyme. These studies have shown that the kinetic characteristics of recombinant glutathione S-transferase (GST)-GK fusion proteins generally correlate with the blood glucose phenotype of the affected pedigree (8, 37, 38). PHHI-GK mutations exhibit a decrease in glucose $S_{0.5}$ and/or an increase in the $K_m$ (6–8, 39). In contrast, mutations from MODY-GK pedigrees are characterized by an increase in glucose $S_{0.5}$ and/or a decrease in $K_m$. However, in some cases, the kinetics of the bacterially expressed mutant enzymes do not exhibit the expected kinetic abnormalities. In these instances, other explanations, such as increased thermostability or loss of regulation by GKRP, need to be invoked in order to explain diminished GK catalysis (4, 40).

To assess whether kinetic measurements alone of bacterially expressed mutant GK enzymes provide sufficient information to predict the blood glucose phenotypes, we generated $gk^{K414E}$ and $gk^{A456V}$ mutant alleles and characterized their $in vivo$ effects. These two particular mutations were chosen because kinetic information is available for both (7, 37). $GK^{K414E}$, which was found in a pedigree with MODY-GK (3), has only a modest impaired relative activity index (GK $K_m$/glucose $S_{0.5}$) (2.5/2.5) and $ATP K_m$ compared with the wild type enzyme of 0.32, as shown in Table 1 (37). By choosing an enzyme with only modest kinetic impairments, we hoped that mice homozygous for this mutation might only be modestly hyperglycemic and therefore viable. On the other hand, we thought that $GK^{A456V}$, which was identified in a patient with PHHI-GK and has a relative activity index of 37.9 (7), would cause marked hypoglycemia in a mouse model, particularly when the mutant allele was present as a homozygous trait.

Surprisingly, both the $gk^{A456V}$ and $gk^{K414E}$ mutant mice were phenotypically different than expected. First, animals that were homozygous for the $gk^{K414E}$ mutation exhibited perinatal lethality, a finding that led us to discover that this mutation results in a thermolabile enzyme. Second, mice that are either heterozygous or homozygous for the $gk^{A456V}$ mutation exhibited only modest hypoglycemia and, surprisingly, exhibited diminished hepatic GK activity. Further analysis revealed that the $gk^{A456V}$ mutant enzyme has a reduced affinity for GKRP, a finding that would explain both the impaired nuclear localization and diminished hepatic GK activity observed in the $gk^{A456V}$ mice. Taken together, these studies indicate that knowledge of how specific point mutations affect GK stability and GKRP binding is essential for accurately predicting $in vivo$ glycemic phenotypes based on the kinetics of bacterially expressed enzymes.

### MATERIALS AND METHODS

**Gene Targeting**—Two gene targeting vectors were made for independently introducing $K414E$ and $A456V$ mutations into the mouse $gk$ gene. The targeting vectors were based on a design that has been described previously (13). PCR-based mutagenesis was performed using the primers 5'-TGGGATGGCCTCCGTGTACGAGCTGCAGCCAGGCTGATCA and 5'-TGACCTCGGGTGCAGCTGGTGACAGGGATCCACACACCATCCCAC to introduce the $K414E$ mutation into exon 9. Similarly, the $A456V$ mutation was introduced into exon 10 using the primers 5'-CTGGTCTCTGGTGATGGCTGCTCAAGAAGGGCTTGCTATG and 5'-CATGCAAAGCTTTGCTTCAGAAGCCACCGCAGAGACCG. Both vectors were linearized with Sall prior to electroporation into a 129S6 mouse ES cell line (13, 41). ES cell clones were selected with 2 μM ganciclovir and 200 μg/ml G418. Chimeric mice were generated by blastocyst microinjections into 3.5-day-old embryos derived from C57Bl/6 mice using clones I7 and 1E10 that bore the $gk^{K414E}$ and $gk^{A456V}$ mutations, respectively. The resulting chimeric mice were bred to either wild type 129S6 mice or to C57Bl/6 animals bearing an EIIa-cre transgene. This resulted in the retention of the pgk-neoR cassette in the 129S6 background and removal of the cassette in the C57Bl/6 background. Offspring from the EIIa-cre transgene matings that did not pass the transgene were subsequently interbred with C57Bl/6J animals for 10 generations to generate a new congenic line for both the $gk^{K414E}$ and $gk^{A456V}$ alleles.

**Mouse Husbandry and Genotyping**—Mice were maintained in a specific pathogen-free state, exposed to a 12-h light/dark cycle, and fed a standard rodent chow (Purina Mills, St. Louis, MO). The mutant $gk$ alleles were distinguished from the wild-type allele by DNA PCR analysis. The $gk^{K414E+ neo}$ and $gk^{A456V+ neo}$ alleles were detected using primer 1 (5'-TGTCCTCAATTGTGCTGTCCTCCA and 5'-AGCCCTCTGTCTCACTACACTGCTTCA), which amplifies a 725-bp fragment of pgk-neo cassette. The wild-type $gk$ allele ($gk(W)$) was detected as a 636-bp fragment using primer 1 and 5'-ATGGTGAGCTGTCACATGAGGAGT.

**Analytical Procedures**—Intraperitoneal glucose tolerance tests were performed on 8–28-week-old mice fasted for 6 h (8 a.m. to 2 p.m.). Animals were injected intraperitoneally with glucose (1 g/kg of body weight). Whole blood was collected from the saphenous vein, and blood glucose was measured using a Hemocue analyzer. Plasma insulin was measured using a sensitive rat insulin radioimmunoassay (RIA) kit (Linco, St. Charles, MO). Plasma glucagon was measured using glucagon RIA kit (Linco). Plasma triglyceride concentrations were measured using a Triglycerides GPO reagent kit (Raichem, San Diego, CA) following a 6-h fast.

**Glucose Ramp Studies**—Islets were isolated by collagenase digestion and cultured for 3 days in RPMI 1640 medium containing 10 mM glucose as previously described (42). The culture medium was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 50 μg/ml streptomycin.

### TABLE 1

| Kinetic constants of bacterially expressed GST fusion proteins |
|---------------------------------------------------------------|
| All values were derived from studies of the human β cell isom | r as a GST-GK fusion protein (7, 37). Means ± S.E. are shown. |
| Parameter                   | Wild type | GST-GK$^{A456V}$ | GST-GK$^{K414E}$ |
| $k_m$ (s$^{-1}$)            | 53.2 ± 0.97 | 84.9 ± 4.5 | 11.0 ± 3.33 |
| Glucose $S_{0.5}$ (mmol/liter) | 8.04 ± 0.28 | 2.53 ± 0.10 | 12.3 ± 3.30 |
| pH (unitless)               | 1.67 ± 0.04 | 1.21 ± 0.03 | 1.67 ± 0.11 |
| $ATP K_m$ (mmol/liter)      | 0.39 ± 0.02 | 0.29 ± 0.03 | 1.73 ± 0.22 |
| Relative activity index     | 1.0       | 37.9       | 0.32       |
| (unitless)                  |           |            |            |
and the islets were incubated at 37 °C in a 5% CO₂, 95% air-humidified incubator. Batches of 100 cultured mouse islets were loaded onto a nylon filter in a chamber and perfused with Krebs-Ringer bicarbonate buffer (115 mmol/liter NaCl, 24 mmol/liter NaHCO₃, 5 mmol/liter KCl, 1 mmol/liter MgCl₂, 2.5 mmol/liter CaCl₂, 10 mM HEPES, pH 7.4) with 0.25% bovine serum albumin at a flow rate of 2 ml/min. Perifusate solutions were gassed with 95% O₂, 5% CO₂ and maintained at 37 °C. Samples were collected every min for insulin assays. Insulin was measured by RIA.

Real Time PCR—Total RNAs were extracted from liver (8–12-week-old mice) using a GenElute Mammalian Total RNA Miniprep kit (Sigma). The total RNAs were treated with RNase-free DNase (Promega, Madison, WI) and then converted into cDNA using a Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR quantification of gene expression was performed using SYBR green (Applied Biosystems) on an Applied Biosystems Prism 7000 thermocycler, and gene expression was calibrated against 18 S RNA. The GK primers were 5′-CACATGAT-CTCTCTGCTACT and 5′-TTCTGATCCTCCTCAGTGA. The GKRPr primers were 5′-GTGAGCATGCCAGAAATGAT and 5′-GCATCCGCTCTCCAGTTT.

Immunoblot Analysis—Animals were anesthetized with 40 mg of Nembutal per kg of body weight and then killed by decapitation. The livers were quickly removed and frozen in liquid nitrogen. 0.2 g of frozen liver tissue was homogenized in 1 ml of lysis buffer (50 mM triethanolamine-HCl, pH 7.3, 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, and 1 pellet of protein inhibitors/10 ml of lysis buffer (Roche Applied Science)). Homogenates were incubated with 200 μl of 25% polyethylene glycol (M, 3350; Sigma) on ice for 15 min. Clarified lysates were obtained by centrifugation at 12,000 rpm for 15 min, and liver extracts were frozen at −70 °C. Islets of Langerhans were isolated by a collagenase technique as described (43), hand-picked, and then sonicated in 50 μl of lysis buffer. Protein concentrations were measured by Bio-Rad protein assay. 10 μg of liver protein extracts and 1 μg of islet protein extracts were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were incubated with the following primary antibodies: sheep anti-GST fusion GK (1:10,000 dilution) (44), rabbit anti-GKRP (1:40,000 dilution) (45), and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (1:80,000 dilution; Abcam) overnight at 4 °C. Membranes were incubated with the following secondary antibodies: Alexa Fluor 680 donkey anti-sheep, Alexa Fluor 680 goat anti-rabbit (1:40,000 dilution; Molecular Probes, Inc., Eugene, OR), and IRDye 800 donkey anti-mouse (1:40,000 dilution; Rockland, Gilbertsville, PA). Visualization and quantification of the immunolabeled proteins was accomplished using the Odyssey™ infrared imaging system (Li-Cor, Lincoln, NE), which measures integrated pixel intensity.

Glucokinase Kinetics—Approximately 200 mg of frozen liver was homogenized in 2 ml of buffer consisting of 50 mM/liter HEPES, 100 mM/liter KCl, 1 mM/liter EDTA, 5 mM/liter MgCl₂, and 2.5 mM/liter dithiothreitol (46). Homogenates were centrifuged at 100,000 × g for 45 min to sediment the microsomal fraction. GK activity was measured in the cytosolic fraction. The supernatants were analyzed for GK in a medium (pH 7.4 at 37 °C) containing 50 mM/liter HEPES, 100 mM/liter KCl, 7.5 mM/liter MgCl₂, 5 mM/liter ATP, 2.5 mM/liter dithiothreitol, 0.5 (for hexokinase), 7.5 (for GK), or 100 mM/liter glucose (total phosphorylating activity), 0.5 mM/liter NAD⁺, and 4 units of glucose-6-phosphate dehydrogenase (Sigma). The reaction was initiated by the addition of ATP, and the reduction of NAD⁺ was assessed by measuring absorbance at 340 nm after 10, 20, and 30 min of incubation time. GK activity (Vₘₐₓ) was determined as the change in absorbance between 10 and 30 min at 100 mM glucose minus the absorbance at 0.5 mM glucose (which measures hexokinase activity). The relative GK activity ratio was determined as the absorbance change between 0.5 and 7.5 mM glucose divided by the GK Vₘₐₓ and normalized to wild type values.

Enzyme Stability Measurements—Thermolability of the GK mutants K414E, A456V, and E300K and wild type GST-GK fusion protein was assessed using protocols previously described (40, 47). The ability of glucose to stabilize GK (48) was measured by incubation with 0 to ~100 mM glucose while maintaining protein concentrations of ~75 μg/ml. GK activity was then determined after incubation at 42.5 °C for 30 min. A GKRP inhibition assay was carried out as previously described (37) using 11.1 mM wild type or mutant GK with glucose at the inflection point of each enzyme so that glucose concentrations are equivalent. Glucose concentrations were 3.0 mM for wild type GK, 2.75 mM for K414E, and 0.612 mM for A456V. ATP concentrations were 1 mM for wild type GK, 2.3 mM for GK-K414E, and 0.61 mM for GK-A456V.

Pancreatic Insulin Content—Pancreata were removed from 20–30-week-old mice, weighed, and homogenized for 45 s with 3 ml of cold acid/ethanol buffer (1% HCl and 75% EtOH). The Polytron probe was rinsed with 3 ml of acid/buffer, and the two buffers were combined and incubated overnight at 4 °C. Lysates were centrifuged at 2400 rpm for 30 min, and the supernatants were put into a 15-ml tube. 1 ml of acid/ethanol buffer was added to the pellet and homogenized for 45 s. The lysate was incubated for 2 h at 4 °C. Centrifugation was performed as before as well as homogenization. The supernatants were combined and frozen at −70 °C. Insulin was measured using a sensitive rat insulin radioimmunoassay kit (Linco, St. Charles, MO).

Histological Analysis—Pancreas was fixed overnight, and liver tissues were fixed for 3 h in 4% paraformaldehyde and processed as described (44). A sheep anti-GST-GK fusion protein antibody (1:1000 dilution) (44) and either a guinea pig anti-human insulin or rabbit anti-glucagon (1:5000; Linco) were used as primary antibodies. A donkey anti-sheep antibody conjugated to biotin, Cy2-labeled donkey anti-guinea pig, or Cy3-labeled donkey anti-rabbit antibodies (1:1000 dilution; Jackson ImmunoResearch, West Grove, PA) were used to detect binding of the primary antibodies. Hepatic GK was detected by a peroxidase system (R.T.U. Vectastain Elite ABC reagent and peroxidase substrate kit diaminobenzidine; Vector Laboratories, Inc.). Transmitted light and fluorescence images were captured using a Zeiss microscope equipped with a high resolution CCD camera.
Statistical Analysis—All results are presented as the means ± S.E. Student's t test was used to test for statistical significance. A p value of less than 0.05 was considered statistically significant.

RESULTS

Generation of MODY-GK (gkK414E/W) and PHHI-GK Mice (gkA456V/W)—Gene targeting was used to introduce either a K414E mutation into exon 9 or an A456V mutation into exon 10 of the mouse gk gene (Fig. 1A). Southern blot (Fig. 1B) and DNA sequence analysis of genomic DNA (Fig. 1C) were performed to confirm that the desired homologous recombination events were correct and the point mutations had been achieved. Chimeric mice generated by blastocyst microinjection were then either crossed with 129S6 to directly place both alleles into an inbred background or mated with EIIa-Cre transgenic mice to remove the neomycin resistance (neoR) cassette and then back-crossing the mutant gk alleles for at least 10 generations to create C57Bl/6J congenic lines. Removal of the neoR cassette was confirmed with PCR analysis (Fig. 1D). The gkK414E/W, gkA456V/W, and gkA456V/A456V mice were viable and had similar plasma triglycerides, free fatty acid levels, and similar or slightly lower body weight when compared with control mice (Table 2). The gkK414E/W mice died within a few days after birth of severe hyperglycemia, consistent with prior gk knock-out studies.

Fasting Blood Glucose and Insulin Levels and Glucose Tolerance Tests of gkK414E/W and gkA456V/W Mice—To assess the effect of the two different genetic mutations in an in vivo setting, fasting plasma glucose concentrations were determined. In both 129S6 and C57Bl/6J genetic backgrounds, the gkK414E and gkA456V mutations caused mild hyperglycemia and hypoglycemia, respectively (Fig. 2A). Four-week-old, fasted gkK414E/W and gkA456V/W mice had an average increase of 43.8% and a decrease of 23.0% in blood glucose concentrations, respectively (Fig. 2A). Basal blood glucose levels of adult
Glucose homeostasis of mutant GK mice. A, blood glucose levels were measured in two genetic backgrounds, C57BL/6J (which lacks the pgk-neo cassette) and 129S6 (which retains the pgk-neo cassette). Four-week-old, 6-h fasted male and female mice were used (gk\(^{K414E}\)-neo/w, n = 10; gk\(^{K414E}\)-neo/W, n = 17; gk\(^{A456V}\)-C57BL/6J, n = 6; gk\(^{A456V}\)-129S6, n = 32; gk\(^{A456V}\)-neo/w, n = 19; gk\(^{A456V}\)-neo/W, n = 15). B, glucose levels of 6-h fasted mutant GK-129S6 mice at 4–32 weeks of age were determined (n = 4–15). C, a glucose tolerance test was performed in 6-h fasted adult 129S6 mice (n = 6 for wild type; n = 5 mutant mice). D, plasma insulin levels after glucose injection were measured in the same mice used in B, 15 min. E, insulin/glucose ratios were calculated from glucose tolerance test experiments shown in C and insulin measurements shown in D. F, insulin/glucose ratios of 6-h fasted adult 129S6 mice (n = 5 for wild type, n = 5 for heterozygous, n = 4 for homozygous mice). **, p < 0.01; *, p < 0.05 as determined using an unpaired two-tailed t test comparing mutant versus control animals.

Intraperitoneal glucose tolerance tests were performed in fasted adult mice. The ability to regulate blood glucose levels in response to a glucose challenge was impaired in gk\(^{K414E}\)-W/W mice but was normal in gk\(^{A456V}\)-W/W mice (Fig. 3C). Basal and glucose-stimulated plasma insulin levels were not significantly different between the gk\(^{K414E}\)-W/W and gk\(^{A456V}\)-W/W mice when compared with wild type mice (Fig. 2D). Intraperitoneal glucose tolerance studies indicated that the acute insulin secretory response to glucose was not affected in either gk\(^{K414E}\)-W/W or gk\(^{A456V}\)-W/W mice (Fig. 2D, 15 min). However, the ratio between plasma insulin and blood glucose levels was significantly lower after a glucose challenge in gk\(^{K414E}\)-W/W mice when compared with wild type mice throughout the 120-min period (Fig. 2E). In contrast, the ratio between plasma insulin concentrations and blood glucose levels was significantly higher in gk\(^{A456V}\)-W/W mice compared with controls prior to the glucose challenge (Fig. 2E, 0 min). Despite lower blood glucose levels, the gk\(^{A456V}\)-W/W mice had similar plasma insulin levels when compared with wild type mice (0.32 ± 0.03 versus 0.36 ± 0.06 ng/ml) and lower insulin levels than gk\(^{A456V}\)-W/W mice (0.45 ± 0.05 ng/ml). Overall, basal insulin/glucose ratios of gk\(^{A456V}\)-W/W mice were similar to gk\(^{A456V}\)-W/W mice (5.6 × 10\(^{-4}\) ± 3.8 × 10\(^{-5}\) versus 5.6 × 10\(^{-4}\) ± 2.5 × 10\(^{-5}\), respectively) (Fig. 2F).

In Vitro Studies of Glucose-stimulated Insulin Secretion in Islets—To assess the effects of the GK point mutations on islet function, we compared the ability of glucose to stimulate insulin secretion from islets isolated from gk\(^{K414E}\)-W/W, gk\(^{A456V}\)-W/W, and gk\(^{A456V}\)-A456V mice. Isolated islets were treated with a 0–25 mM glucose ramp, which elicited a significant insulin release at 6.0 mM glucose in control islets (Fig. 4A). In contrast, islets from gk\(^{A456V}\)-W/W and

**TABLE 2**

Metabolic parameters

|                | gk\(^{W}\)/W | gk\(^{K414E}\)/W | gk\(^{A456V}\)/W | gk\(^{A456V}\)/A456V |
|----------------|------------|----------------|----------------|-------------------|
| Body weight (g) | 25.7 ± 0.5 | 25.1 ± 0.7     | 25.7 ± 1.0     | 23.7 ± 0.5*       |
| Plasma triglycerides (mg/dl) | 89.6 ± 5.5 | 95.4 ± 6.2     | 82.8 ± 9.8     | 90.5 ± 5.7       |
| Plasma free fatty acid (mmol/liter) | 0.44 ± 0.02 | 0.43 ± 0.04    | 0.45 ± 0.04    | 0.43 ± 0.03      |

* Only male mice were used.
**p < 0.005 when compared with wild type mice.
GK A456V/A456V mice were more sensitive to glucose and secreted insulin in response to 2.5 and 4.0 mM glucose, respectively (Fig. 4A). On the other hand, GK K414E/W mice were less sensitive to glucose and secreted insulin in response to 8.0 mM glucose (Fig. 4A).

Hepatic GK Expression, Activity, and Localization in gk A456V/W, gk K414E/W, and gk A456V/A456V Mice—In addition to being essential for glucose-induced insulin secretion in the islets, GK also determines the rate of glucose utilization and glycogen synthesis in hepatocytes (13, 49). As previously shown in transgenic mice with increased GK gene copy number, an increase in GK activity causes an increase in whole body glucose turnover rates (11, 49, 50). Conversely, a reduction in hepatic GK activity leads to a decrease in hepatic glucose uptake (13). To explore the effects of the mutant enzymes on hepatic function, we first measured hepatic GK activities in fasted adult mice. As expected, GK activity was reduced by 43.0% in gk K414E/W mice (0.0138 ± 0.002 versus 0.0242 ± 0.009 μmol/mg of protein/min of control livers) (Fig. 3A). Surprisingly, GK activity was also found to be reduced by 38.0 and 71.9% in gk A456V/W and gk A456V/A456V mice, respectively (0.015 ± 0.001 and 0.0068 ± 0.0004 μmol/mg of protein/min, respectively). Confirmation of a reduced mutant GK activity was obtained by measuring the GK activity ratio between the phosphorylation rate at 7.5 and 100 mM glucose. The ratio of hepatic GK activity relative to wild type mice was similar in the gk K414E/W, gk A456V/W, and gk A456V/A456V animals (1.08 ± 0.08, 1.15 ± 0.03, and 1.42 ± 0.27, respectively, versus 1.00 ± 0.13 of gk W/W) (Fig. 3B). To assess whether decreased GK activity coincided with a reduction in GK mass, Western blot analysis was performed. Extracts from the livers of fasted gk K414E/W and gk A456V/W mice had 50 and 33% less GK protein, respectively, when compared with control extracts (0.5 ± 0.06 of gk K414E/W and 0.67 ± 0.09 of gk A456V/W versus 1.0 ± 0.06 of gk W/W) (Fig. 3C). Reduced GK A456V levels were confirmed by a larger decrease in protein levels found in gk A456V/A456V mice (0.37 ± 0.04; Fig. 3C). GK mRNA levels were similar among mutant mice (Fig. 3D).

GKRP is an important intracellular regulator of hepatic GK activity. Thus, to determine whether GKRP expression was...
altered in the GK mutant mice, GKRP protein levels were determined by Western blot analysis and found to be similar in gK414E/W, gK456V/W, and gK414E/A456V mice when compared with wild type controls (Fig. 3E). There was a small but significant decrease in GKRP mass in gK456V/A456V mice when compared with gK456V/W mice (1.30 ± 0.2 of gK456V/A456V versus 0.82 ± 0.1 of gK456V/W) (Fig. 3E). GKRP mRNA levels were also similar among all groups of mice (Fig. 3F). Thus, these results indicate that the reduction of mutant GK activity and protein levels cannot be explained by a reduction in GKRP.

Because of the critical role that the interaction between GKRP and GK plays in the liver, we also analyzed the intracellular localization of GK and GKRP in hepatocytes from animals with the various mutant alleles by immunohistochemistry. As expected, both GK and GKRP were identified in the nuclei of the fasted animals (Fig. 5). However, the amount of nuclear GK immunoreactivity in mice with two gK456V mutant alleles was visibly lower than that of the control animals (Fig. 5, A versus D), consistent with the reduction of both GK activity and mass by activity measurements and Western blot analysis.

To explore why there was a reduction in GK protein, we measured the thermolability of both GK414E and GK456V when expressed in bacteria as GST-GK fusion proteins. GST-GK456V had an activity profile similar to or higher than the wild type GST-GK enzyme as the temperature was increased, and the activity was abruptly decreased when the temperature reached 47.5 °C (Fig. 6A). In contrast, GST-GK414E was found to be as thermolabile as GST-GK456V, a known thermolability mutant (Fig. 6A). In addition, the stabilizing effect of glucose on mutant and wild type GST-GK proteins was also determined at 42.5 °C, which is the highest temperature at which there is the least variation of Kcat between the wild type and mutant enzymes. As shown in Fig. 6B, decreasing the concentrations of glucose slightly reduced the activity of both the mutant and wild type GST-GK enzyme. In contrast, GST-GK456V had a markedly reduced GK activity when glucose was reduced (Fig. 6B). Furthermore, we also determined the ability of human GKRP to inhibit the activity of the mutant enzymes. Both wild type GST-GK and GST-GK414E showed a reduction in activity with increasing concentrations of human GKRP with and without sorbitol 6-phosphate (S-6-P) (Fig. 6, C and D). However, GST-GK456V was less responsive to the inhibition by GKRP (Fig. 6, C and D). Given that GK and GKRP must bind to each other in order for nuclear localization to occur, this finding indicates that the A456V mutation disrupts binding to the regulatory protein and provides an explanation for the impaired nuclear GK localization that was observed in both the gK456V/W and gK456V/A456A mice.

Islet GK Expression, Morphology, and Insulin Content Measurements in gK414E/W and gK456V/W Mice—
To determine whether the reduction of mutant GK expression occurs only in liver, Western blot analysis of protein extracts from isolated islets was performed. The gK414E/W mice had lower islet GK protein levels, consistent with diminished stability of this mutation, but GK protein levels of isolated islets from gK456V/A456V mice were similar to those of control...
islets (Fig. 4D). Finally, to assess whether either GK mutation altered morphology of islets, immunohistochemical staining for insulin, glucagon, somatostatin, and pancreatic polypeptide was performed using adult pancreatic tissues from animals that had been fed a normal diet. As shown in Fig. 4B, islet architecture appeared to be normal insofar as glucagon-expressing cells were located at the islet periphery and insulin-expressing cells were found in the core of islets. Moreover, total insulin content levels of gk-K414E/W and gk-A456V/W pancreas were similar to those of the control (Fig. 4C). These results indicate that both insulin content and islet structure were unaffected in the two proteins in the liver. Although mice that are homozygous for the gk-K414E allele died shortly after birth of severe hyperglycemia, thereby mimicking both the two reported cases of PND-GK in humans and prior global GK knock-out mice. Since the gk-K414E mutation selected for this study appeared only to modestly alter the kinetics of GK, the perinatal lethality suggested that this mutation was having additional effects that were not made evident by simply assessing enzyme kinetics. For this reason, we assessed the thermal stability of this mutation and discovered that the enzyme was just as labile as GST-GKE300K, a known thermolability mutant.

Mice carrying a single gk-A456V mutant allele exhibited hyperglycemia that, based on the fact that the animals were viable, appears to be comparatively less severe than the phenotypic consequences of PHHI-GK in humans (6–8, 39). Although the basis for this difference needs to be explored further, there are a number of species-specific differences that could contribute. For instance, there is only 88% similarity between human and rodent GKRP, whereas GK itself is highly conserved (51). Moreover, human GKRP is a more potent inhibitor of GK than the rodent isoform and also has a higher affinity for ligands, such as fructose 6-phosphate, that modulate the interaction of these two proteins in the liver. Although mice that are homozygous for the gk-A456V mutant allele are only modestly more hyperglycemic than animals with a single mutant allele, it is not possible to extrapolate this finding to humans, since a case report of a human with biallelic activating mutations of GK has not been reported.

It is widely thought that changes in insulin secretion are the root cause of both the hyperglycemia and hypoglycemia in both MODY-GK and PHHI-GK. However, mice with either the gk-K414E or gk-A456V mutation exhibited basal plasma insulin levels that differed only slightly from the control animals. Although there was a significant difference in the ratios of the plasma insulin and blood glucose levels, our measurements of basal insulin concentrations were not statistically different and showed only a trend toward an increase in plasma insulin in gk-A456V/W mice and a small decrease in plasma insulin in gk-K414E/W mice. Despite the similar basal insulin concentrations, we deduce that there is a small difference in the basal plasma insulin concentrations based on the leftward and rightward shifts of the insulin secretion thresholds observed using islets from the gk-A456V and gk-K414E mutant mice, respectively.

Previously, we also observed normal plasma insulin levels in mice that had an additional copy of the entire gk gene locus as a P1 phase-derived transgene (49). However, these animals had a reduction in the levels of islet GK protein. Thus, at the time we speculated that the lack of a change in the plasma insulin concentration may have been the result of hypoglycemia-induced

**DISCUSSION**

The goal of this study was to assess whether the kinetic measurements of bacterially expressed mutant GK enzymes provide sufficient information to accurately predict the blood glucose phenotype. To do so, we generated and characterized mice that either contain a gk-A456V mutation that activates GK or a gk-K414E mutation that impairs GK activity. We addressed the issue of genetic background, which has previously made it difficult to perform side-by-side comparisons, by breeding mice containing the mutant alleles directly into a 129S6 genetic strain to generate two new inbred lines or by back-crossing variants that lacked a downstream neo cassette into animals of the C57Bl/6J strain to create two congenic lines. By carefully controlling the genetic backgrounds, we were able to more accurately assess the physiological impact of each point mutation.

**Blood Glucose Phenotypes**—As expected, mice bearing a single copy of the gk-K414E mutant allele exhibited modest hyperglycemia, thereby mimicking MODY-GK in humans. Mice that were homozygous for the gk-K414E allele died shortly after birth of severe hyperglycemia, thereby mimicking both the two reported cases of PND-GK in humans and prior global GK knock-out mice. Since the gk-K414E mutation selected for this study appeared only to modestly alter the kinetics of GK, the perinatal lethality suggested that this mutation was having additional effects that were not made evident by simply assessing enzyme kinetics. For this reason, we assessed the thermal stability of this mutation and discovered that the enzyme was just as labile as GST-GKE300K, a known thermolability mutant.

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Mice with Glucokinase K414E and A456V Mutations
down-regulation of pancreatic GK (49). However, in this study, we isolated islets and directly measured the amount of islet GK and found that islet GK is expressed at similar levels in both the controls and gkA456V animals. Hence, the lower plasma glucose levels observed in heterozygous and homozygous gkA456V mice may also reflect an increase in glucose uptake by insulin-sensitive tissues, such as muscle and fat. However, the marked down-regulation of hepatic GK in these animals suggests that the liver is not a major site of glucose uptake.

Islet Phenotypes—Individuals with MODY-GK have a defect in glucose-stimulated insulin release and exhibit a rightward shift in the glucose concentration-response curve (52). Previously modeling predicted both a rightward and a leftward shift of the glucose-stimulated insulin release threshold from islets containing mutant GK^K414E^ and GK^A456V^, respectively (7, 37, 53). The perfusion studies we have performed here using isolated islets have validated this prediction by clearly demonstrating that islets from gk^K414E/W^ mice are less sensitive to glucose stimulation, whereas both gk^A456V/W^ and gk^A456V/A456V^ islets are markedly more sensitive.

Some PHHI patients have been reported to have abnormally large and hyperfunctional islets in the setting of severe hypoglycemia (39). However, immunohistochemical analysis of adult pancreas of gk^A456V/W^ mice demonstrated that these mice have normal islet morphology. In addition, total insulin content levels of gk^A456V/W^ pancreas are similar to those of control pancreas. The less severe hypoglycemic phenotype of gk^A456V/W^ mice might partially explain why islet morphology and insulin content are within the normal range.

Liver Phenotypes—As expected, the gk^K414E/W^ mouse had a decrease in hepatic GK activity. However, based on the relative GK activity ratios, the wild type enzyme appears to be the predominant form present in these animals. This would only be expected if the mutant enzyme were less stable and did not accumulate to any significant degree. Surprisingly, both the gk^A456V/W^ and gk^A456V/A456V^ mice also had a reduced hepatic GK activity and similar relative GK activity ratios when compared with wild type mice. Since the gk^A456V/A456V^ mouse should have exhibited a higher relative GK activity ratio than the wild type animals, it is possible that measurements were not entirely accurate due, perhaps, to the very low activity values.

In any case, the most surprising finding of these studies is the marked down-regulation of both hepatic GK activity and mass in gk^K414E/W^ and gk^A456V/W^ mice. There are at least two mechanisms by which this could occur. First, GK^A456V^ might be an unstable protein. Indeed, previous studies of bacterially expressed mutant GST-GK fusion proteins have clearly shown that some are thermolabile (4, 37, 47, 54). However, the thermolability studies showed that GST-GK^K414E^ mutant is less stable than the wild type enzyme, GST-GK^A456V^ was found to be as stable as the wild type enzyme. Moreover, there was a similar amount of the GK^A456V^ enzyme in islets isolated from both the heterozygous and homozygous gk^A456V^ mutant animals. Thus, this mechanism fails to explain the reduced expression of GK in the livers of gk^A456V^ mutant animals.

Second, the down-regulation of hepatic GK^A456V^ may be caused by disruption of the interaction between GKRP and GK. Indeed, studies of GKRP knock-out mice have shown that GKRP plays an important role in maintaining normal amounts of hepatic GK. Mice lacking both GKRP alleles have more than 65% reduction of GK (35, 36). However, in our studies, the amount of GKRP was not unaffected. Thus, a reduction of GKRP itself cannot be invoked as the cause of the reduced hepatic GK protein levels. Rather, we suggest that an alteration in the binding of GK to GKRP is the principal cause of the reduction in hepatic GK.

At least three factors must be considered when attempting to explain diminished interaction between GK and GKRP proteins. First, the intracellular concentrations of glucose, fructose 1-phosphate, and fructose 6-phosphate are known to play key roles. However, although knowledge of the concentrations of these intracellular metabolites would be helpful in understanding the observed phenotype, they are not readily measured in animal models. Second, the affinity of the GK interaction with GKRP is also a key factor. Previously, the inhibitory effect of GKRP on GST-GK^A456V^ activity was found to be similar to that of wild type GK (7). Our experiments indicate that the GST-GK^A456V^ mutant is less responsive to human GKRP in the absence and presence of sorbitol 6-phosphate than either the wild type enzyme or the GK^K414E^ mutant. Indeed, while this manuscript was under review, Heredia et al. (55) reported that binding of GK^A456V^ to GKRP is reduced by 76-fold. Third, the conformational state of GK should also be considered. Structural studies of GK^A456V^ have shown that the mutated residue is located in the allosteric activating domain of the protein (7). Given that the V62M mutation, which is also located in the allosteric activating domain, has been shown to diminish the interaction of GK with GKRP, the conformation of the enzyme is likely to be another factor that affects binding of GK to GKRP (40). Indeed, since GK can exist in either an open or closed form, and since the activated enzyme exists predominantly in the closed form, the conformational state of the enzyme may also directly affect the binding of GK to GKRP.

Although our knowledge of how GKRP regulates GK in the liver is incomplete, these studies provide additional evidence that the interactions between these two proteins are important for modulating the amount of GK in the liver. Indeed, our results, as well as those obtained previously from the analysis of GKRP knock-out mice, clearly indicate that the binding of GK to GKRP (which is readily apparent by the nuclear localization of GK) and the amount of GK in the hepatocyte are correlated (35, 36). When this protein-protein association is impaired, either by the absence of GKRP or metabolic and/or kinetic alterations of GK, the amount of GK in the liver is significantly lowered. Moreover, since changes in the amount or kinetics of GK have a direct effect on glucose uptake and utilization (13, 49) and since the binding of GK to GKRP is influenced by key intracellular metabolites, it is possible that the regulated binding of these two proteins creates a feedback loop whose role is to help modulate the amount of hepatic GK activity by altering the rates of protein turnover.

Conclusions—By introducing two human disease-associated GK mutations into the mouse, we have gained a new appreciation of the key role that both GK stability and GKRP binding play in modulating GK activity and how this consequently affects the blood glucose concentration. The gk^K414E^ mutation
causes hyperglycemia because of a decrease in GK activity in both the islet and liver for which there are no effective compensatory mechanisms. On the other hand, the \(g^a456v\) mutation causes hypoglycemia because of increased GK activity in the islet, which leads to a leftward shift in the threshold for glucose-stimulated insulin secretion. However, down-regulation of the amount of hepatic GK, due to a decreased interaction between GK and GKRP, appears to greatly attenuate the impact of this mutation, diminished nuclear sequestration of GK due to this mutation, and hepatic GK appears to increase enzyme turnover, therefore resulting in lower than expected hepatic GK activity. Moreover, we extrapolate from these studies that the blood glucose phenotype of PHHI-GK patients is mainly important. Thus, the availability of genetically defined animals, such as mice with Glucokinase K414E and A456V Mutations, may be invaluable for eliciting the mechanisms by which glucose metabolism is modulated in modulating \(\beta\) cell mass in response to insulin resistance produced by high fat feeding (56).

Finally, we anticipate that these mice will be useful for a variety of additional studies. Indeed, a recent report relied on mice that were haploinsufficient for the \(\beta\) cell HK isoform to identify a dominant role for glucose in modulating \(\beta\) cell mass in response to insulin resistance produced by high fat feeding (56).

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