

FIG. 1. Results of OGTTs (A) and intravenous glucagon stimulation tests (B). ■, Proband before treatment; ○, the mother. Upper and lower normal limits are represented by dotted lines in A. S, serum.

mother had the same mutation and asymptomatic fasting hypoglycemia.

RESEARCH DESIGN AND METHODS

Subjects. The proband, a boy from nonconsanguineous and reported healthy Caucasians, had a history of severe neonatal hypoglycemia. Born at term but small for gestational age (birth weight 2,400 g and length 46 cm), he had a blood glucose of 0.8 mmol/l on the first day of life and was treated with intravenous glucose and steroids. Diazoxide (up to $8 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) was given from 1 to 8 months of age. During treatment, fasting blood glucose was maintained in a range of 2.6 to 3.2 mmol/l. Attempts to discontinue diazoxide treatment during the first half year of life resulted in blood glucose levels below 2.0 mmol/l and clinical signs of hypoglycemia. At follow-ups during 1–4 years of age, psychomotor development was found to be normal. From 8 months to 4 years of age, fasting blood glucose remained in the range of 2.4–3.0 mmol/l. Frequent meals reduced the incidence of the mild clinical symptoms of hypoglycemia during childhood. From 11 to 14 years of age, his BMI increased from 24.1 to 34.0 kg/m^2 . At 14 years of age, the proband experienced three hypoglycemic attacks associated with convulsions and unconsciousness. His blood glucose was between 2.1 and 2.5 mmol/l on admittance after oral glucose loads. Pubic hair scored Tanner IV, and testicular volume was 5/12 ml. Two electroencephalogram (EEG) examinations within 1 month of the severe attacks were abnormal, with multifocal low activity and occasional sharp waves. A third EEG 3 months after the last coma episode was normal and so was the computed tomography (CT) scan of the brain.

The 42-year-old mother never had any symptoms of hypoglycemia, but her fasting blood glucose was low at 2.9–3.5 mmol/l. The father, the brother, and the mother's other family members never had hypoglycemic symptoms and had normal fasting glucose levels. The mother's mother, however, presented with type 2 diabetes at age 72 years, with a blood glucose of 28.7 mmol/l and an HbA_{1c} of 17% (normal range 4.3–6.3%).

Clinical laboratory procedures. Insulin, C-peptide, and proinsulin concentrations were analyzed by the two-site time-resolved immunofluorometric Delphia method (16), except for neonatal insulin, which was determined by radioimmunoassay (RIA). Normal adult Delphia fasting values (± 2 SD) are serum insulin 12–77 pmol/l, serum C-peptide 130–760 pmol/l, and serum proinsulin 2–23 pmol/l (17). The analysis of C-peptide includes the low concentration of proinsulin due to cross-reaction.

Oral glucose tolerance tests (OGTTs) were performed with 1.75 g glucose per kilogram body weight according to World Health Organization criteria, including measurement of blood glucose, serum C-peptide, serum proinsulin, and serum insulin concentrations at 0, 30, 60, 120, and 150 min. Normal adult Delphia values of serum insulin, serum C-peptide, and serum proinsulin (± 2 SD) in response to an OGTT are shown in Fig. 1 (17).

Intravenous glucagon tests ($15 \mu\text{g}/\text{kg}$; maximally 1 mg) were performed with determination of blood glucose, serum C-peptide, serum proinsulin, and serum insulin at 0, 2, 4, 6, 8, and 10 min (18).

Identification of the PHHI gene. All SUR1, Kir6.2, and GK exons including intron-exon boundaries were amplified by PCR and screened for mutations by single-strand conformation polymorphism (SSCP) (SUR1 and Kir6.2) (19) or by denaturing gradient gel electrophoresis (DGGE) (GK) (20). All sequence variations detected by SSCP or DGGE were subjected to DNA direct sequenc-

ing using the BigDye terminator sequencing kit (Perkin Elmer Applied Bio Systems, Foster City, CA) on an Applied Bio Systems DNA sequencing apparatus 373A (Perkin Elmer).

Kinetic analysis. Recombinant human islet A456V GK was generated using principles and practical procedures previously described (21). The enzyme was expressed in the form of a glutathionyl S-transferase (GST) fusion protein to facilitate purification. The kinetic analysis of GST-GK A456V was performed using the protocols developed during the study of GST-GK V455M and other GK mutations (22). The results were compared with those obtained with wild-type GST-GK preparations newly made for the present investigation. The relative activity index, an expression of the proposed enzyme's in situ phosphorylation capacity based on expression at 5 mmol/l blood glucose, was calculated according to the following (22):

$$\text{Relative activity index} = \frac{k_{\text{cat}}}{S_{0.5}^{\text{nH}}} \times \frac{2.5}{2.5 + \text{ATP}K_m} \times \frac{5^{\text{nH}} \times 2}{5^{\text{nH}} + S_{0.5}^{\text{nH}}}$$

An intracellular ATP concentration of 2.5 mmol/l is assumed in these calculations. The relative activity index is normalized to a basal blood glucose of 5 mmol/l to account for GK expression.

Structural analysis. Structural analysis of activating mutations in the glucokinase enzyme was performed using the structural model of Mahalingam et al. (23). The location of activating mutations in a putative "allosteric" activator site is indicated.

Mathematical modeling. The kinetic data were used to calculate the glucose threshold for GSIR using a mathematical model described previously (22). However, the impact of blood glucose levels on GK expression for both alleles was here considered in contrast to earlier modeling studies, which ignored the altered expression of the mutants and relied entirely on the adaptation of the wild-type allele. For this purpose, the following expression coefficient was used for either allele: $e = (S^{\text{nH}} \times 2)/(S^{\text{nH}} + S_{0.5}^{\text{nH}})$ instead of an empirical factor of 0.2 per mmol/l glucose change. S refers to the glucose level at threshold, nH is the Hill coefficient for cooperativeness with glucose, the numerical value of 2 indicates that half-maximal induction is achieved at glucose $S_{0.5}$, and $S_{0.5}$ refers to the concentration of glucose needed to achieve the half-maximal rate of phosphorylation. Because of the lower glucose $S_{0.5}$ of A456V, its expression at low glucose is less reduced than that of the wild-type, which falls to negligible levels.

RESULTS

Clinical data. In the neonatal period, investigations for the cause of hypoglycemia showed no urine ketone bodies, and urine screening for diagnostic amino acids, organic acids, and intermediates of carbohydrate metabolism was also negative. While on prednisolone, repeated determinations of serum insulin detected values of <2.0, 4.2, and 8.2 mU/l, respectively (using the RIA method with a normoglycemic reference value of <14 mU/l), with corresponding blood glucose values ranging from 2.5 to 2.7 mmol/l. Intramuscular glucagon stimulation at 26 days of age showed a normal glucose response of a 2.8 mmol/l increase at 15 min but showed low basal (2.8 mmol/l) and 60-min (2.3 mmol/l) values. The proband's fasting blood glucose was low both during and after diazoxide treatment (2.4–3.2 mmol/l), but frequent meals reduced the incidence of mild hypoglycemic symptoms. From 11 to 14 years of age, his BMI increased from 24.1 to 34.0 kg/m².

At age 14 years, the proband had no urine ketones during severe hypoglycemic attacks. Hyperinsulinism was recorded with fasting serum levels of 224 pmol/l insulin, 1,519 pmol/l C-peptide, and 29 pmol/l proinsulin at a concurrent blood glucose of 3.1 mmol/l. Serum/plasma ammonia, cortisol, IGF-I, thyroid-stimulating hormone, and lactate were normal. It was determined by CT scan, routine and endoscopic ultrasound examination of the liver and pancreas were normal. Treatment with diazoxide was resumed (2 mg · kg⁻¹ · day⁻¹) initially with satisfactory results. However, because of repeated hypoglycemic attacks, the diazoxide dose was increased to 12 mg · kg⁻¹ · day⁻¹. After adding chlorothiazide at 10 mg · kg⁻¹ · day⁻¹,

it was possible to reduce diazoxide to 5 mg · kg⁻¹ · day⁻¹. In time, obesity was moderately reduced to a BMI of 30.0 kg/m². After 6 months of treatment, his HbA_{1c} was 3.8% but gradually reached normal values. Lipid profiles during this treatment revealed moderately high fasting serum triglycerides of 2.07–2.64 mmol/l (normal range 0.46–1.86 mmol/l), whereas fasting cholesterol, LDL cholesterol, and direct HDL cholesterol were within the normal range.

The 42-year-old mother never had any symptoms of hypoglycemia. Her BMI was 23.3 kg/m². Her serum levels of insulin, C-peptide, and proinsulin were in the range of normoglycemics, but the fasting blood glucose was low at 2.9–3.5 mmol/l, indicating relative hyperinsulinemia. Her plasma ammonia concentration was normal. Fasting serum HDL cholesterol was high at 2.27 mmol/l (normal range 0.78–1.74 mmol/l), whereas LDL cholesterol, cholesterol, and triglycerides were normal.

Stimulation tests. An OGTT and an intravenous glucagon stimulation test were performed on the proband, before drug treatment, and on his mother (Fig. 1). In the proband, at completion of the OGTT and with a blood glucose of 1.6 mmol/l, the only clinical sign of severe hypoglycemia was sweaty hands. He had vastly exaggerated responses of serum levels of insulin, C-peptide, and proinsulin, whereas the mother had a blunted or delayed peptide response to the OGTT.

The intravenous glucagon stimulation test showed pronounced β -cell response with large early increases in serum levels of insulin, C-peptide, and proinsulin in the proband compared with moderate increases in the mother. In both case subjects, the increase in blood glucose from low fasting levels suggested the presence of significant glycogen stores and a normal glucose mobilization from the liver.

Screening candidate genes. No pathogenetic variants were found in the SUR1 or Kir6.2 genes in a search by SSCP. The molecular scanning of the GK gene by DGGE detected abnormally migrating bands in exon 10. A heterozygous mutation substituting valine for alanine at codon 456 (A456V) was found after direct sequencing. The A456V mutation was also found in the mother of the proband but not in the normoglycemic brother or the maternal grandmother (note that the maternal grandfather was deceased). In 80 normal control subjects, no abnormally migrating bands in exon 10 were detected.

Biochemical characterization of A456V. The mutant enzyme was expressed as a GST fusion protein, and the purified enzyme was then subjected to kinetic analysis (Table 1). The enzyme showed an increased affinity for glucose (about threefold), and the Hill coefficient was lowered. The k_{cat} , or turnover number, was significantly increased (~1.6-fold). K_m for ATP was in the normal range and was elevated by high glucose, indicating normalcy of ATP/glucose interactions. The two potential inhibitor molecules of GK (i.e., stearyl-CoA as an example of long-chain acyl-CoA and GK regulatory protein) were at least as effective with the mutant as with the wild-type enzyme (not shown), arguing against the possibility that deinhibition might be the cause of GK activation in vivo. The effective activation of mutant GK is expressed by the relative activity index of 38-fold, extrapolating to a marked enhancement of β -cell glucose usage.

TABLE 1
Kinetic characteristics of GK A456V

Kinetic parameter	Wild-type GK	A456VGK
k_{cat} (s^{-1})	53.2 ± 0.97	84.9 ± 4.5
Glucose $S_{0.5}$ (mmol/l)	8.04 ± 0.28	2.53 ± 0.10
nH (unitless)	1.67 ± 0.04	1.21 ± 0.03
ATP K_m (mmol/l)		
At glucose $S_{0.5}$	0.24 ± 0.02	0.15 ± 0.01
At 10 times the glucose $S_{0.5}$	0.39 ± 0.02	0.29 ± 0.03
Relative activity index (unitless)	1.0	37.9 ± 1.23

Data are means \pm SE. The results are the means of the kinetic analysis of four independent expressions of wild-type and mutant GST-GK. Differences between the mutant and wild-type enzyme are statistically significant for all parameters studied ($P \leq 0.05$).

Structural analysis. Structural analysis of A456V, along with other naturally occurring (V455M) or artificially obtained (D158A and Y214A) mutations that increase the glucose affinity and/or k_{cat} , showed that both spontaneous and designer mutants are located in a circumscribed domain of the enzyme molecule remote from the substrate binding cleft and are thus not part of either the glucose or the MgATP binding sites. This result suggests an allosteric activator domain of the protein (Fig. 2).

Mathematical modeling. The physiological consequences of the changed kinetic characteristics of the A456V mutation on GSIR and thus glucose homeostasis are illustrated by the mathematical modeling depicted in Fig. 3. The threshold for GSIR is reached at $\sim 25.7\%$ of the maximal β -cell glucose phosphorylation rate. Assuming comparable expression control for the mutant and the wild-type allele, heterozygosity for the A456V mutation results in a greatly reduced apparent threshold for GSIR of ~ 1.5 mmol/l contrasting with the physiological 5 mmol/l. In the fasting proband and his mother, blood glucose was ~ 1 mmol/l above this calculated threshold maintained at a level of ~ 2.5 mmol/l associated with increased (proband) or normal (mother) serum C-peptide and insulin concentrations.

DISCUSSION

Spontaneous activating and inactivating mutations of the GK gene coupled with autosomal dominant inheritance

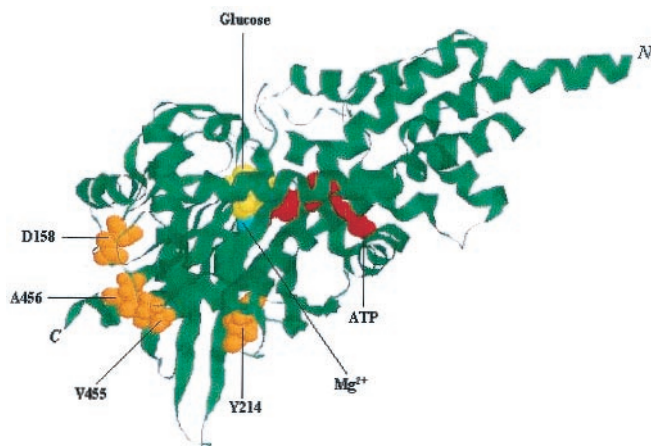


FIG. 2. Allosteric activation domain of GK. The structural GK model shows the location of the activating mutations D158A, Y214A, V455M, and A456V in a circumscribed allosteric domain distinct from the binding cleft for glucose and MgATP. This particular orientation of the structure was chosen to emphasize the large molecular distance of the putative activator region from the substrate binding site.

patterns result in very different phenotypes, including severe PHHI, mild MODY2, and severe PND consistent with the GK glucose sensor paradigm (10,15). The analysis

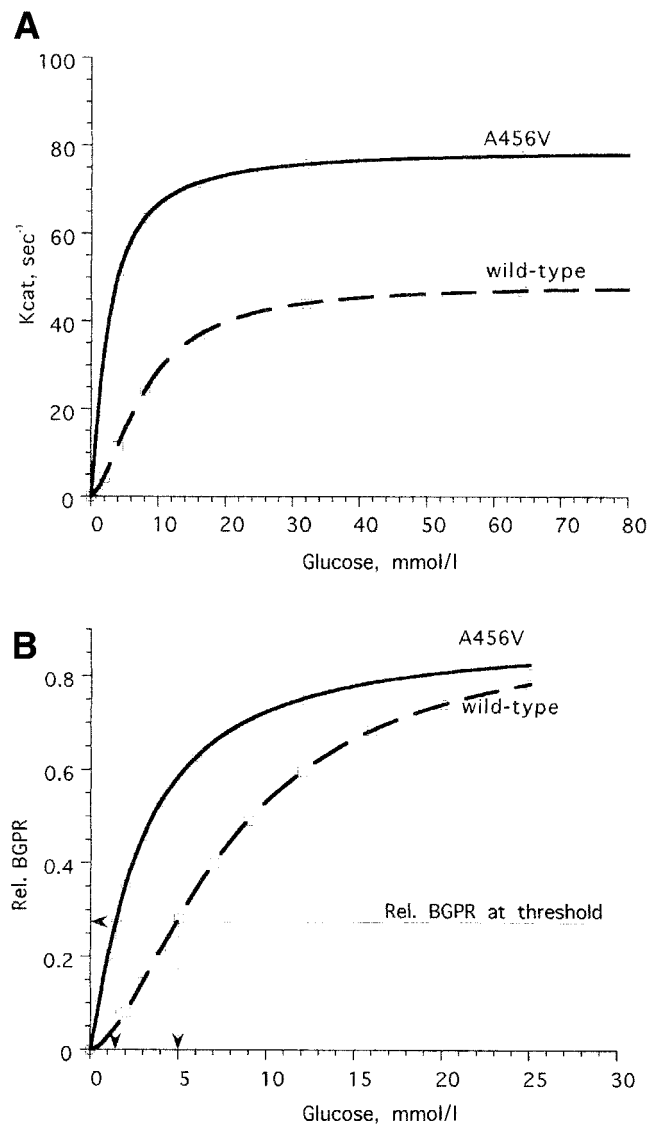


FIG. 3. Threshold shift of GSIR due to A456V. A comparison of wild-type and mutant enzyme kinetics is shown in A, and the relative β -cell glucose phosphorylation rate (rel. BGPR) is plotted in B as a function of blood glucose. The wild-type/wild-type threshold for GSIR is by definition 5 mmol/l (GSIR-5) and, in this study, is reached at $\sim 25.7\%$ of the hypothetical maximum of the BGPR. The apparent GSIR-5 for A456V is calculated as 1.5 mmol/l based on adaptation of both GK alleles to low glucose when the rel. BGPR is $\sim 25.7\%$.

of the present case with a novel activating mutation A456V offers an opportunity to discuss the far-reaching implications of the glucokinase glucose sensor paradigm for our understanding of glucose homeostasis and the treatment of hypoglycemia and diabetes. It might be theoretically and practically useful to apply the term “glucokinase disease” to collectively refer to these various syndromes.

The discovery of spontaneous and man-made activating mutants of GK is indeed remarkable. The synthetic mutation D158A, first recognized by Veiga-da-Cunha et al. (24) in 1996, has been used as a control enzyme in a series of biochemical genetic studies without appreciating that it has an abnormally high affinity for glucose. V455M was discovered in 1998 in a family afflicted by GK-linked PPHI (8).

The activated state of the synthetic mutation Y214A was described in a study of human islet GK in 2000 (25). The new spontaneous mutant A456V was identified during attempts to find a molecular genetic basis of the present case of PPHI. Activation of GK catalysis by the point mutations D158A, Y214A, V455M, and A456V is the result of lowering the glucose $S_{0.5}$, lowering the Hill coefficient for the substrate glucose, and increasing the k_{cat} , separately or in combination. The activation results in a near-hyperbolic, high- k_{cat} and low-glucose $S_{0.5}$ enzyme that contrasts with the catalytically less active sigmoidal wild-type.

The model structure of GK reveals that D158A, Y214A, V455M, and A456V are located in a well-circumscribed domain of the enzyme clearly remote from the binding sites of the substrates glucose and MgATP, which suggests the existence of an allosteric activator site of GK.

Two other mutations with lowered glucose $S_{0.5}$, N166R, and K296M are located near the substrate binding site, but the molecular basis of this change is probably very different from the mechanism proposed here, which involves allosteric mutations operating from a location distant from the substrate binding site.

The postulated existence of an allosteric activator site for GK implies that there might exist endogenous physiological activator molecules for GK in tissues that express the enzyme (that is, the liver, the pancreatic β -cells, enteric endocrine cells, and neurons of the hypothalamus), with the possibility of acute or chronic dual control by activators and inhibitors, a molecular mechanism found to operate in many enzymes of high control strength for metabolic pathways. If that were the case, impairment of such control could be of significance for glucose homeostasis. It is also conceivable that the newly discovered GK activator drugs interact with this site (26,27).

The striking contrast in the clinical presentation of mother and son who both express the A456V mutation of GK requires comment. The quantitatively most impressive difference between the two are found in the BMI and the fasting serum levels of insulin, C-peptide, and proinsulin, whereas blood glucose concentrations are in a comparable range of 2.5–3.0 mmol/l (Fig. 1). The reader is reminded of a similar situation in the first reported family with PPHI-GK (8), in which the proband was as obese and severely hyperinsulinemic as the present test case subjects, whereas his sister, also heterozygous for the V455M allele of GK, was of normal weight, had relatively low

serum insulin levels, and her clinical symptoms were less pronounced than those in her brother. Weight gain usually occurs in subjects with primary hyperinsulinemia of any origin as a consequence of increased food intake to maintain euglycemia. However, if the obesity observed in our proband at adolescence was just secondary to hypoglycemia and not a causal factor, the aggravation of the hypoglycemia remains unexplained. We hypothesize that factors related to adipose tissue mass may increase pancreatic GK expression in addition to augmentation of β -cell volume (28–31), resulting in the exaggerated insulin secretion that is difficult to compensate by counterregulatory mechanisms and frequent meals. The same interaction may account for the better glucose tolerance in overweight/obese children with inactivating glucokinase mutations compared with lean children (20).

Another interesting feature is the potential development of type 2 diabetes with age in PPHI (8,32). In the V455M family, the carrier mother of the proband had diet-controllable hypoglycemia from adolescence but developed diabetes at the age of 48 years. In transgenic mice with a Kir6.2 mutation and hypersecretion of insulin, β -cells undergo apoptosis (33), perhaps initiated by the increased intracellular calcium concentration (34). In the present β -cell stimulation tests, the proband's exaggerated responses of serum insulin and C-peptide was contrasted by the blunted or moderate peptide responses of the mother. The follow-up of the A456V proband and his mother will elucidate whether any progression to impaired GSIR will occur.

The highly variable clinical manifestations of individuals with GK V455M or A456V show the great difficulty of diagnosing activating GK mutations and suggest that more PPHI-GK patients may be diagnosed in the future. It should be noted that, until now, PPHI-GK has been considered to be a disease presenting beyond infancy in light of the V455M family. Our proband presented with neonatal hypoglycemia from the first hours of life. Moreover, he was small for gestational age, whereas most PPHI neonates present large for gestational age, as in diabetic fetopathy. In contrast, our proband may have been small for gestational age because of his mother's low blood glucose levels during pregnancy. The combination of reduced glucose substrate in this low-weight child and his reduced threshold for GSIR may have been responsible for the aggravated hypoglycemia in the neonatal period.

With the advancement of the biochemical genetic analysis of PPHI-GK in the V455M and A456V families, the relevance of this information for the management of hypoglycemic and hyperglycemic states becomes increasingly apparent. Because extrapancreatic factors (for example, overeating and obesity) may be critical in the pathogenesis of clinical symptoms of hypoglycemia, as observed here and previously (29–31), it may be necessary to pay increased attention to dietary aspects in designing a suitable treatment plan for some PPHI patients. This approach is supported by the experience that carbohydrate feeding protects against the hyperinsulinemic response of a protein-rich meal in PPHI-GDH (35).

Several investigators have speculated that activation of the GK system may be useful for lowering blood glucose in type 2 diabetic patients and have indeed shown that this is

possible by overexpressing GK in hepatocytes (36–38). The rationale of this approach has been criticized, however, on the basis of experiments with normal rats. Malfold overexpression of GK in the liver of these animals using viral vector technology caused the blood glucose to fall to subphysiological levels, but this treatment was associated with a marked increase of blood lipids, which would greatly limit its usefulness (38). However, the laboratory data of the individuals with persistent GK-induced hypoglycemia described here show that fasting serum lipid levels are affected only marginally, if at all. Because the goal of any diabetes treatment using GK activation would be to avoid hypoglycemia of a severity seen in patients who have an activating GK mutation, the danger of dyslipidemia is probably further reduced. These speculations are far from being an idle exercise considering the recent discovery of potent pharmacological GK activators (26–27).

The present report of a second activating GK mutation together with the recent publication of two subjects with PND (15) highlights again the preeminent role played by GK in glucose homeostasis. Mathematical modeling using the biochemical data of such case subjects shows that the glucose threshold for GSIR is precisely controlled by GK over a wide range, resulting in severe hypersecretion or hyposecretion of insulin actually observed *in vivo*. The results of the biochemical genetics of the present and a previous case of PPHI-GK also demonstrate that GK might indeed be an ideal drug target. Because pharmacological GK activation has been accomplished recently, GK research promises to complete the ideal full circle, starting with early discoveries of basic molecular physiology, moving on to human pathology, exploring new therapeutic approaches, and finally feeding back to fundamental science.

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