Cell-biological assessment of human glucokinase mutants causing maturity-onset diabetes of the young type 2 (MODY-2) or glucokinase-linked hyperinsulinaemia (GK-HI)

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Mutations in the glucokinase (*GK*) gene cause type-2 maturity-onset diabetes of the young type 2 (MODY-2) and GK-linked hyperinsulinaemia (GK-HI). Recombinant adenoviruses expressing the human wild-type islet GK or one of four mutant forms of GK, (the MODY-2 mutants E70K, E300K and V203A and the GK-HI mutant V455M) were transduced into glucose-responsive insulin-secreting β -HC9 cells and tested functionally in order to initiate the first analysis *in vivo* of recombinant wild-type and mutant human islet GK. Kinetic analysis of wild-type human GK showed that the glucose $S_{0.5}$ and Hill coefficient were similar to previously published data *in vitro* ($S_{0.5}$ is the glucose level at the half-maximal rate). E70K had half the glucose affinity of wild-type, but similar enzyme activity. V203A demonstrated decreased catalytic activity and an 8-fold increase in glucose $S_{0.5}$ when compared with wild-type human islet GK. E300K had a

glucose $S_{0.5}$ similar to wild-type but a 10-fold reduction in enzyme activity. E300K mRNA levels were comparable with wild-type GK mRNA levels, but Western-blot analyses demonstrated markedly reduced levels of immunologically detectable protein, consistent with an instability mutation. V455M was just as active as wild-type GK, but with a markedly reduced $S_{0.5}$. The effects of the different GK mutants on glucose-stimulated insulin release support the kinetic and expression data. These experiments show the utility of a combined genetic, biochemical and cell-biological approach to the quantification of functional and structural changes of human GK that result from MODY-2 and GK-HI mutations.

Key words: adenovirus, β -cells, kinetics, overexpression, secretion.

INTRODUCTION

Glucokinase (GK), also called hexokinase type IV or D, is found in the liver, where it participates in the control of glucose uptake, and in the pancreatic β -cells, where it serves as glucose sensor and regulates insulin release [1,2]. The enzyme may also be expressed in selected neuroendocrine cells of the gastrointestinal tract and the brain [3]. Unlike the other members of the hexokinase family, GK is a 50-kDa monomer, lacks feedback inhibition by physiological levels of glucose 6-phosphate, and has a 100–1000-fold lower affinity for its substrate, D-glucose. Another unique feature of the enzyme is its co-operative kinetics with its substrate glucose, as indicated by a Hill coefficient of 1.8.

Hyperglycaemia in a subpopulation of individuals with maturity-onset diabetes of the young (MODY), a clinically well-distinguished familial form of non-insulin-dependent diabetes mellitus, is caused by mutations in the *GK* gene. These patients demonstrate moderately elevated fasting and post-prandial glucose concentrations, impaired glucose tolerance and autosomal dominant inheritance [4]. To date, close to 100 mutations in the *GK* gene have been identified, and are classified as MODY type 2 (MODY-2), to distinguish it from the other four MODY forms [1,2,5]. Known *GK* changes include missense mutations, nonsense

mutations and others, which consist of base-pair deletions that result in frameshift mutations or elimination of splicing sites [4–7]. It has been demonstrated that many of these mutations cause a decrease in V_{max} , an increase in the glucose $S_{0.5}$ or a change of the ATP K_m , either present alone or in combination ($S_{0.5}$ is the glucose level at the half-maximal rate; the $S_{0.5}$ is specific for an allosteric enzyme which produces a sigmoidal curve). Gidh-Jain et al. [8] have made an attempt to explain these kinetic alterations on the basis of structural features of the GK protein. Additionally, Kesavan et al. [7] have provided the first direct evidence that certain amino acid substitutions may result in protein instability as the singular cause of the enzyme defect. The crucial role of GK as glucose sensor of the β -cell was reinforced by the recent discovery of autosomal dominant GK-linked hyperinsulinaemia (GK-HI). A *GK* mutation (V455M) was found that lowers the glucose $S_{0.5}$ from the normal 8.4 to 2.9 mM without affecting any other kinetic feature of the enzyme [6].

These studies now suggest two broad reasons that need to be addressed to determine physiological relevance. First, it remains to be determined whether the mutant GK enzymes expressed in pancreatic β -cells are indeed modified in precisely the same manner as in *Escherichia coli* during expression of recombinant

Abbreviations used: GK, glucokinase; MODY-2, maturity-onset diabetes of the young type 2; GK-HI, GK-linked hyperinsulinaemia; GST, glutathione S-transferase; E1, early region 1; MOI, multiplicity of infection; RLK, rat liver GK; RIGK, rat islet GK; HIGK, human islet GK; CHX, cycloheximide; LacZ, β -galactosidase.

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GK generated in the form of glutathione S-transferase (GST)–GK-fusion protein. Secondly, it remains to be determined whether the glucose metabolism and secretory function of β -cells expressing mutant enzymes are altered, as predicted from the kinetic data. Thus several questions need to be addressed. Do changes of a single amino acid affect mRNA or protein expression? Do these mutations result in the synthesis of unstable proteins that might be degraded rapidly by proteolytic enzymes, or do they result in the expression of protein molecules that are inactive? Are glucose metabolism and glucose-stimulated insulin release from transfected β -cells consistent with the kinetic changes observed for the enzyme molecules?

To address these issues, in the present study we have analysed normal human islet GK (HIGK) protein and four different missense mutants (E70K, E300K, V203A and V455M). To this end, we have employed an adenoviral expression system to examine the metabolic and functional changes that result in the β -HC9 cells transiently expressing these mutants. Recombinant adenoviruses are suitable expression vectors that can be grown and isolated at high titres for efficient transduction of cells in culture [9].

Because of the cellular heterogeneity in pancreatic islets, experiments utilizing whole islets may frequently yield inconclusive results [10,11]. To circumvent this problem in studying the effects of GK mutations on glucose sensing and insulin secretion specifically by β -cells, we have employed the β -HC9 cell line. The β -HC9 cell line was developed from individual hyperplastic pancreatic islets of transgenic mice expressing the simian-virus-40 tag under the control of the rat insulin promoter [12]. Glucose uptake and GK-mediated phosphorylation are similar in the β -HC9 cells and normal β -cells of the islet. In addition, insulin secretion by the β -HC9 cells responds to glucose concentrations in the same physiological range as in pancreatic β -cells [13].

In the present study, we utilized first-generation adenoviruses, deleted in E1 (early region 1; dl7001) or E1 and E3 (sub360) gene regions but retaining all relevant genes in the E2, E3 and E4 regions. The vectors were engineered to express β -cell and liver isoforms of rat GK in order to overexpress for analysing their effects on glucose-stimulated insulin release and glucose usage. This approach was used further to characterize several mutant HIGK proteins in an attempt to elucidate the molecular alterations that cause hyperglycaemic and hypoglycaemic phenotypes in humans.

MATERIAL AND METHODS

Original plasmids

Both the liver and β -cell isoforms of the rat GK cDNA were obtained from Dr. Mark Magnuson at Vanderbilt University (Nashville, TN, U.S.A.). The open reading frames of the liver GK (RGKL) and β -cell GK (RGKB) isoforms were provided in the expression vector pCMV4. This vector contains a cytomegalovirus promoter, a translational augmentor from alfalfa mosaic virus, a linker primer site for *KpnI* and *XbaI* at either end of the 5' and 3' ends, respectively, and a 3' splice and polyadenylation signal from the human growth-hormone gene, as well as a bacterial origin of replication and ampicillin-resistance gene [14]. The HIGK clone originated from Dr. Alan Permutt's laboratory (Washington University, St. Louis, MO, U.S.A.), and its mutants (HIGK-E70K, HIGK-E300K, HIGK-V203A and HIGK-V455M) were generated by Dr. Mark Magnuson, as described previously by Liang et al. [15]. They were spliced into a pGEX-3X expression vector (Pharmacia Biotech, Uppsala, Sweden) containing an ampicillin-resistance gene.

Viral construction

The recombinant adenoviruses H5.020CMVRLGK, H5.010CMVRLGK, H5.020CMVHIGK, H5.020CMVHIGK-E70K, H5.020CMVHIGK-E300K, H5.020CMVHIGK-V203A and H5.020CMVHIGK-V455M were constructed with a multi-purpose plasmid, pAdCMVlink.1. A 2.2-kb *ScaI*-restricted fragment from pGEX-3X was ligated to pAdCMVlink.1 at the *EcoRV* site to generate pAdCMVHIGK, pAdCMVHIGK-E70K, pAdCMVHIGK-E300K, pAdCMVHIGK-V203A and pAdCMVHIGK-V455M. To construct pAdCMVRLGK and pAdCMVRLGK, the pCMV4RLGK and pCMV4RIGK plasmids were doubly digested with *KpnI* and *XbaI*. The plasmid pAdCMVlink.1 was cut with *KpnI* and *SpeI*. Ligation was performed in each case with T4 DNA ligase (Life Technologies, Grand Island, NY, U.S.A.). Homologous recombination between the pAdCMVGK plasmids and sub360 (H5.020) or dl7001 (H5.010) viral DNA generated recombinant adenoviruses containing cDNA encoding both liver and β -cell isoforms of rat GK, wild-type HIGK and the following mutations: HIGK-E70K, HIGK-E300K, HIGK-V203A and HIGK-V455M. In addition to deletion of the E1 region (1.0–9.6 map units), rendering the virus replication-incompetent, a small portion of the E3 region in sub360 (map units 78.4–86) was deleted. To ensure functional protein prior to co-transfection with DI7001 or sub360, tissue-culture plates (60 mm) containing HeLa cells were transiently transfected with each plasmid using lipofectamine (1 μ g of DNA per 8 μ l of lipofectamine). Western-blot analysis confirmed the presence of immunoreactive protein (results not shown). No differences were noted in the GK recombinants using either sub360 or dl7001.

These vectors were propagated in 293 cells, a human embryonic kidney cell line that supplies the E1 in *trans*, allowing the recombinant adenoviruses to replicate. The cells were infected and then harvested just prior to lysis. Adenoviral supernatants were banded twice by caesium chloride-density ultracentrifugation and purified over a Sephadex G-50 column (Sigma, St. Louis, MO, U.S.A.). Viral stocks were stored in 10 % glycerol in Hepes-buffered saline, pH 7.8, at -80°C until use. Viral concentrations were determined using a Beckman DU-640 spectrophotometer by measuring absorbance at 260 nm.

Cell transduction

β -HC9 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies), 1 % penicillin/streptomycin solution (Sigma) and 15 % fetal bovine serum (Life Technologies). Media were replaced every 2–3 days. β -HC9 cells were used between passages 20 and 30. The particle-to-plaque-forming-unit ratio for each virus was determined by a plaque assay performed on 293 cells. Cells were infected in Dulbecco's modified Eagle's medium (with no fetal bovine serum) for 1–2 h, followed by the addition of fetal bovine serum to a final concentration of 15 %. Experiments utilizing the rat liver GK (RLGK) and rat islet GK (RIGK) viruses were performed at multiplicities of infection (MOIs) of 10 or 25 and harvested 24 or 96 h post-infection. Experiments involving HIGK and its mutants were all done at MOI 100 and harvested 24 h post-infection.

GK activity in cell extracts

Cells were infected with each of the above recombinant adenoviruses at varying MOIs. They were then harvested at various times by washing twice with PBS, followed by mechanical removal of the monolayer using a cell lifter (Fisher, Malvern, PA, U.S.A.). Cells were lysed with a mechanical homogenizer in

a homogenizing buffer, pH 7.8 (20 mM K_2HPO_4 /1 mM EDTA/110 mM KCl/5 mM 1,4-dithiothreitol). Samples were centrifuged at 10000 *g* for 30 min at 4 °C. The supernatant was collected and assayed for GK activity at 30 °C with an NAD⁺-coupled spectrophotometric assay using glucose-6-phosphate dehydrogenase from the bacterium *Leuconostoc mesenteroides* (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Supernatants were assayed over a range of glucose concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 mM. The data fitting was carried out with a non-linear optimization program contained in Kaleidagraph, which is based on the Levenberg–Marquardt algorithm [16]. GK activity was corrected for hexokinase activity by subtracting a baseline activity obtained at 0.78 mM glucose, at which point the hexokinase activity was nearly saturated.

Glucose-stimulated insulin-release assay

Insulin-release assays analysing the effect of overexpressing rat GK were performed 96 h post-infection. The assays comparing the effect of overexpressing HIGK and its mutants were carried out 24 h post-infection. Prior to the insulin-release assay, tissue-culture medium was removed from each plate and the cells were rinsed three times with 1 ml of modified Hank's buffer. They were then preincubated in 1 ml of this buffer for 30 min. Insulin secretion was measured in β -HC9 cells incubated in modified Hanks buffer [124 mM NaCl/4.6 mM KCl/1 mM KH_2PO_4 /5 mM $NaHCO_3$ /10 mM Hepes (free acid)/10 mM Hepes (sodium salt)/0.25% BSA (pH 7.35–7.40)] for 60 min with varying concentrations of glucose at 37 °C. Aliquots of buffer were then spun through oil to remove detached cells and assayed for insulin by a single-antibody radio-immunoassay with anti-insulin serum obtained from Miles (Elkhart, IN, U.S.A.) and rat insulin as standard (Lilly, Indianapolis, IN, U.S.A.) [17].

Glucose usage

Glucose usage was measured using a radiometric method with modifications [18]. Cells cultured in a 12-well plate were first incubated with modified Hank's buffer in the absence of glucose for 60 min at 37 °C in a shaking water bath. Then, incubation fluid was replaced by 400 μ l of the same buffer containing different glucose concentrations (0.3–30 mM) as well as 4 μ Ci of 5- 3H]glucose. After another 60-min incubation, the reaction was stopped with 40 μ l of HCl (1 M) and a 50- μ l aliquot was transferred to a diffusion tube to perform the diffusion step at 34 °C overnight. After corrections for incomplete equilibration of the 3H_2O and the blank of the tritiated sugar stock solution, glucose usage was calculated according to Ashcroft et al. [19].

Glucose oxidation

Glucose oxidation was measured using published methods with modification [20]. Cells cultured in 25-cm² flasks were fed with 2 ml of modified Hanks buffer containing different glucose concentrations (ranging from 0.3 to 30 mM) and 3 mCi/ml of [U - ^{14}C]glucose. Each flask was sealed with a rubber stopper. After 90 min of incubation, 400 μ l of 1 M HCl was injected. In the meantime, 200 μ l of Hyamine hydroxide (Research Products International, Mount Prospect, IL, U.S.A.) was injected through the rubber stopper to impregnate a filter paper that was preloaded in a centre well of the flask. Flasks were further shaken at room temperature for 5 h. The filter, together with the centre well, were then transferred into a scintillation vial to count $^{14}CO_2$ for calculation of the rate of glucose oxidation. A 50- μ l aliquot

of medium was also counted for calculation of specific radioactivity.

Northern- and Western-blot analyses

Total RNA was extracted from β -HC9 cells infected with wild-type and mutant GK viruses using TRIzol reagent (Life Technologies). Total RNA (13 μ g per lane) was separated on 1.2% agarose/formaldehyde gels, blotted on to Hybond-N nylon membrane (Amersham, Cleveland, OH, U.S.A.), and hybridized overnight with a random-primer [^{32}P]dCTP-labelled *Cla*I- and *Sph*I-restricted internal fragment of GK cDNA (700 bp). Blots were washed and then exposed to X-ray film with an intensifying screen at –80 °C.

Homogenate (20 μ g) was resolved by SDS/PAGE (10% gel) and electrotransferred on to nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.). The membrane was incubated with a sheep anti-rat GST–GK-fusion protein antibody (provided kindly by Dr. Mark Magnuson) at a 1:1000 dilution. A donkey anti-sheep IgG horseradish peroxidase-conjugated molecule (Sigma) was used as the secondary antibody (1:1000 dilution). Immunoreactivity was detected using an ECL[®] Western-blotting-detection reagent (Amersham, Cleveland, OH, U.S.A.).

RESULTS

Kinetic characteristics of wild-type rat GK proteins expressed in β -HC9 cells

Particle-to-plaque-forming unit ratios for AdCMVRLGK and AdCMVRLGK were determined to be 30:1 and 40:1 respectively. Kinetic properties of GK expressed in β -HC9 cells of intermediate passage (20–30) were determined spectrophotometrically (see Table 1).

Table 1 Enzymic properties of wild-type and mutant forms of GK

Infections of β -HC9 cells were carried out in Dulbecco's modified Eagle's medium for 1–2 h, at which time fetal bovine serum was added to achieve a final concentration of 15%. Cells were harvested at different time points and kinetic analysis was performed on supernatants containing soluble cellular protein. The means $\pm$ S.E.M. of the protein concentrations in the soluble cellular protein extracts (mg/ml) of the GK enzymes were as follows: LacZ, 2.11; RIGK, 1.34 $\pm$ 0.19; RLKG, 1.71 $\pm$ 0.15; HIGK, 2.20 $\pm$ 0.23; E70K, 2.22 $\pm$ 0.21; E300K, 2.58 $\pm$ 0.28; V203A, 2.45 $\pm$ 0.23; and V455M, 2.21 $\pm$ 0.15. Activity values for AdCMVRLGK and AdCMVRIGK were from cells that were infected at an MOI of 25 for 96 h. Activities reported for AdCMVHIGK, AdCMVHIGK-E70K, AdCMVHIGK-E300K, AdCMVHIGK-V203A and AdCMVHIGK-V455M were determined at an MOI of 100 and 24 h post-infection. Approximate hexokinase contributions to the total soluble glucose-phosphorylating activities were as follows: HIGK, 4%; E70K, 2%; E300K, 50%; V203A, 16%; and V455M, 6%. AdCMVLacZ expressed an irrelevant transgene and was used as the control virus. The kinetics results for AdCMVLacZ are the means of two experiments. The other results are derived from three or more experiments and represent means $\pm$ S.E.M. A unit is defined as the amount of enzyme that generated 1 μ mol of product per min at 30 °C. Kinetic data for GST–GK were obtained from Kesavan et al. [7].

	$S_{0.5}$ for glucose (mM)	Hill coefficient for glucose (<i>h</i>)	Activity (units/mg of protein)
Purified GST–GK	8.21 $\pm$ 0.55	1.78 $\pm$ 0.02	67.87 $\pm$ 0.89
Rat GK-transduced β -HC9 cells			
AdCMV LacZ	8.5	1.5	5.25 $\times 10^{-4}$
AdCMV RIGK	5.76 $\pm$ 0.23	1.84 $\pm$ 0.05	(6.86 $\pm$ 3.18) $\times 10^{-2}$
AdCMV RLKG	5.75 $\pm$ 0.33	1.85 $\pm$ 0.07	(5.73 $\pm$ 3.39) $\times 10^{-2}$
HIGK-transduced β -HC9 cells			
AdCMV HIGK	6.36 $\pm$ 0.63	2.34 $\pm$ 0.19	(1.77 $\pm$ 0.39) $\times 10^{-2}$
AdCMV HIGK-E70K	10.3 $\pm$ 0.45	2.36 $\pm$ 0.26	(3.93 $\pm$ 1.39) $\times 10^{-2}$
AdCMV HIGK-E300K	6.51 $\pm$ 0.38	2.49 $\pm$ 0.59	(1.45 $\pm$ 0.12) $\times 10^{-3}$
AdCMV HIGK-V203A	46.3 $\pm$ 1.7	1.82 $\pm$ 0.18	(4.29 $\pm$ 0.64) $\times 10^{-3}$
AdCMV HIGK-V455M	3.35 $\pm$ 0.32	2.33 $\pm$ 0.15	(1.17 $\pm$ 0.093) $\times 10^{-2}$

Figure 1 The overexpression of GK results in increased glycolysis

Glucose-usage curves for both rat liver and β -cell isoforms of GK are shown. Cells were infected at an MOI of 10 for 96 h. Four experiments were performed. Data reported are means $\pm$ S.E.M. (A) The full dose-response curves; and (B) the glucose levels from 0.3 to 5.0 mM.

Previously published data on recombinant GST-GK were used as a comparison for the data obtained [5–7]. AdCMVLacZ (where LacZ is β -galactosidase)-infected β -HC9 cells, which served as a negative control, showed very low endogenous GK activity. Liver and β -cell isoforms of rat GK had a 25% lower apparent $S_{0.5}$ for glucose compared with the recombinant human GST-GK protein from *E. coli*. The Hill coefficient was approximately 1.8, indicating the co-operative nature of the reaction mechanism. The specific activity of GK from virally infected cells was much lower than that of the purified protein, reflecting the small amount of virally expressed GK protein in the excess of cellular proteins present in the lysate, compared with the virtually pure GST-GK-fusion protein.

RIGK/RLKG overexpression alters glucose metabolism and glucose-stimulated insulin release in β -HC9 cells

Glucose usage was determined in β -HC9 cells transduced with rat GK viruses at an MOI of 10 using the production of $^3\text{H}_2\text{O}$

Figure 2 Glucose oxidation in β -HC9 cells overexpressing RIGK

β -HC9 cells were infected at an MOI of 10 for 24 h. The cells were then incubated with [^{14}C]glucose, and conversion into $^{14}\text{CO}_2$ was analysed. Data are means $\pm$ S.E.M. of four separate experiments. (A) The full dose-response curves. (B) Shows that enhanced glucose oxidation in cells overexpressing GK is demonstrable only at levels below 3 mM. GOPDH, α -glycerophosphate-dehydrogenase.

from 5- ^3H]glucose as a measure of the rate of glycolysis. Glucose usage was measured following 96 h of viral exposure. β -HC9 cells transduced with RLKG and RIGK viruses had very similar glucose-concentration-dependency curves. They metabolized glucose at a significantly higher rate (3-fold) at high glucose than uninfected control cells or virally transduced cells expressing the irrelevant transgene *LacZ* (Figure 1).

The production of $^{14}\text{CO}_2$ was markedly increased in cells transduced with the rat pancreatic GK virus at glucose concentrations lower than 3 mM when compared with mock infection or cells transduced with cytosolic α -glycerophosphate dehydrogenase, used as an irrelevant recombinant adenovirus. At glucose concentrations above 3 mM, glucose oxidation of cells transduced with the RIGK virus decreased with increasing concentrations of glucose, in contrast with the opposite response in the controls (Figure 2). These results suggest that factors other than GK partially determine the rate of glucose oxidation.

β -HC9 cells transduced with a virus expressing an irrelevant transgene, *E. coli LacZ*, demonstrated that these cells secreted increasing amounts of insulin in response to increasing concentrations of extracellular glucose. Overexpression of RIGK led to a 5-fold increase in insulin release at glucose concentrations between 0 and 10 mM (Figure 3). Insulin secretion plateaued at glucose concentrations above 10 mM. Overexpression of GK thus resulted in a marked leftward shift of the concentration-

Figure 3 The up-regulation of GK and glucose-stimulated insulin secretion

Insulin-release curves for the rat β -cell isoform GK and an irrelevant virus at an MOI of 25 and 96 h post-infection are shown. Data are from three experiments in terms of means $\pm$ S.E.M.

dependency curve. The plateau at 10 mM suggests that factors other than GK become rate-limiting at higher concentrations of glucose. This underscores the important fact that GK serves as the rate-limiting enzyme in the glycolytic pathway within very narrow physiological limits, which were probably exceeded by far in this overexpression study.

Kinetic characteristics of wild-type and mutant HIGK

Guided by the knowledge gained in the model studies, we next examined wild-type HIGK and four spontaneous human mutants using the adenoviral expression system. Expression of the wild-type HIGK, like both rat pancreatic and liver GK, caused a 25% reduction of the $S_{0.5}$ for glucose as compared with the GST-GK-fusion protein (Table 1). Mutant GKs displayed changes in their kinetic parameters compared with controls. HIGK-E70K was twice as active as HIGK, but glucose affinity was almost half that of wild-type. V203A was less active than wild type, and the glucose $S_{0.5}$ was 46.3 mM compared with 6.4 mM for wild-type. HIGK-V455M displayed similar activity to wild-type, but its $S_{0.5}$ for glucose was reduced from 6.4 to 3.4 mM, indicating that this mutant has a greater affinity for glucose. The other mutant investigated in this study, HIGK-E300K, had a normal $S_{0.5}$ value for glucose; however, it exhibited a 10-fold decrease in activity compared with the wild type.

Glucose-induced insulin release by cells infected with mutant GKs

The kinetic studies suggested that mutant GKs had altered kinetic characteristics when expressed in β -HC9 cells. Therefore, our next goal was to analyse if secretory functions of β -HC9 cells, and by inference metabolic properties, were also affected by the mutations. β -HC9 cells infected with wild-type AdCMVHIGK and AdCMVHIGK-E70K had 2–3-fold greater insulin secretion than mock-infected cells between 0 and 10 mM glucose (Figure 4). There was a decline in insulin secretion at glucose concentrations above 10 mM. Of the other mutants,

Figure 4 Insulin-release patterns for the native and four mutant forms of human β -cell GK

Infection (24 h) of β -HC9 cells at an MOI of 100 was followed by an insulin-release assay. Data represent the means $\pm$ S.E.M. of three separate experiments. Two panels are used for clarity of presentation.

HIGK-E300K demonstrated a barely detectable increase in insulin secretion above that of mock-infected cells. Its insulin-release curve showed practically the same glucose-dependent insulin-secretion profile as uninfected cells. HIGK-V203A had increasing insulin secretion at all concentrations of glucose. At 10 mM glucose, it had 2-fold greater insulin secretion than the mock control. HIGK-V455M had peak insulin secretion at 3 mM glucose that was about three times greater than the control. At glucose concentrations above 3 mM, insulin secretion decreased in the presence of increasing amounts of glucose.

Analysis of transcriptional and translational control of wild-type and mutant HIGK protein expression

To obtain basic information about the time course of GK expression, β -HC9 cells were infected at an MOI of 10 with the rat β -cell isoform of the GK adenovirus and incubated for 48 h. Cycloheximide (CHX), at a concentration that was selected in a series of experiments to effectively block new protein synthesis in these cells (results not shown), was then added to the plates. GK activity was assayed at 0, 4, 8, 12, 24, 36 and 48 h following the addition of, or in the absence of, CHX. In the presence of CHX

Figure 5 GK protein expression after the arrest of protein synthesis

β -HC9 cells were infected with the wild-type β -cell isoform of rat GK at an MOI of 10. Post-infection (48 h), 5 μ g/ml CHX was added to the cells for a final concentration of 17.7 μ M. Cells were harvested at various time points and GK activity was determined in extracts of cells maintained in the absence or presence of CHX. Single data points are given and represent the rates observed in extracts using a standardized protocol.

the enzyme activity gradually decreased over time (Figure 5). In fact, the enzyme activity at 24 h after the addition of CHX was approximately half the activity at the start of CHX treatment, indicating that the half-life of the GK protein may be about 24 h. On the other hand, the activity obtained in the absence of CHX gradually increased over 24 h, at which time it peaked, and then decreased over the next 24 h. These results indicate that the peak expression of the rat GK protein via adenoviral infection of β -HC9 cells occurred at about 72 h post-inoculation.

To analyse further human GK protein expression, β -HC9 cells were infected, harvested at 24, 36 and 48 h, and then assessed by Western blotting. Protein expression of wild-type HIGK, HIGK-E70K and HIGK-V455M appeared stable over 48 h. However, this was not the case for the other mutants. AdCMVHIGK-E300K-infected β -HC9 cells had a 3-fold reduction in GK protein expression at 24 h post-infection compared with the wild type (Figure 6). At 36 h, the level was about 8% of wild type and, 48 h post-infection, there was little HIGK-E300K protein detectable. These results were observed consistently in a series of

Figure 7 mRNA expression of human native and mutant islet GK

Total RNA was isolated from virally transduced β -HC9 cells with an MOI of 100 at 24 h post-infection. Samples were hybridized with a 700-bp internal fragment of the GK cDNA. β -Actin was used to quantify the RNA loaded and to normalize the amount of GK expression. The intensities of GK and actin expression were quantified using the model GS-700 imaging densitometer (Bio-Rad). Results of a single experiment are shown in the top panel. The bottom panel demonstrates the means $\pm$ S.E.M. from three separate experiments.

experiments, which is corroborated by the lack of GK activity in HIGK-E300K-infected cells at 48 h (results not shown). HIGK-V203A had protein expression similar to the normal protein; however, there was a slight but reproducible retardation in the

Figure 6 Western-blotting analysis of wild-type and mutant GK expression

Cell lysates were collected 24, 36 and 48 h post adenoviral infection at an MOI of 100. Total protein (20 μ g) was loaded per lane. A polyclonal antibody to a GST-GK-fusion protein was used to quantify GK expression over time. The intensity of GK expression was measured using a model GS-700 imaging densitometer (Bio-Rad, Hercules, CA, U.S.A.). Results of a single comprehensive experiment are shown.

migration of its protein on the SDS/polyacrylamide gel (Figure 6).

In response to the differences in protein expression between mutants, we sought to identify the stage in protein synthesis where the differences arose. Total RNA was isolated from β -HC9 cells infected with each virus at an MOI of 100 for 24 h. Based on the CHX experiment discussed above (see Figure 6), virally infected cells should still have been synthesizing protein at 24 h post-infection. The membrane was probed with a 2-kb β -actin probe, in order to normalize total RNA loaded, and a 700-bp GK probe. GK mRNA expression of E70K, E300K and V203A was very similar (Figure 7). The data suggest that decreased activity and protein expression of E300K are not due to diminished levels of mRNA.

DISCUSSION

Choice of the cell system for cell-biological study of mutant GK

In this report, we sought to expand the understanding of the molecular basis for MODY-2 and GK-HI caused by GK mutations. Specifically, we examined how different mutant GK enzymes overexpressed in intact β -cells modify glucose metabolism and secretory function of the cells. This investigation is an essential complement to previous studies of the kinetic and biophysical properties of recombinant mutant GK proteins expressed in *E. coli*. The β -HC9 cell line was chosen because it has been used previously to characterize glucose metabolism and insulin release [12,13,21] and is characterized by a normal concentration-dependency curve for glucose-stimulated insulin release. The kinetic characteristics of glucose usage, glucose oxidation and glucose-induced oxygen consumption are controlled by glucose phosphorylation. These findings are in line with the concept that the kinetics of glucose metabolism, beginning with phosphorylation in the cytosol and culminating in mitochondrial generation of metabolic coupling factors, are determined by the major glucose-phosphorylating enzyme GK.

In order to validate this test system, recombinant adenoviruses expressing rat isoforms of β -cell GK and liver GK were used to transfect intermediate-passage β -HC9 cells. The effects of overexpression of the enzyme were studied by analysis of glucose usage, glucose oxidation and insulin release. GK overexpression led to a nearly 100-fold increase in GK phosphorylating activity over basal levels, consistent with previous reports [10,11]. One limitation of using adenoviral vectors is the high level of expression, which results in enzyme activities far in excess of levels *in vivo*. However, transducing cells with a lower MOI results in less GK protein production, in part due to fewer transduced cells, which compromises interpretation. One way to study graded induction would be with an inducible promoter system, such as the 'tet-on' system of Gossen et al. [22], but this approach might be insufficient for screening a large number of different mutants, as needed here.

As noted in Figure 1, a plateau effect was seen at glucose concentrations above 10 mM, which suggests that, at high glucose concentrations, other downstream components of the pathway become rate-limiting instead of GK, as is true in the setting *in vivo*. The effect of GK overexpression on glucose oxidation was also very pronounced. Glucose oxidation increased at glucose concentrations between 0.3 and 3.0 mM in cells overexpressing GK, but was apparently inhibited at higher glucose concentrations (Figure 2). A comparison of the results in Figures 1 and 2 shows that with increased flux through GK oxidative metabolism of glucose cannot keep pace with glycolysis. This may be the result of the limiting cofactors ADP and P_i . As might be predicted from the GK activity, glucose usage and glucose-

oxidation data, a leftward shift in the insulin-release curve is brought about by overexpression of GK in β -HC9 cells. The magnitude of the increase was approximately 5-fold at 10 mM glucose, a value somewhat higher than, but consistent with that previously reported for, INS-1 cells [10].

Our results, demonstrating that overexpressing GK increases glucose usage and glucose-stimulated insulin release in β -cells, are in agreement with those of Wang and Iynedjian [10,23]. When a recombinant adenovirus expressing RLGK under the control of a high-level constitutive cytomegalovirus promoter was used to transduce isolated rat islets, no increases in either glucose usage or insulin release were noted, despite a 20-fold increase in GK activity [11]. The metabolic differences observed in these experiments and ours suggest that isolated islets do not seem to provide a suitable system to assess GK-expression studies. The results from the present study suggest that the β -HC9 cell line might be very useful for characterizing wild-type as well as mutant GK enzymes.

Effects of GK mutant-enzyme overexpression on β -HC9 cell function

Our analysis of wild-type as well as four MODY-2- and GK-HI-associated mutant isoforms of HIGK in β -HC9 cells revealed distinct functionality for individual mutants. Previous studies had suggested that HIGK-E300K was thermally unstable, as demonstrated by reduced activity compared with the wild type at temperatures above 45 °C, which probably reflects critical structural defects of the mutant protein [7,15]. The results of our study suggest that the mutant protein is unstable *in vivo* as well. First, the enzyme was very difficult to detect in extracts of β -HC9 cells. Attempts to overexpress the HIGK-E300K protein did not result in greater insulin secretion compared with background levels. Western-blot analyses demonstrated that there was only minimal protein expression at 24 h and practically none at 48 h post-infection. Northern-blot analyses confirmed that HIGK-E300K and wild-type HIGK produced similar amounts of GK mRNA. Thus our results, together with published data on purified recombinant E300K *in vitro*, strongly suggest that the E300K mutation causes instability and increased proteolytic breakdown of this protein. It is likely therefore that mutation causes disease by causing a lack of functional protein. Further studies targeted at exploring the degradative pathway of E300K protein are required.

In contrast with the results for the E300K mutant, E70K was as stable as wild-type human GK protein when assessed in the intact cell under physiological conditions. Both protein and RNA expression were similar for the wild-type and mutant protein. Interestingly, previous studies *in vitro* found this mutant to be thermally unstable. Our results do, however, indicate that HIGK-E70K has a reduced affinity for glucose, as indicated by its nearly 2-fold higher $S_{0.5}$ value. These results therefore support the hypothesis that HIGK-E70K causes MODY because of its decreased affinity for the substrate and possible small changes of catalytic capacity rather than because of protein instability.

SDS/PAGE analysis has shown previously that GK mutant proteins expressed in a bacterial system migrated to the same position as the wild-type protein [7,8]. However, this was not the case here, when eukaryotic cells were used as an expression system. HIGK-V203A migrated at a slightly slower rate (perhaps suggesting a somewhat higher molecular mass or conformational changes in the protein) than the wild type and the other mutants that we studied (Figure 6). This discrepancy may be due to post-translational processing. Whereas the bacterial system merely

expresses the cDNA cloned into it, the β -HC9 cells not only express the protein but are also capable of post-translational modifications. However, differences in migration could also be due to the amino acid substitution itself, which could account for a conformational change in the tertiary structure and thus lead to less binding by SDS to the mutant protein and alteration of the migration velocity. Such anomalies in SDS/PAGE usually remain consistent from one experiment to the next [24]. Further experiments need to be done to determine how the amino acid substitution of HIGK-V203A affected the post-translational modification (if any) or structural features of the GK protein. The present data for the enzyme and the insulin-release pattern of glucose-stimulated β -HC9 cells are consistent with the clinical results and reports about kinetics with recombinant V203A GK.

The HIGK-V455M mutation causes an increased affinity for glucose, which correlates with its hypoglycaemic phenotype. The HIGK-V455M insulin-release curve is shifted significantly to the left with a peak at 3 mM, as compared with 10 mM or higher for the wild type and MODY-2 mutants. Thus it takes very low glucose concentration to induce insulin release. In a physiological setting, the blood glucose concentration is maintained at 5–8 mM; therefore, at such glucose concentrations it is understandable why this mutation results in hyperinsulinaemia.

Conclusion

In summary, recombinant adenoviruses have been used to study the effects of high-level islet GK and liver GK expression in β -HC9 cells. Overexpression of the enzyme of nearly 100-fold resulted in a 5-fold enhancement of insulin release in response to low glucose, i.e. a leftward shift of the respective dose-response curves. The present data are in accord with the widely held view that GK is the rate-limiting step in β -cell glucose metabolism and serves as glucose sensor under physiological conditions. However, when the GK enzyme is overexpressed far above its normal range and when the modified cells are then functionally tested with glucose, other metabolic steps and factors become limiting, depending on the glucose concentration during the test. Nevertheless, the cell-biological system used here provided an opportunity to evaluate GK mutants *in vivo*.

The present study illustrates that a four-phase strategy is needed to fully characterize GK mutants that cause hyper- or hypoglycaemia. (i) Demonstration of the linkage of the phenotype to the GK locus. (ii) Identification of the biochemical nature of the causative GK mutation. (iii) Characterization of the recombinant mutant GK as to kinetic constants (k_{cat} , glucose $S_{0.5}$, Hill coefficient for glucose, ATP K_m , thermal stability and other relevant parameters). (iv) Expression of the mutant in the β -HC9 cell line or any other suitable β -cell line (e.g. INS-1 or MIN-6) for enzyme kinetic analysis, for studies of expression by Western- and Northern-blot analysis and for determining the particular insulin-release pattern.

We have accomplished this for wild-type HIGK and the four mutants E70K, V203A, E300K and V455M. The application of a cell-biological approach is demonstrated to be of critical importance to fully characterize these particular GK mutants. Without this step, our database would be incomplete on MODY-2 and GK-HI.

The number of GK mutants is approaching 100 and will most likely increase even further with time. Since the GK protein plays a pre-eminent role in glucose homeostasis on par with insulin and the insulin receptor, since the enzyme is a promising drug target and since its biochemical and biophysical characteristics are of unique interest for basic science, it is crucial to continue vigorously the detailed quantitative exploration of the abundant treasures of spontaneous GK mutants of MODY-2 and GK-HI, using complementary biochemical, biophysical and cell-biological approaches, as practiced in the studies of this series (Liang et al. [15], Kesavan et al. [7], and E. A. Davis, A. Cuesta-Muñoz, M. Raoul, C. Buettger, I. Sweet, M. Moates, M. A. Magnuson and F. M. Matschinsky, unpublished work).

This research was funded by NIDDK grants 19525 and 22122 (F.M.M.), R01 DK47811 (S.E.R.) and DK 49814 project 3 (F.M.M. and S.E.R.). We thank Dr. Kevin Foksett, Dr. D. J. Sidjanin and Dr. Dolena Ledee for their critical and helpful reviews of this work prior to submission.

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