

# Susceptibility of Glucokinase-MODY Mutants to Inactivation by Oxidative Stress in Pancreatic $\beta$ -Cells

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**OBJECTIVE**—The posttranslational regulation of glucokinase (GK) differs in hepatocytes and pancreatic  $\beta$ -cells. We tested the hypothesis that GK mutants that cause maturity-onset diabetes of the young (GK-MODY) show compromised activity and posttranslational regulation in  $\beta$ -cells.

**RESEARCH DESIGN AND METHODS**—Activity and protein expression of GK-MODY and persistent hyperinsulinemic hypoglycemia of infancy (PHHI) mutants were studied in  $\beta$ -cell (MIN6) and non- $\beta$ -cell (H4IIE) models. Binding of GK to phosphofructo-2-kinase, fructose-2,6-bisphosphatase (PFK2/FBPase2) was studied by bimolecular fluorescence complementation in cell-based models.

**RESULTS**—Nine of 11 GK-MODY mutants that have minimal effect on enzyme kinetics in vitro showed decreased specific activity relative to wild type when expressed in  $\beta$ -cells. A subset of these were stable in non- $\beta$ -cells but showed increased inactivation in conditions of oxidative stress and partial reversal of inactivation by dithiothreitol. Unlike the GK-MODY mutants, four of five GK-PHHI mutants had similar specific activity to wild type and Y214C had higher activity than wild type. The GK-binding protein PFK2/FBPase2 protected wild-type GK from oxidative inactivation and the decreased stability of GK-MODY mutants correlated with decreased interaction with PFK2/FBPase2.

**CONCLUSIONS**—Several GK-MODY mutants show posttranslational defects in  $\beta$ -cells characterized by increased susceptibility to oxidative stress and/or protein instability. Regulation of GK activity through modulation of thiol status may be a physiological regulatory mechanism for the control of GK activity in  $\beta$ -cells. *Diabetes* 60:3175–3185, 2011

**G**lucokinase (GK) plays a central role in blood glucose homeostasis serving as the glucose sensor for glucose-stimulated insulin secretion (GSIS) by the pancreatic  $\beta$ -cell and as the major regulator for glucose uptake by hepatocytes (1–3). Studies using murine tissue-specific knockout models established that both hepatic and pancreatic  $\beta$ -cell GK contribute to blood glucose homeostasis (4,5). However, although liver-specific knockout causes mild hyperglycemia, the  $\beta$ -cell GK knockout model is phenotypically similar to the global GK knockout with severe hyperglycemia and neonatal mortality, indicating a critical role for GK in blood glucose homeostasis in murine models (4,5).

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The role of GK in blood glucose homeostasis in man is supported by the naturally occurring mutations that cause diabetes or hyperinsulinemia. Heterozygous mutations that decrease enzyme activity cause maturity-onset diabetes of the young (GK-MODY) characterized by mild hyperglycemia (6), whereas homozygous or compound heterozygous mutations manifest as the more severe phenotype of permanent neonatal diabetes mellitus (7). In contrast, heterozygous mutations that increase enzyme activity cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (8). Despite the small number (<15) of identified mutations in PHHI (9), the clinical phenotype is widely heterogeneous ranging from mild to severe hypoglycemia (9,10). This phenotype variability can be largely explained by the effect of the mutation on enzyme kinetics determined in vitro on purified protein (9,10). In contrast, the GK-MODY phenotype is fairly homogeneous (9,11) despite the diverse effects on enzyme kinetics ranging from defects in maximal activity, affinity for glucose and/or ATP through to minimal changes in enzyme activity (9,12). Detailed kinetic analysis represents only one aspect of the complex regulation of GK. Other factors relating to the  $\beta$ -cell environment must be considered to explain the effect of some mutants in vivo. Analysis of these mutants in a cell-based model offers a useful tool to investigate these mechanisms (13).

Regulation of GK at the posttranslational level differs in the liver and pancreatic  $\beta$ -cells (14). In hepatocytes, GK is regulated by binding to the liver regulatory protein (GKRP) (15), which modulates GK activity by sequestration of inactive GK in the nucleus in the fasted state and release of active GK into the cytoplasm in the fed state (16). Binding of GK to GKRP also stabilizes the protein as shown by decreased liver protein expression in the GKRP-null mice (17). The posttranslational regulation of GK in pancreatic  $\beta$ -cells is less well understood.  $\beta$ -Cells do not express liver GKRP, and accordingly GK does not localize to the nucleus (16). GK activity/localization in  $\beta$ -cells is regulated by binding to other interacting partners, including neuronal nitric oxide (NO) synthase (18), the proapoptotic factor BAD (19), and the bifunctional enzyme phosphofructo-2-kinase, fructose-2,6-bisphosphatase (PFK2/FBPase2) (20). The importance of these interactions in the posttranslational regulation of GK activity remains to be fully elucidated.

In the current study, we used 11 MODY mutants that have minimal effect on enzyme kinetics in vitro, to investigate the cellular regulatory mechanisms of GK in  $\beta$ -cells and to test the hypothesis that GK-MODY mutants show compromised activity in pancreatic  $\beta$ -cells.

## RESEARCH DESIGN AND METHODS

**Reagents.** The pCMV-Tag3C vector and site-directed mutagenesis kits were obtained from Agilent Technologies (Berksire, U.K.); jetPEI-Hepatocyte was from MP Biomedicals (Solon, OH); GK activator (21) and its stereoisomer were from Axon MedChem (Groningen, the Netherlands); Lipofectamine 2000,

monochlorobiamine (MCB), 4-amino-5-methylamino-2',7'-difluoro fluorescein diacetate (DAF-FM diacetate), and 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) were from Invitrogen (Paisley, U.K.); myc-antibody (910E) was from Santa Cruz Biotechnology (Santa Cruz, CA); fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody was from Jackson ImmunoResearch (West Grove, PA); GK antibody was from Mark Magnuson (Vanderbilt University School of Medicine, Nashville, TN) (22); FBPAse2 vector was from Alex Lange (University of Minnesota Medical School, Minneapolis, MN); and the yellow fluorescent protein (YFP)-fragment vectors were from Tom Kerppola (University of Michigan Medical School, Ann Arbor, MI). All other reagents were from Sigma-Aldrich (Poole, U.K.).

**Generation of myc-GK constructs.** An NH<sub>2</sub>-terminal myc-tag was added to human pancreatic  $\beta$ -cell GK (wild-type [WT] and mutant) by excision of GK from the pGEX-3 $\times$  plasmid (23) and insertion into the pCMV-Tag3C vector using *Bam*HI/*Eco*RI sites. The C230S mutation (690–692, TGC to TCC) was introduced into the GK-WT and V62M-mutant using site-directed mutagenesis with specific primers (forward: 5'-GTG GGC ACG GGC TCC AAT GCC TGC TAC-3'; reverse: 5'-GTA GCA GGC ATT GGA GCC CGT GCC CAC-3'). Sequences were confirmed by DNA sequencing and protein translation by Western blotting.

**Cell culture and transfection.** Cells were cultured at 25 mmol/L glucose unless otherwise stated. MIN6 (p20–27) cells were cultured as described previously (13). H4IIE and Cos1 were cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum, 75 mg/L penicillin, and 50 mg/L streptomycin. Cell monolayers in 24-well plates were transfected with 0.2–0.6  $\mu$ g plasmid DNA and 0.6–1.8  $\mu$ L Lipofectamine 2000 and cultured for 24 h.

**Hepatocyte immunostaining.** Hepatocytes were isolated from male Wistar rats and were suspended in minimum essential medium containing 5 mmol/L glucose with 5% (vol/vol) newborn calf serum, 75 mg/L penicillin, and 50 mg/L streptomycin and seeded on gelatin-coated coverslips in 24-well plates (24). Three hours after attachment, hepatocytes were transiently transfected with 4  $\mu$ g plasmid DNA and 12  $\mu$ L jetPEI-Hepatocyte for 5 h and then cultured overnight. Cells were fixed with ice-cold acetone and immunostained using an anti-myc antibody (1:50 dilution) and a FITC-conjugated anti-mouse secondary antibody (1:100 dilution). Nuclei were stained with 10  $\mu$ mol/L Hoechst dye 33342. Immunofluorescence was visualized using a Nikon E400 microscope ( $\times$ 63 oil immersion objective). Five images were taken in three independent experiments. The nuclear-to-cytoplasmic ratio for each image was determined by measurement of the mean pixel intensity of nuclei (including nucleoli) and cytoplasmic areas using Image ProPlus Analysis Software. Expression of results as a ratio corrects for drifts in fluorescence intensity between transfected cells.

**GK activity and immunoreactivity.** GK activity in MIN6 and H4IIE cells was determined at 50 mmol/L glucose on 13,000 g supernatants as described previously (13). Glucose-phosphorylating activity was determined at 0.5 and 50 mmol/L glucose, representing low- $K_m$  and total hexokinase activity, respectively, and GK activity was determined from the difference between these values. GK immunoreactivity was determined by SDS-PAGE using a 10% polyacrylamide gel and immunoblotting (13) with a GK antibody (22). Under these conditions, myc-tagged GK (52 kDa) could be distinguished as a distinct band from that of endogenous GK (50 kDa).

**Cellular glutathione, NO, and hydrogen peroxide determination.** For determination of total glutathione (GSH) content, an enzyme-recycling assay was used (25). In brief, cells were extracted into 2.5% sulfosalicylic acid and centrifuged at 13,000g. GSH content was measured on the supernatant by incubation with 110 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 70 mmol/L NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 6.3 mmol/L EDTA, 300  $\mu$ mol/L NADPH, 40  $\mu$ M 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.4 units/mL glutathione reductase at A<sub>412</sub>. For GSH depletion assays, GSH was determined using MCB (26). Cells were cultured with diamide, sodium nitroprusside (SNP), or menadione (MEN) as stated for 1 h before a 10-min incubation with 75  $\mu$ mol/L MCB in a Krebs-HEPES buffer containing 116 mmol/L NaCl, 20 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, 1 mmol/L NaPi, and 0.8 mmol/L Na<sub>2</sub>SO<sub>4</sub>, pH 7.4, and 1% FBS. NO and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content was determined in cells incubated with 30  $\mu$ mol/L DAF-FM diacetate (NO) or 30  $\mu$ mol/L H<sub>2</sub>DCFDA (H<sub>2</sub>O<sub>2</sub>) in Krebs-HEPES buffer for 10 min before the addition of diamide, SNP, or MEN as stated for 1 h. GSH depletion, NO, and H<sub>2</sub>O<sub>2</sub> were measured using a SpectraMax M5e Reader (Molecular Devices) at excitation (Ex)385 nm, emission (Em)478 nm (cutoff 475 nm); Ex495 nm, Em515 nm (cutoff 505 nm); and Ex488 nm, Em510 nm (cutoff 495 nm), respectively.

**Bimolecular fluorescence complementation assay.** The bimolecular fluorescence complementation (BiFC) assay relies on the complementation of two nonfluorescent fragments of YFP when brought together by the interaction of proteins fused to each fragment (27). Sequences encoding amino acid residues of 1–155 or 156–264 of YFP, designated YN and YC, respectively (27), were generated by PCR and ligated into the pCMV-Tag3C vector. Human  $\beta$ -cell GK (WT or mutant) was fused to the NH<sub>2</sub> terminus of YN (GK-YN), and the bisphosphatase domain of PFK2/FBPase2 was fused to the NH<sub>2</sub> terminus of YC (FBPAse2-YC). The GK interaction interface (SLKVWT, 5'-TCC CTG AAA

GTC TGG ACT-3') was deleted using site-directed mutagenesis with specific primers (forward: 5'-CAT CCG GTC TCA AGG CAT CAG CAG CCA CAT GAA GAG GAC CAT TC-3'; reverse: 5'-GAA TGG TCC TCT TCA TGT GGC TGC TGA TGC CTT GAG ACC GGA TG-3'). Sequences were confirmed by DNA sequencing and protein translation by Western blotting. For imaging analysis, Cos1 cells were transfected with 1  $\mu$ g GK-YN, 1  $\mu$ g FBPAse2-YC, and 0.2  $\mu$ g full-length monomeric red fluorescent protein using Lipofectamine 2000 for 24 h. After 3-h complementation, fluorescence was visualized in living cells using either a Nikon TE300 or a Nikon TE2000 inverted fluorescence microscope ( $\times$ 60/100 objective). Fluorescence was visualized at the following: YFP, Ex500 nm, Em535 nm; and monomeric red fluorescent protein, Ex584 nm, Em607 nm. Nuclei were stained with 10  $\mu$ mol/L Hoechst dye 33342 (Ex350 nm, Em460 nm).

**Structural modeling.** The GK mutants were localized to the previously published WT GK crystal structure in both the open and closed conformations (cocrystallized with glucose and an allosteric activator) (28) using the Swiss-PDB Viewer software.

**Statistical analysis.** This was performed by ANOVA followed by the Bonferroni test or  $\chi^2$  analysis using the Prism analysis program.

## RESULTS

### Selection of GK mutants based on enzyme kinetics.

Approximately 80 of the 600 mutations identified in the *Gck* gene have been kinetically characterized in vitro using recombinant glutathione *S*-transferase-GK fusion proteins and the predicted threshold for GSIS calculated using mathematical modeling (9,12). A threshold for GSIS of >6.5 mmol/L glucose is consistent with the diabetic phenotype. In this study, we selected 11 GK-MODY mutants with a predicted GSIS <6.5 mmol/L for cellular analysis (Table 1). The mutants selected include V62M and G72R where there is discordance (29–31) in the effect of these mutations on enzyme kinetics. Possible explanations for the differences are discussed elsewhere (32). We used five PHHI mutants with a predicted GSIS of <5 mmol/L as controls.

GK has a bipartite structure with a flexible hinge allowing changes in conformation from super open /open (less active) to closed (more active) conformations upon binding of glucose (1–3). The location of the mutated residues in the superopen and closed conformations (28) was determined by structural modeling. The activating mutations (T65I, W99R, Y214C, V455M, and A456V) localize to the hinge region (Supplementary Fig. 1) as demonstrated previously (29). However, the 11 MODY mutants did not localize to a common location.

**Location of GK-MODY mutants in hepatocytes.** In hepatocytes, GKRP determines the cellular location and protein stability of GK (15–17). We first determined the subcellular location of the selected MODY mutants in hepatocytes. With the exception of V62M and G72R, which showed only cytoplasmic staining (Fig. 1) (13,29,30), all other MODY mutants showed nuclear localization and a similar nuclear-to-cytoplasmic ratio as WT (Fig. 1), indicating interaction with GKRP. We infer that the MODY phenotype for the majority of these mutants is unlikely to be a result of the dysregulation of subcellular location in hepatocytes.

**Catalytic activity of GK-MODY mutants in pancreatic  $\beta$ -cells.** We next determined the specific activity of the MODY mutants in  $\beta$ -cells using varying plasmid concentrations of myc-tagged GK in the MIN6 model (Fig. 2A). The specific activity of GK was determined from the slope of GK activity (corrected for endogenous activity) against corresponding GK immunoreactivity (Fig. 2B–I, Table 1, and Supplementary Table 1). Nine of 11 (A53S, V62M, G72R, H137R, S263P, G264S, M298K, E300K, and K414E) MODY mutants had a lower specific activity than WT (>35%),

TABLE 1  
Kinetic characteristics and cellular stability of GK mutants

Phenotype		Relative activity index (unitless)	Threshold for GSIS (mmol/L)	MIN6 GK activity:IR slope	H4IIE GK activity:IR slope
WT		1.00	5.00	1.00	1.00
A53S	MODY	0.88	5.00	0.30 ± 0.18*P	ND
V62M	MODY	2.59#	4.00	0.35 ± 0.13*	1.05 ± 0.15
E70K	MODY	0.65	6.10	0.85 ± 0.33	ND
G72R	MODY	1.01#	5.30	0.34 ± 0.13*	1.34 ± 0.15
H137R	MODY	0.69	5.75	0.19 ± 0.20*	ND
S263P	MODY	0.64	6.00	0.07 ± 0.15*P	0.34 ± 0.13*
G264S	MODY/PNDM	0.95	5.40	0.32 ± 0.08**	1.59 ± 0.42
R275C	MODY	1.1	5.00	1.03 ± 0.39	ND
M298K	MODY	0.44	6.50	0.04 ± 0.04***	0.27 ± 0.03**
E300K	MODY	0.66	5.80	0.20 ± 0.16*P	0.54 ± 0.17*
K414E	MODY	0.62	5.30	0.07 ± 0.17*P	ND
T65I	PHHI	3.11	3.10	1.33 ± 0.90	ND
W99R	PHHI	4.14	2.80	1.29 ± 0.31	0.95 ± 0.27
Y214C	PHHI	17.98	1.40	1.94 ± 0.17*	ND
V455M	PHHI	5.23	2.70	0.99 ± 0.22	ND
A456V	PHHI	17.06	1.45	0.74 ± 0.22	ND

Data for the relative activity index and threshold for GSIS are compiled (12). The slopes of each individual data set from Fig. 2 and Supplementary Fig. 2 ( $n = 3$ ) were calculated using linear regression analysis and are normalized to the respective WT control within each experiment. PNDM, permanent neonatal diabetes mellitus; IR, immunoreactivity; ND, not determined. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.005$  relative to WT; #discrepancy of kinetic parameters between independent studies (29–31); P, low protein expression in the specific activity assay, which limits the validity of the calculation for these mutants.

indicating enzyme inactivation (Fig. 2*B–F* and Table 1). None of the five PHHI mutants had a significantly lower specific activity than WT. T65I, W99R, and V455M had similar activity as WT; A456V had an activity 74% of WT; and Y214C had a specific activity twice that of WT (Fig. 2*C* and Table 1).  $\chi^2$  Analysis confirmed a significant difference in specific activity distribution between the MODY and PHHI mutants ( $P = 0.0075$ ).

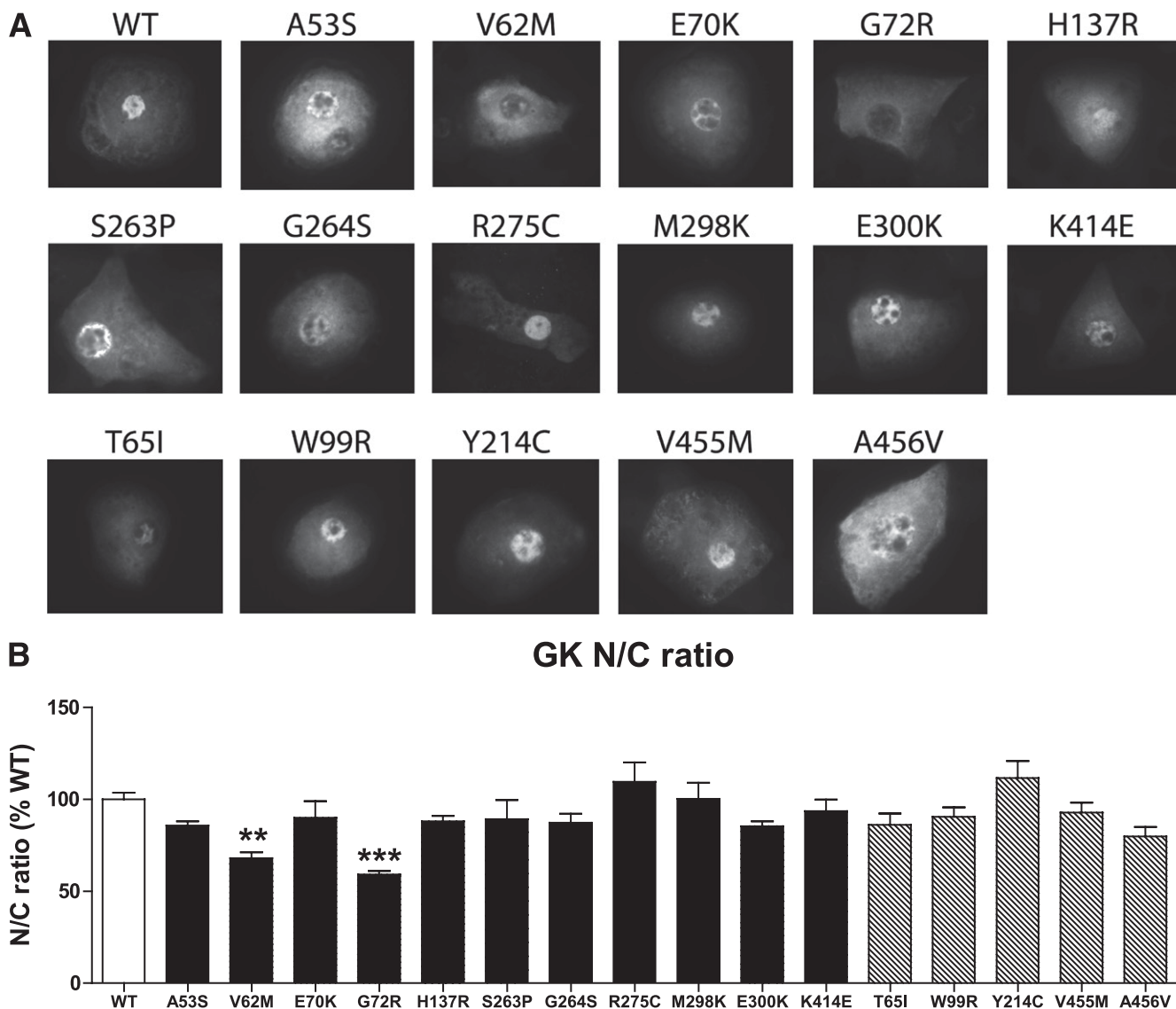
**Protein stability of GK-MODY mutants in pancreatic  $\beta$ -cells.** Several MODY mutants expressed lower levels of immunoreactive protein than WT (Fig. 2). We therefore determined protein stability during inhibition of protein synthesis with cycloheximide (12 h). There was no effect of cycloheximide on endogenous or WT GK protein (Fig. 3*A* and *B*). Five MODY mutants (A53S, H137R, S263P, E300K, and K414E) displayed marked protein instability compared with WT (>50%). Four MODY mutants (V26M, G72R, G264S, and M298K) and all five PHHI mutants showed similar protein stability as WT (Fig. 3*C*). Thus, with few exceptions, protein instability correlated with low specific activity (Fig. 3*D*).

**Catalytic activity of GK-MODY mutants in hepatoma cells.** To determine whether the low catalytic activity of the MODY mutants is specific to the pancreatic  $\beta$ -cell six mutants with a low specific activity in MIN6 were expressed in a hepatoma cell model (H4IIE). Mutants with a specific activity 30–40% of WT in MIN6 (V62M, G72R, and G264S) showed similar specific activity to WT in H4IIE (Table 1 and Supplementary Fig. 2), indicating that the instability of these mutants is cell type dependent. Mutants with a specific activity <20% of WT in MIN6 (S263P, M298K, and E300K) also showed low specific activity in H4IIE (Table 1 and Supplementary Fig. 2).

**Sensitivity of GK-MODY mutants to oxidative stress.** Pancreatic  $\beta$ -cells have lower antioxidant defenses than hepatocytes and are particularly vulnerable to cellular damage by oxidative stress (33,34). The total GSH content of MIN6 was 80% lower than in hepatocytes or H4IIE (Fig. 4*A*).

We therefore tested whether depletion of GSH using di-amide and increased oxidative stress induced with SNP or MEN further compromises the activity of MODY mutants (Fig. 4*B–D*). Four GK-MODY mutants (V62M, G72R, S263P, and G264S) with decreased specific activity in MIN6 but sufficient catalytic activity to enable reliable measurement were selected for these studies. Oxidative stress induced with MEN but not SNP decreased the activity of WT enzyme by 20% (Fig. 4*E* and *F*), and the PHHI mutant W99R showed similar activity to WT. However, all four MODY mutants showed significantly greater inactivation by SNP and MEN (Fig. 4*E* and *F*), indicating increased inactivation by oxidative stress. There was no detectable difference in immunoreactive protein under these conditions (data not shown). Oxidative stress induced by MEN also decreased the activity of endogenous GK in MIN6, which was prevented by pre-incubation with the antioxidant *N*-acetyl cysteine (Fig. 4*G*). The inactivation of endogenous GK by reactive oxygen species was partially prevented by 24-h treatment with a pharmacological GK activator (GKA-R) that favors the closed (active) conformation (Fig. 4*H*).

**Role of cysteine residues in the oxidation of GK-MODY mutants.** GK is extremely sensitive to thiol group modification by thiol reactive agents such as alloxan, with consequent inactivation of catalytic activity (35–37). This inactivation is either reversible with dithiothreitol (DTT) or irreversible (38,39). To investigate the role of thiol group modification in the catalytic instability of the mutants, the effects of varying concentrations of alloxan and DTT was determined. DTT caused a twofold increase in the activity of WT (Fig. 5*A*) and the PHHI mutant W99R (Fig. 5*B*). However, it caused a significantly greater fractional activation of all four MODY mutants (Fig. 5*B*), indicating that the low specific activity is in part a result of reversible modification of thiol residues. The four MODY mutants, but not W99R, showed a greater sensitivity to inactivation by alloxan as shown by the lower  $IC_{50}$  values (Fig. 5*C* and *D*), further confirming enhanced sensitivity to



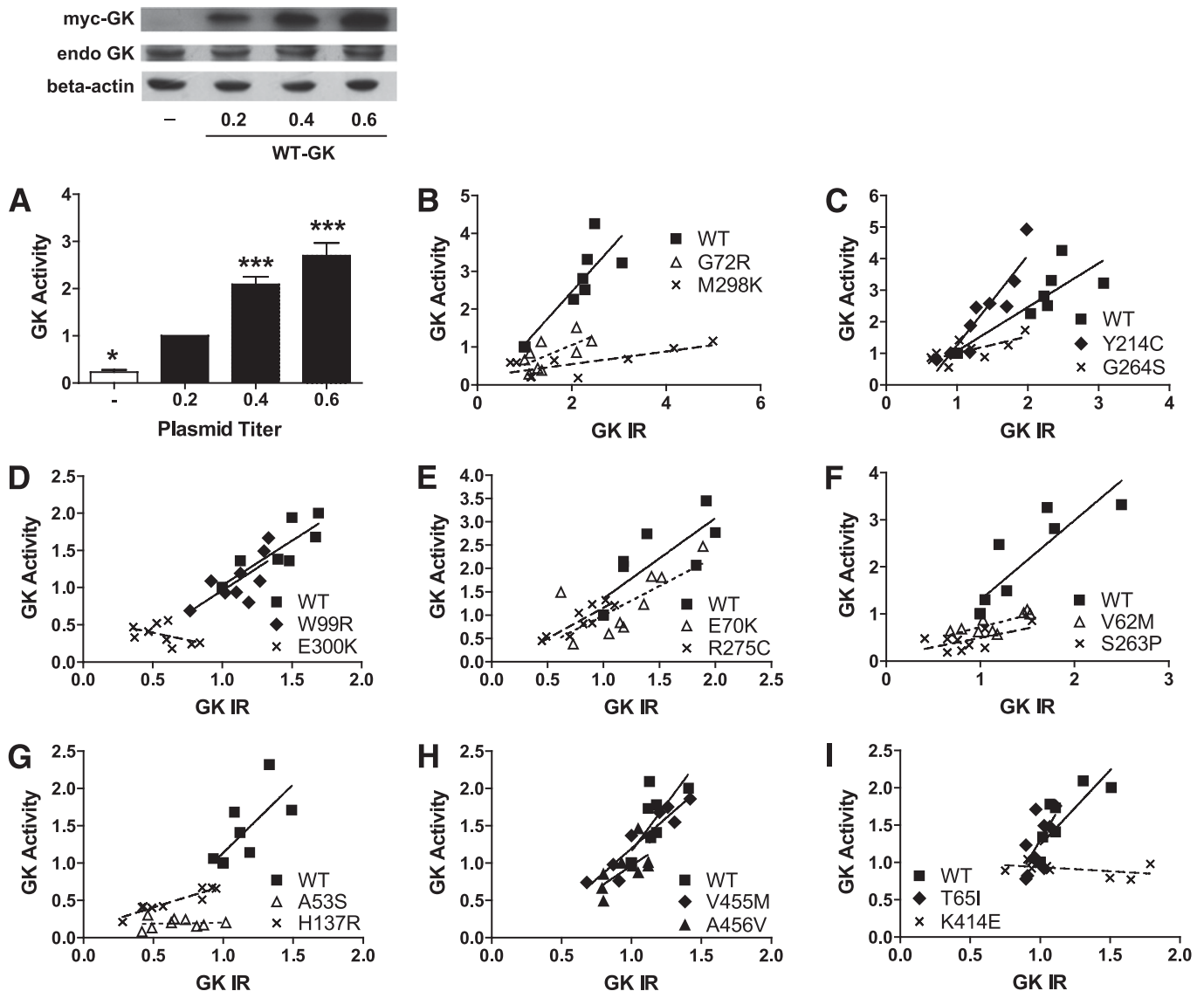
**FIG. 1.** Location of mutant GK in hepatocytes. Primary hepatocytes were transfected with myc-tagged GK-WT or mutant GK using JetPei-hepatocyte reagent and incubated for 5 h. After overnight incubation in minimum essential medium with 5 mmol/L glucose, cells were fixed using ice-cold acetone and immunostained for myc-GK using an anti-myc antibody. *A*: Images are representative of 15 cells obtained from three independent experiments. *B*: The nuclear-to-cytoplasmic (N/C) ratio was calculated as described in RESEARCH DESIGN AND METHODS and is expressed as %WT. Means are  $\pm$  SEM of 15 cells from three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

oxidation (37,38). Because cysteine 230 (Cys230) is highly susceptible to alloxan modification (38), we tested the effect of mutating this residue either alone or in combination with V62M in the alloxan sensitivity assay. The C230S mutant was less sensitive to alloxan than WT (Fig. 5E) as reported previously (38). However, the double mutant C230S-V62M had similar sensitivity to alloxan and oxidative stress as V62M, although it was less sensitive to SNP inactivation (Fig. 5E–G). This indicates that residues other than Cys230 are involved in the sensitivity of V62M to oxidative stress.

#### Interaction of GK-MODY mutants with PFK2/FBPase2.

In the pancreatic  $\beta$ -cell, GK activity is regulated by binding to PFK2/FBPase2 (20). To determine whether this interaction protects GK from oxidative inactivation, PFK2/FBPase2 was coexpressed with WT GK in MIN6. PFK2/FBPase2 protected GK from inhibition by alloxan, with a shift in  $IC_{50}$  from 1.1 to 3.5 mmol/L (Fig. 6A) and reversal

of the inhibition by 0.5 mmol/L alloxan (Fig. 6B). We next investigated the interaction of MODY mutants with PFK2/FBPase2 using the BiFC assay (27). Because this assay is critically dependent on the level of protein expression, the GK-PFK2/FBPase2 interaction could only be tested on mutants with minimal effect on protein stability, i.e., V62M, G72R, and G264S (Fig. 6D). A direct interaction between WT GK and the bisphosphatase domain FBPase2 was evident from YFP staining (Fig. 6C). Total loss of interaction between GK and a FBPase2 variant lacking the GK binding domain (SLKVWT) confirmed the specificity of the signal with WT FBPase2. Three of three MODY mutants tested (V62M, G72R, and G264S) showed decreased interaction with FBPase2, while the PHHI mutant W99R showed similar interaction to WT (Fig. 6D). Similar levels of protein expression of the mutants were confirmed by immunoblotting (Fig. 6D). These data suggest that GK binding to PFK2/FBPase2 may stabilize GK activity in  $\beta$ -cells.



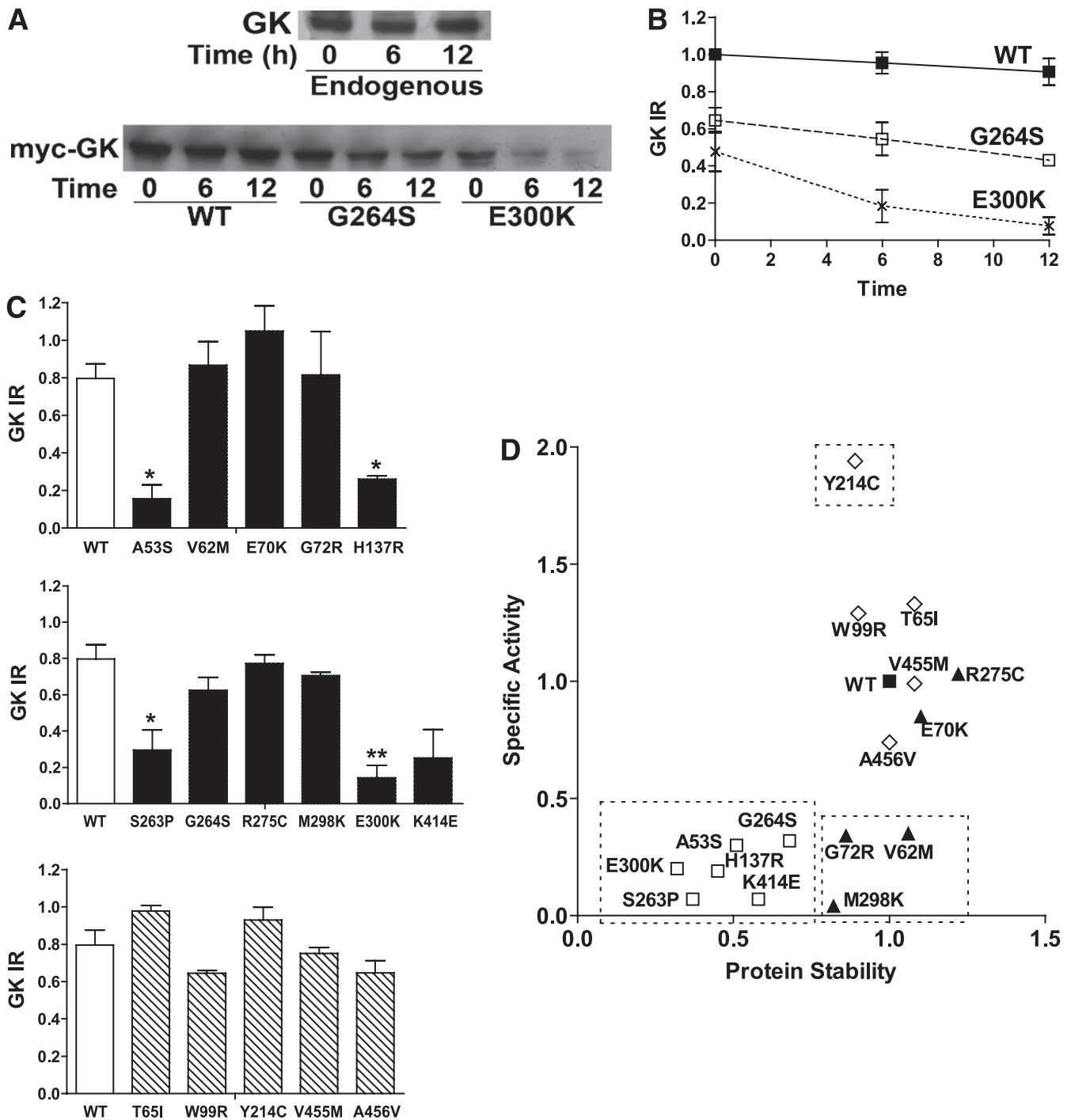
**FIG. 2.** Cellular stability of mutant GK in MIN6  $\beta$ -cells. MIN6 cells were transiently transfected with WT or mutant GK at increasing cDNA titers (0.2, 0.4, and 0.6  $\mu$ g/well) and cultured for 24 h. GK activity was determined via a spectrophotometric method and endogenous GK activity ( $1.33 \pm 0.05$  mU/mg protein) subtracted from overexpressed GK-WT and mutant activity. GK-WT was expressed  $5.9 \pm 0.9$ -,  $10.5 \pm 1.4$ -, and  $12.9 \pm 1.7$ -fold above endogenous activity ( $n = 24$ ). Immunoreactivity (IR) was determined via Western blotting and quantified by densitometry. **A:** Representative activity and immunoblot for GK-WT, endogenous GK expression, and  $\beta$ -actin. GK activity is expressed relative to myc-WT titer 1 (0.2  $\mu$ g). Means are  $\pm$  SEM. \* $P < 0.05$ , \*\*\* $P < 0.01$ ;  $n = 9$ . Immunoblots are representative of nine experiments. **B–I:** GK activity is plotted against GK immunoreactivity. Results are expressed relative to myc-WT titer 1 (0.2  $\mu$ g). The slope of the graph was determined by linear regression analysis;  $n = 3$  plasmid titers from three independent experiments.

## DISCUSSION

The mild diabetic phenotype of GK-MODY is remarkably homogeneous despite the variable effects of causative mutations on enzyme kinetics (9,11,12). In cases where the mutation is markedly inactivating ( $<10\%$  of WT), the mild phenotype is explained by compensatory upregulation of the WT allele induced by the mild hyperglycemia (40). However, alternate explanations are required for the diabetic phenotype when the mutation has minimal influence on enzyme kinetics (Table 1). Possible mechanisms include protein instability that manifests either as thermolability *in vitro* (29,30,41), decreased activity/protein in cell based assays (13,42) or *in vivo* (43), or decreased interaction between liver GK and GKRP with consequent decreased hepatic GK protein (43). The critical role of pancreatic  $\beta$ -cell

GK in glucose homeostasis is evident from the similar phenotype of the murine  $\beta$ -cell GK knockout to the global knockout (4,5). In this study, we asked whether GK-MODY mutants that have minimal effect on enzyme kinetics *in vitro* show abnormal activity in a  $\beta$ -cell environment.

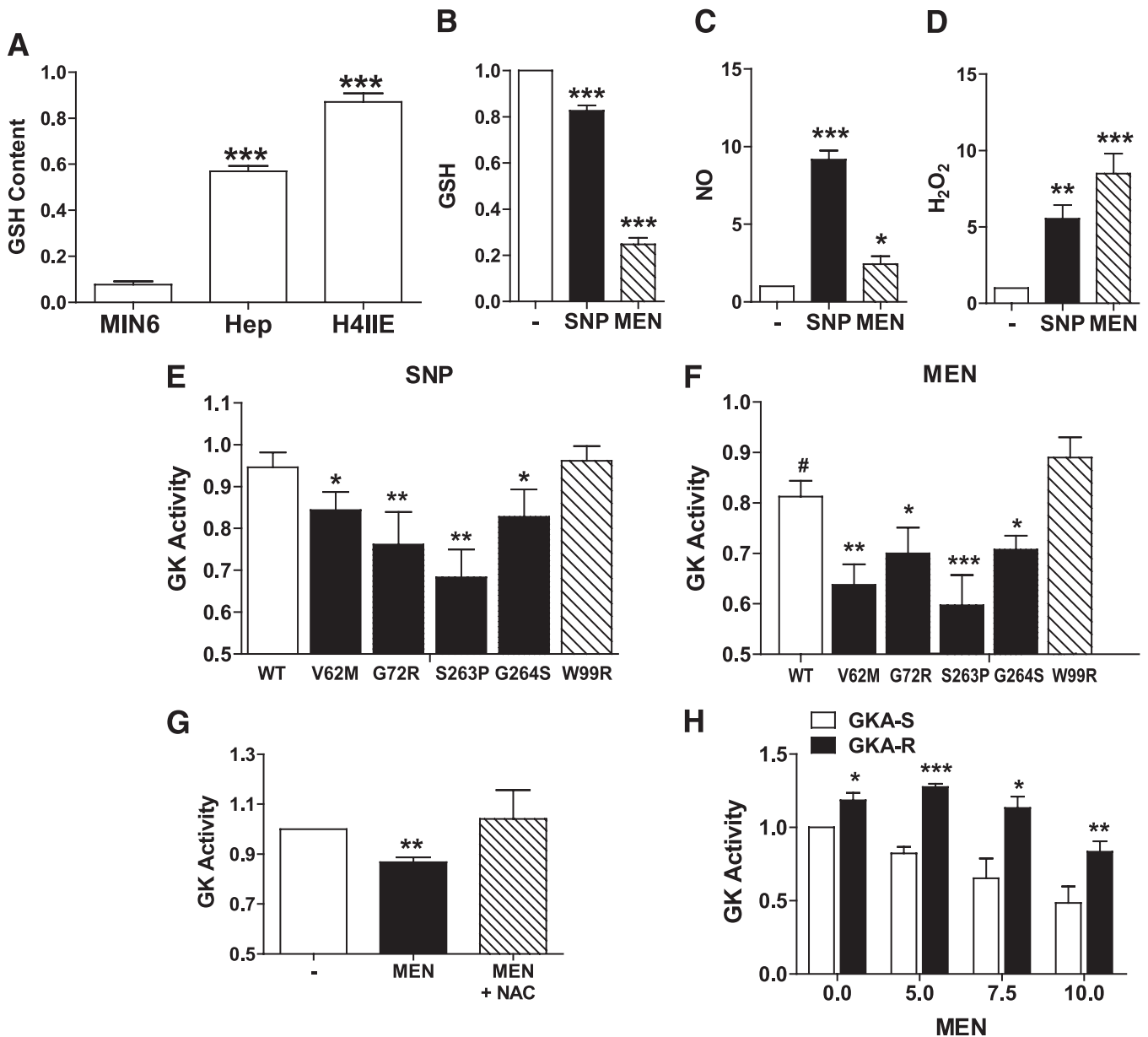
Two key findings emerged from this study. First, the majority of the MODY mutants but none of the PHHI mutants showed enzyme inactivation in  $\beta$ -cells. This inherent instability may contribute to the diabetic phenotype. Second, although there was heterogeneity among the MODY mutants in the compromised specific activity and protein stability, a subset of MODY mutants with decreased specific activity in  $\beta$ -cells but not hepatoma cells was more susceptible to inactivation by oxidative stress. These findings indicate the importance of the  $\beta$ -cell environment and oxidative stress in the MODY phenotype (Table 2).



**FIG. 3.** Protein stability of mutant GK in MIN6  $\beta$ -cells. MIN6 cells were transiently transfected with WT or mutant GK (0.6–1.2  $\mu$ g/well) and cultured for 24 h. After transfection, protein synthesis was inhibited using 2  $\mu$ mol/L cycloheximide, and GK immunoreactivity (IR) was determined at 0 time and at 6 and 12 h. **A:** Immunoblot of endogenous GK and overexpressed GK representative of three independent experiments. **B:** Densitometry results are expressed relative to IR at time 0. Means are  $\pm$  SEM of three independent experiments. **C:** Change in mutant GK immunoreactivity after 12 h. Results are expressed relative to IR at time 0. Means are  $\pm$  SEM of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . **D:** Specific activity (Table 1) plotted against protein stability (C).

Two of the 11 MODY mutants (E70K and R275C) studied in the  $\beta$ -cell model showed similar specific activity to WT. Thus, other explanations are required for the diabetic phenotype in these two cases. The remaining nine MODY mutants (A53S, V62M, G72R, H137R, G264S, S263P, M298K, E300K, and K414E) had a specific activity less than 35% of WT when expressed in MIN6. The majority of these mutants showed either lower protein levels compared with

WT (A53S, V62M, G72R, H137R, S263P, G264S, and E300K) or marked (A53S, H137R, S263P, E300K, and K414E) protein instability. Decreased enzyme activity/protein may be sufficient to explain the diabetic phenotype since a mouse model homozygous for K414E died shortly after birth, consistent with a severe  $\beta$ -cell deficit of GK protein (43). Three MODY mutants with very low specific activity (<20% of WT) in  $\beta$ -cells (S263P, M298K, and E300K) also showed low

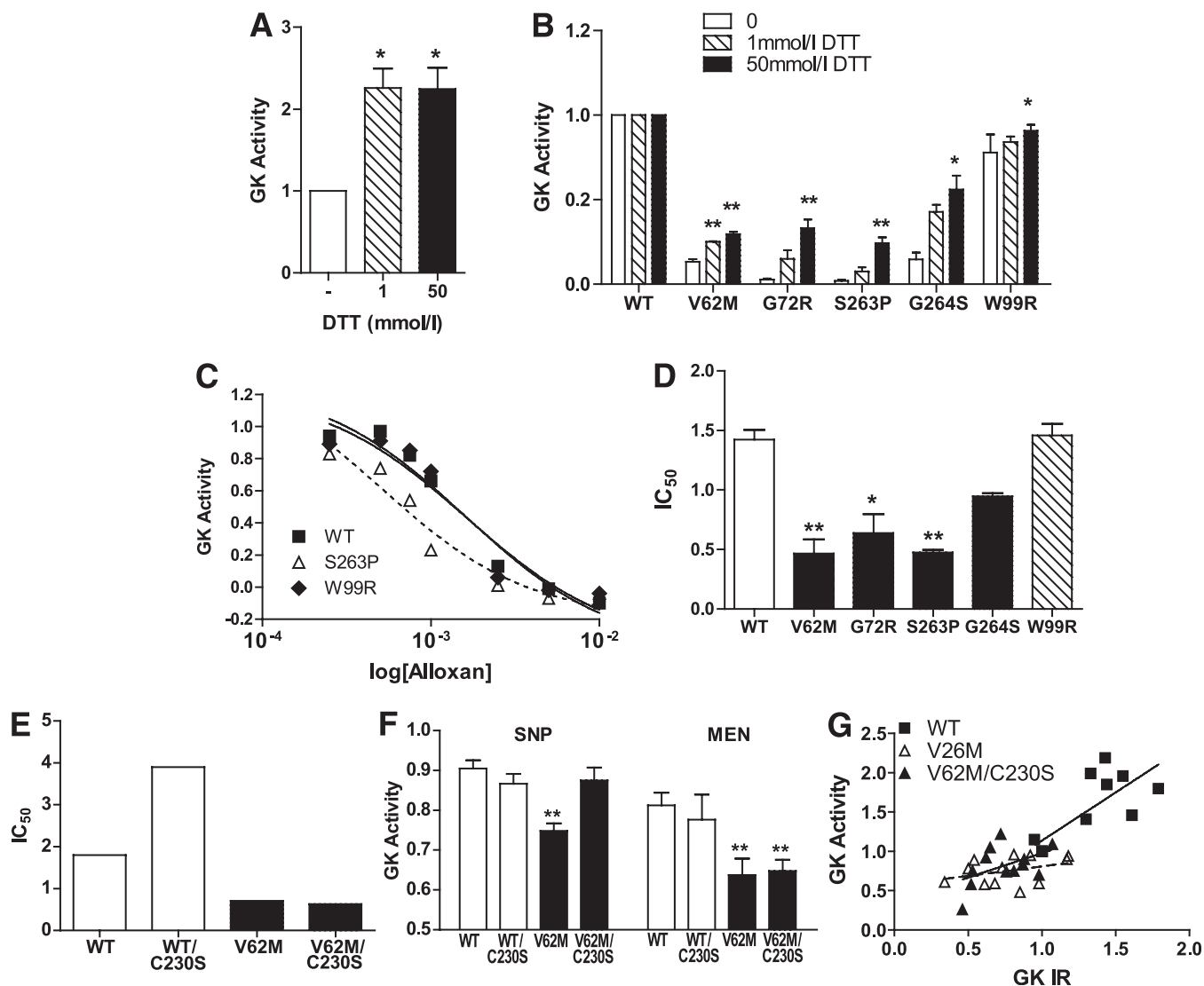


**FIG. 4.** Sensitivity of WT and mutant GK to elevated oxidative stress. **A:** GSH content of MIN6, primary hepatocytes (Hep), and H4IIE cells was determined using an enzyme-recycling assay. Results are expressed as nanomoles per milligram protein. Means are  $\pm$  SEM of three independent experiments. \*\*\* $P < 0.005$  relative to MIN6. **B–F:** MIN6 cells were transiently transfected with 0.6  $\mu\text{g}/\text{well}$  (WT, W99R) or 1.2  $\mu\text{g}/\text{well}$  (V62M, G72R, S263P, and G264S) of myc-GK and cultured for 24 h. Cells were treated with 100  $\mu\text{mol}/\text{L}$  diamide plus 100  $\mu\text{mol}/\text{L}$  SNP or 10  $\mu\text{mol}/\text{L}$  MEN at 5 mmol/L glucose for 1 h. **B–D:** Parallel incubations were performed for determination of GSH, H<sub>2</sub>O<sub>2</sub>, and NO and expressed as fold change relative to control. Means are  $\pm$  SEM of four independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$  relative to absence of SNP/MEN. **E and F:** GK activity was determined using a spectrophotometric method and expressed relative to absence of SNP/MEN. Means are  $\pm$  SEM of four (SNP) or five (MEN) independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$  relative to WT; # $P < 0.05$  relative to absence of MEN. **G:** Untreated MIN6 cells were cultured in the presence of 2 mmol/L *N*-acetyl cysteine (NAC) for 24 h followed by a 1-h incubation with 100  $\mu\text{mol}/\text{L}$  diamide and 5  $\mu\text{mol}/\text{L}$  MEN. GK activity was determined using a spectrophotometric method and expressed relative to absence of MEN/NAC. Means are  $\pm$  SEM of four independent experiments. \*\* $P < 0.01$ , effect of MEN. **H:** Untreated MIN6 cells were cultured in the presence of 10  $\mu\text{mol}/\text{L}$  GKA-S (inactive) or GKA-R (active) for 24 h followed by incubation with 100  $\mu\text{mol}/\text{L}$  diamide and increasing concentrations of MEN (5, 7.5, and 10  $\mu\text{mol}/\text{L}$ ) for 1 h. GK activity was determined using a spectrophotometric method and expressed relative to control. Means are  $\pm$  SEM of four independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , effect of GKA-R.

specific activity in hepatoma cells. These mutants were shown previously to be thermolabile in vitro (9,41). Three MODY mutants with low specific activity (30–40% of WT) in  $\beta$ -cells (V62M, G72R, and G264S) showed comparable specific activity with WT in the hepatoma model. These mutants were shown previously to be relatively thermostable in vitro (29,30). G264S was identified in permanent neonatal diabetes mellitus in conjunction with a GK splice site mutation (IVS8+2T>G) and was associated with marked

hyperglycemia, consistent with severely compromised GK activity (44). The lower stability of G264S in the  $\beta$ -cell model compared with hepatoma cells supports the validity of the  $\beta$ -cell model for predicting the phenotype of MODY mutants and highlights the importance of the  $\beta$ -cell environment in modulating GK activity.

Pancreatic  $\beta$ -cells have lower antioxidant defenses than hepatocytes and are more vulnerable to oxidative stress (33,34). Three sets of evidence from this study support

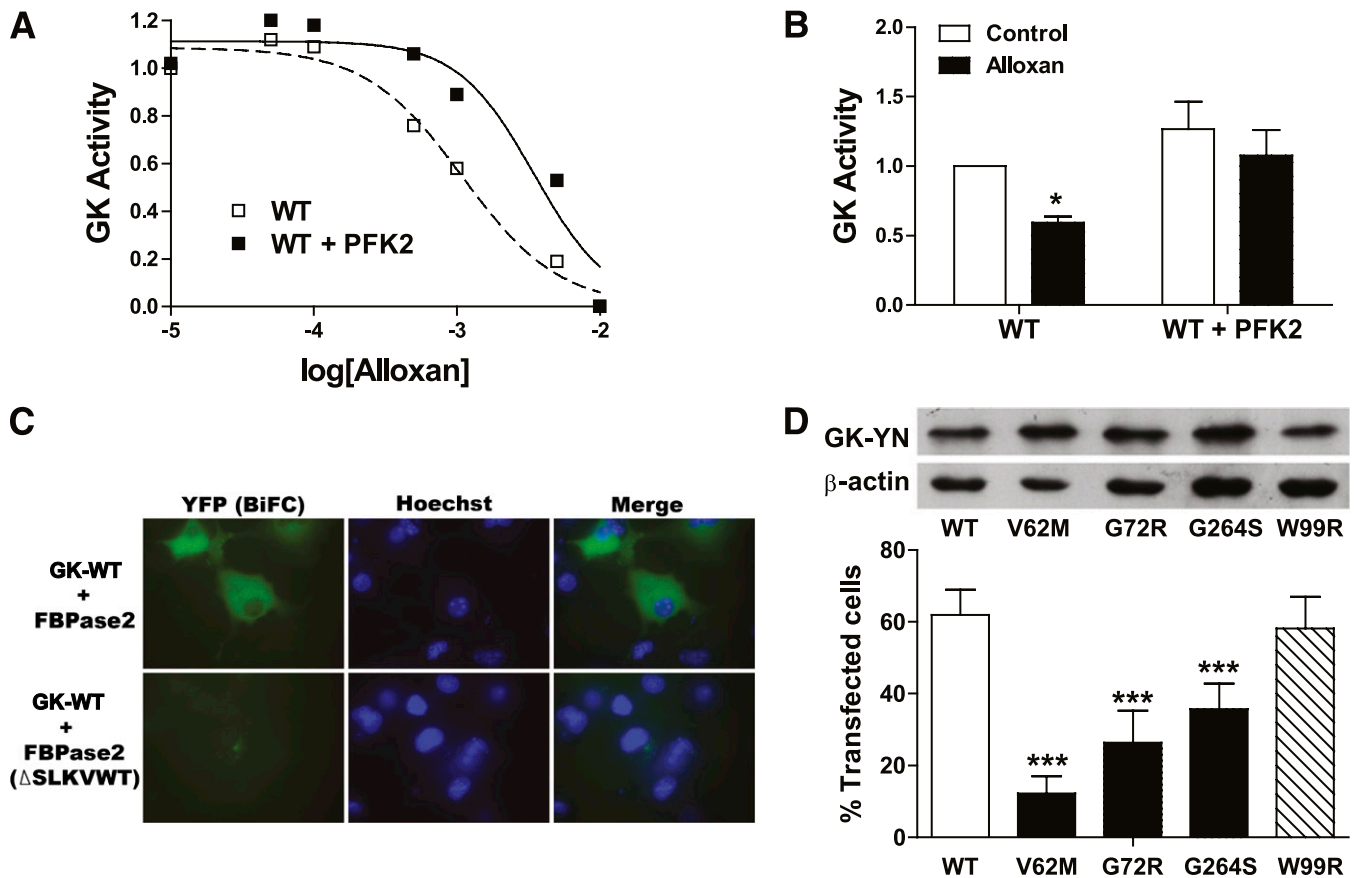


**FIG. 5.** Role for cysteine residues in the oxidation of GK mutants. *A–D:* MIN6 cells were transiently transfected with 0.6  $\mu\text{g}/\text{well}$  (WT, W99R) or 1.2  $\mu\text{g}/\text{well}$  (V62M, G72R, S263P, and G264S) of myc-GK and cultured for 24 h. *A* and *B:* Cell lysate was either untreated or treated with 1 or 50 mmol/L DTT for 5 min. GK activity was determined on the lysate and expressed relative to the absence of DTT (*A*) or relative to WT (*B*). Means are  $\pm$  SEM of four independent experiments. *A:* \* $P < 0.05$ , relative to absence of DTT. *B:* \* $P < 0.05$ , \*\* $P < 0.01$ , relative to WT. *C* and *D:* Cells were permeabilized with 0.04 mg/mL digitonin and the cell lysate incubated with increasing concentrations of alloxan (0.25, 0.5, 0.75, 1, 2.5, 5, and 10 mmol/L) in the absence of glucose for 5 min. *C:* GK activity was determined at 25 mmol/L glucose and plotted against alloxan concentration on a log plot. Results are representative of three experiments. *D:* The inhibitory constant for alloxan ( $\text{IC}_{50}$ ) was calculated by nonlinear regression analysis and is expressed as micromoles per liter. Means are  $\pm$  SEM of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , relative to WT. *E* and *F:* MIN6 cells were transiently transfected with 0.6  $\mu\text{g}/\text{well}$  (GK-WT, WT/C230S) or 1.2  $\mu\text{g}/\text{well}$  (V62M or V62M/C230S) of myc-GK and cultured for 24 h. *E:* The  $\text{IC}_{50}$  for alloxan was calculated as above;  $n = 1$ . *F:* Oxidative stress was induced as in Fig. 4 and GK activity expressed relative to absence of SNP/MEN. Means are  $\pm$  SEM of six independent experiments. \*\* $P < 0.01$ , relative to GK-WT. *G:* MIN6 cells were transiently transfected with WT or mutant GK at increasing cDNA titers (0.2, 0.4, and 0.6  $\mu\text{g}/\text{well}$ ) and cultured for 24 h. GK activity is plotted against GK immunoreactivity as in Fig. 2;  $n = 3$  plasmid titers from four independent experiments.

a role for oxidative stress as a predisposing factor to the instability of several MODY mutants (Table 2). First, three of six MODY mutants showed enzyme inactivation in  $\beta$ -cells but not hepatoma cells. Second, these mutants showed greater inactivation than WT by oxidative stress. Third, the low specific activity of these mutants was partially reversed by DTT. This indicates both reversible and irreversible inactivation of the enzyme. GK is more sensitive to oxidation of thiol groups than other hexokinase isoforms (35,36). Oxidation of thiol groups locks the enzyme in a restricted conformation, thereby affecting enzyme activity. This is in part a result of the formation of disulfide bonds (37,38). Tippett and Neet (35) proposed that physiological

changes in GSH concentration may regulate GK activity through conformational changes of the enzyme induced by modification of thiol residues. The finding that endogenous GK is inactivated by elevated oxidative stress and that this inactivation is attenuated by a GK activator that favors the closed (active) conformation is consistent with this hypothesis (35). This supports a model whereby endogenous GK activity is inhibited by thiol oxidation in conditions of GSH depletion and/or reactive oxygen species production. The sensitivity of GK to posttranslational modifications induced by cell stress is supported by a recent study reporting irreversible tyrosine nitration of GK following chronic ethanol exposure (45).





**FIG. 6.** Interaction between GK and PFK2/FBPase2. **A** and **B**: MIN6 cells were transiently transfected with 0.6  $\mu\text{g}/\text{well}$  GK-WT without or with 0.6  $\mu\text{g}/\text{well}$  PFK2/FBPase2. Cells were permeabilized with 0.04 mg/mL digitonin, and the cell lysate was incubated with increasing concentrations of alloxan for 5 min as above. GK activity was determined on lysates at 10 mmol/L glucose. **A**: GK activity was determined and plotted against alloxan concentration on a log plot. Results are representative of three independent experiments. **B**: Results are expressed relative to GK-WT in the absence of 0.5 mmol/L alloxan. Means are  $\pm$  SEM of four independent experiments. \* $P < 0.05$ , effect of alloxan. **C**: Cos1 cells grown in glass chambers were transfected with 1  $\mu\text{g}/\text{well}$  GK-YN and either 1  $\mu\text{g}/\text{well}$  FBPase2-YC or FBPase2(SLKVWT deletion)-YC for 24 h. The BiFC complex was then matured for 3 h at 30°C. Images are representative of 20 fields from three independent experiments. **D**: Cos1 cells grown in glass chambers were transfected with 1  $\mu\text{g}/\text{well}$  GK-YN (WT, V62M, G72R, W99R, and G264S), 1  $\mu\text{g}/\text{well}$  FBPase2-YC, and 0.2  $\mu\text{g}/\text{well}$  full-length monomeric red fluorescent protein (mRFP) protein for 24 h, and the BiFC complex was matured for 3 h at 30°C. The frequency of cells expressing either mRFP or YFP in each field was calculated (20 fields), and the YFP-positive cells were expressed as a percentage of mRFP-positive cells. \*\*\* $P < 0.005$ , relative to WT, representative of three experiments. Immunoblot showing GK-YN and  $\beta$ -actin is representative of three experiments. (A high-quality digital representation of this figure is available in the online issue.)

The increased susceptibility of MODY mutants to oxidation may be explained by loss of binding to a protective protein. The GK interaction partner PFK2/FBPase2 binds to the closed (active) conformation of GK at elevated glucose and prevents the transition to the superopen (less active) conformation, thereby increasing the enzyme activity (20,46,47). However, another explanation could be protection of GK from inactivation through modification of thiol residues. Two sets of evidence support this mechanism. First, overexpression of PFK2/FBPase2 protected WT GK from inactivation by alloxan. Second, MODY mutants with decreased specific activity and increased sensitivity to oxidative stress showed decreased interaction with PFK2/FBPase2. It is noteworthy that overexpressed PFK2/FBPase2 did not affect the activity of endogenous GK (results not shown) indicating a saturating effect of endogenous PFK2/FBPase2 as reported previously (13).

A key finding from the current study was the higher specific activity of the Y214C PHHI mutant relative to WT. Enzyme kinetics and tryptophan fluorescence measurements indicate that Y214C adopts a more compact conformation than WT (32). Because other PHHI mutants also have a more compact conformation than WT, the unique

stability of Y214C suggests that small changes in the conformation of Y214C relative to other PHHI mutants or other additional factors may have a major influence on enzyme stability. Regardless of the explanation, the lower stability of WT relative to Y214C is consistent with a role for thiol status in the control of endogenous GK activity as proposed by Tippett and Neet (35). This mechanism may be altered by the Y214C mutation. This would explain the severe phenotype of the patient with the Y214C mutation (48), compared with the much milder phenotype of the A456V mutation (9,49), despite similar enzyme kinetics (Table 1).

In summary, the current study shows that several GK-MODY mutants that cause diabetes despite minimal influence on enzyme kinetics and show compromised catalytic stability and/or protein stability in  $\beta$ -cells but to varying degrees. This variation may reflect the complex regulation of GK activity by several interacting proteins, which may alter both subcellular localization and GK stability (18–20). Common to several MODY mutants is increased susceptibility to oxidative stress compared with WT or PHHI mutants, which may be in part explained by decreased binding to PFK2/FBPase2. The elevated stability of the Y214C mutant and inactivation of endogenous GK activity

TABLE 2  
Summary of cellular defects of GK mutants

	Specific activity in MIN6	Protein stability in MIN6	Specific activity in H4IIE	Inhibition by SNP	Inhibition by MEN	Activation by DTT	Inhibition by alloxan	Interaction with PFK2	Interaction with GKRP
A53S	↓	↓	ND	↑*	ND	ND	↑*	ND	As WT
V62M	↓	As WT	As WT	↑	↑	↑	↑	↓	↓
E70K	As WT	As WT	ND	ND	ND	ND	ND	ND	As WT
G72R	↓	As WT	As WT	↑	↑	↑	↑	↓	↓
H137R	↓	↓	ND	↑*	ND	ND	↑*	ND	As WT
S263P	↓	↓	↓	↑	↑	↑	↑	ND	As WT
G264S	↓	As WT	As WT	↑	↑	↑	↑	↓	As WT
R275C	As WT	As WT	ND	ND	ND	ND	ND	ND	As WT
M298K	↓	As WT	↓	↑*	ND	ND	↑*	ND	As WT
E300K	↓	↓	↓	↑*	ND	ND	ND	ND	As WT
K414E	↓	↓	ND	ND	ND	ND	ND	ND	As WT
T65I	As WT	As WT	ND	ND	ND	ND	ND	ND	As WT
W99R	As WT	As WT	As WT	As WT	As WT	As WT	As WT	As WT	As WT
Y214C	↑	As WT	ND	As WT*	ND	ND	As WT*	ND	As WT
V455M	As WT	As WT	ND	ND	ND	ND	ND	ND	As WT
A456V	As WT/↓	As WT	ND	ND	ND	ND	ND	ND	As WT
A53S	↓	↓	ND	↑*	ND	ND	↑*	ND	As WT

The differences between GK mutants and GK-WT are expressed as either decreased or increased relative to WT. Results are based on data from specific activity in MIN6 (Fig. 2 and Table 1), protein stability in MIN6 (Fig. 3), specific activity in H4IIE (Supplementary Fig. 2 and Table 1), inhibition by SNP/MEN (Fig. 4), activation by DTT (Fig. 5), inhibition by alloxan (Fig. 5), interaction with PFK2 (Fig. 6), and interaction with GKRP (Fig. 1). \*Data in Supplementary Fig. 3.

by oxidation supports a role for the regulation of endogenous WT GK through modulation of thiol status as a potential physiological mechanism for the control of GK activity.

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K.S.C. researched data. F.M.M. provided GK-MODY mutants and reviewed and edited the manuscript. L.A. contributed to discussion and reviewed and edited the manuscript. C.A. researched data and wrote the manuscript.

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