Glucokinase mutations associated with non-insulin-dependent (type 2) diabetes mellitus have decreased enzymatic activity: Implications for structure/function relationships

(gene mutations/enzyme structure/function)


*Department of Physiology and Biophysics, State University of New York, Stony Brook, NY 11794; †Howard Hughes Medical Institute and Departments of Biochemistry and Molecular Biology and Medicine, University of Chicago, Chicago, IL 60637; ‡Centre d’Etude du Polymorphisme Humain, 27 rue Juliette Dodu, 75010 Paris, France; §Department of Pharmacology, John Radcliffe Hospital, and Diabetes Research Laboratories, Radcliffe Infirmary, Oxford OX3 9DU, United Kingdom; ¶Laboratory of Botany, National Institute of Health, Bethesda, Maryland; §Department of Pathology and Bacteriology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom; †Rolf Luft Center for Diabetes Research, Department of Clinical Genetics, Karolinska Hospital, S-104 01 Stockholm, Sweden; and **Department of Pharmacology, Jefferson Cancer Institute, Philadelphia, PA 19107

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ABSTRACT The glycolytic enzyme glucokinase plays an important role in the regulation of insulin secretion and recent studies have shown that mutations in the human glucokinase gene are a common cause of an autosomal dominant form of non-insulin-dependent (type 2) diabetes mellitus (NIDDM) that has an onset often during childhood. The majority of the mutations that have been identified are missense mutations that result in the synthesis of a glucokinase molecule with an altered amino acid sequence. To characterize the effect of these mutations on the catalytic properties of human β-cell glucokinase, we have expressed native and mutant forms of this protein in Escherichia coli. All of the missense mutations show changes in enzyme activity including a decrease in Vₘₐₓ and/or increase in Kₘ for glucose. Using a model for the three-dimensional structure of human glucokinase based on the crystal structure of the related enzyme yeast hexokinase B, the mutations map primarily to two regions of the protein. One group of mutations is located in the active site cleft separating the two domains of the enzyme as well as in surface loops leading into this cleft. These mutations usually result in large reductions in enzyme activity. The second group of mutations is located far from the active site in a region that is predicted to undergo a substrate-induced conformational change that results in closure of the active site cleft. These mutations show a small ~2-fold reduction in Vₘₐₓ and a 5- to 10-fold increase in Kₘ for glucose. The characterization of mutations in glucokinase that are associated with a distinct and readily recognizable form of diabetes is a key step towards identification of key amino acids involved in glucokinase catalysis and localized functionally important regions of the glucokinase molecule.

The phosphorylation of glucose in mammalian tissues is mediated by a family of hexose phosphotransferases (1–3). These include glucokinase and hexokinases I, II, and III. Glucokinase is expressed in insulin-secreting pancreatic β cells and hepatocytes and is characterized by a high Kₘ and specificity for glucose and a relative lack of product inhibition by glucose 6-phosphate compared to the hexokinases (4–6). It plays an important role in regulating and integrating glucose metabolism in both tissues (3, 6–8). In pancreatic β cells, glucokinase, which represents about 20% of the total glucose phosphorylating activity, and the GLUT2 glucose transporter are believed to act in concert as the "glucose-sensing apparatus" modulating insulin release in response to changes in plasma glucose concentration (3, 9, 10). By contrast, glucokinase provides about 80% of the total glucose phosphorylating activity in mammalian liver. In this tissue, the expression of a glucokinase phosphorylating activity that has a high Kₘ for glucose and relative lack of inhibition by glucose 6-phosphate serves to maintain a gradient for glucose entry into the hepatocyte, which is particularly important following a meal when plasma glucose levels are elevated (4, 7).

We have recently shown that mutations in the glucokinase gene can cause an autosomal dominant form of non-insulin-dependent diabetes mellitus (NIDDM) with onset usually before 25 years of age (11–13). Mutations in the glucokinase gene have been found in about 60% of French families with early-onset NIDDM (refs. 11 and 13; P. E. F., unpublished data). They have also been found in British (12), Swedish (H. L., unpublished data), and African-American (G. I. B., unpublished data) patients with NIDDM and thus are not restricted to a particular ethnic or racial group. Although many mutations in glucokinase have now been identified, the consequences of these mutations on enzyme activity are not yet known. The object of this study was to express various mutant forms of human β-cell glucokinase in bacteria and to compare their kinetic properties with those of the native protein. The results are consistent with the hypothesis that gene dosage is the mechanism for glucokinase gene mutation-induced glucose intolerance in this form of NIDDM.

EXPERIMENTAL PROCEDURES

Materials. Glucosamine-Septarose was prepared as described by Miwa et al. (14).

Construction of pET Expression Plasmids for Human β-Cell and Liver Glucokinase. A 2.6-kb human pancreatic β-cell glucokinase cDNA clone, pGK-20 (15), was used to generate the construct pEhGK-WT. An NdeI site was generated at the 5' end using PCR with an oligonucleotide that had a 1-bp mismatch (GGC TGG TGT GCA TAT GCT GGA CGA CAG). The insert in the pET 3a expression construct included the protein coding region of the cDNA as well as the 3' untranslated region.

The sequences of human β-cell and liver glucokinase differ at their NH₂ termini (13, 16). The human liver glucokinase expression plasmid pEhGK was constructed from an Nsi I-EcoRI fragment of the β-cell glucokinase cDNA (encoding amino acids 16–466) and two pairs of complementary oligonucleotide linkers. The 5' linkers (NdeI/PstI) encoding the 16 N-terminal amino acids and the translation initiation site

Abbreviation: NIDDM, non-insulin-dependent diabetes mellitus.
were phosphorylated, annealed, and ligated to the Nde I site of the pET 3a vector and the Pst I site of the cDNA.

Site-Directed Mutagenesis of Human β-Cell Glucokinase. In vitro mutagenesis of human β-cell glucokinase was carried out using the Altered Sites in vitro mutagenesis system (Promega). All mutations were confirmed by DNA sequencing.

Bacterial Expression and Purification of Native Human β-Cell and Liver Glucokinases and β-Cell Human Glucokinase Mutants. Native and mutant human glucokinases were expressed in Escherichia coli essentially as described (17, 18). Glucokinase was purified 20-fold from extracts of E. coli in four steps, including (NH₄)₂SO₄ precipitation (45–65%), gel filtration on a Sephadex G-100 column, chromatography on a glucosamine-Sepharose column, and FPLC Mono Q-Sepharose chromatography. The latter step was necessary to remove a small amount of low KM hexokinase activity in the cell extract that could confound kinetic studies. The purified protein was homogeneous as judged by SDS/PAGE. Native β-cell glucokinase had a specific activity of 100 units/mg, which is similar to that reported for the purified rat liver enzyme (19). The purified recombinant protein was also subjected to NH₂-terminal sequence analysis. The protein, analyzed through 12 cycles, gave the expected sequence: Met-Leu-Asp-Asp-Arg-Ala-Arg-Met-Glu-Ala-Ala-Lys-

Modeling of Human β-Cell Glucokinase Structure. A simple model of human β-cell glucokinase was generated using the known crystal structure of yeast hexokinase (20–23).

RESULTS

Expression of Native and Mutant Forms of Human Glucokinase in E. coli. Mutations in the glucokinase gene associated with early-onset NIDDM have been found in 9 of the 12 exons (Fig. 1) and include splicing, nonsense, and missense mutations, the latter of which provide an opportunity to address structure/function relationships in this important regulatory enzyme of glycosis. To date, there has been only one study employing site-directed mutagenesis to investigate structure/function relationships of glucokinase (18). In that study, mutation of Asp-205 → Ala in the rat liver enzyme resulted in a 500-fold decrease in the Kₘ of the reaction with no change in the Kₘ for glucose or ATP. This finding is consistent with this carboxylate group hydrogen bonding to the phosphoryl acceptor and functioning as a general base catalyst (20–23).

The human β-cell form of glucokinase was readily expressed in E. coli (Fig. 2A) using the T7 RNA polymerase-based expression system (24); easily detectable levels of glucokinase activity were found in the soluble fraction of cell extracts after 15 min of induction with isopropyl β-D-thiogalactopyranoside and activity increased nearly 50-fold after 2 hr. The native and mutant forms of β-cell glucokinase were expressed at similar levels with the exception of the truncated form of the enzyme, which has a Glu-279 → amber mutation and lacks residues 279–465. We were unable to detect expression of this nonsense protein presumably because it was unstable and rapidly degraded. Like the native enzyme, all of the mutant forms were recovered in the soluble fraction and could be readily purified to homogeneity.

Effects of Missense Mutations on β-Cell Activity. The mutations showed a wide range of effects on Vₘ₉₅ and Kₘ for glucose with no detectable effect on affinity for ATP (Table 1). Since the kinetic parameters of the β-cell and liver isoforms are very similar (Table 1), we expect that these mutations will have the same effect on the activity of liver glucokinase. However, this issue needs to be addressed directly. The higher efficiency of bacterial expression of liver glucokinase compared to the β-cell isoform has been reported by others (25) for the corresponding rat isoforms and suggests that the first 15 NH₂-terminal amino acids affect stability and/or proper folding of glucokinase in E. coli cytosol.

Structure/Function Analysis of Glucokinase Mutations. We have used the three-dimensional structure of the related protein yeast hexokinase B to develop a model of human β-cell glucokinase (13, 20–23). As shown in the alignment in Fig. 3, there are 140 identical residues (~30% identity) between human β-cell glucokinase and yeast hexokinase B, and we predict that the human β-cell glucokinase will have the same arrangement of α-helices and β-strands as observed in the crystal structure of yeast hexokinase. The locations of the 11 missense mutations are indicated in Fig. 4, which represents a model of the open form of human β-cell glucokinase. Glucokinase, like hexokinase, is predicted to fold into a small domain and large domain, which are separated by a deep cleft (21). When glucose binds at the bottom of the cleft, there is a large conformational change that closes the cleft between the two domains and is essential for yeast hexokinase B catalysis. In the open form of glucokinase, Asn-204, Asp-205, Asn-235, Glu-256, and Glu-290 are predicted to form hydrogen-bond interactions with hydroxyls of bound glucose (Fig. 4 and ref. 23).

As shown in Fig. 4, many of the mutations map to the region of the active site cleft or to surface loops leading into the cleft. Three of the mutations involve amino acids that are predicted to be near the active site, Val-203, Thr-228, and Glu-256 (Figs. 3 and 4; Table 1), and may form part of the glucose binding site. Val-203 is next to Asn-204 and Asp-205, both of which are predicted to contact glucose. Mutation of Val-203 → Ala caused a 200-fold decrease in Vₘ₉₅. Since this residue is lle in yeast hexokinase B (Fig. 3), the hydrophilic character of the amino acid in this position may be very

FIG. 1. Schematic structure of the human glucokinase gene and localization of mutations identified in patients with NIDDM. The β-cell isoform of glucokinase is encoded by exons 1a and 2–10 (13). Two different glucokinase transcripts have been identified in human liver mRNA; the major transcript is encoded by exons 1b and 2–10, and the minor transcript is encoded by exons 1b, 1c, and 2–10. Exons 1a, 1b, and 1c encode the 15, 16, and 14 NH₂-terminal residues of the different glucokinase isoforms. Except for these amino acids, the sequences of the three isoforms are otherwise identical. The β-cell, liver₁β, and liver₁α transcripts encode proteins of 465, 466, and 464 amino acids, respectively. In this report amino acids are designated relative to the sequence of human β-cell glucokinase. The single-letter abbreviations for amino acids are used. "X" indicates a nonsense mutation. The splicing mutations are as follows: K262+2Gdel15 is a 15-bp deletion that removes the T of the GT in the splice donor site of intron 4 and the following 14 bp resulting in a GG; G227–2A→T is a mutation of the splice acceptor site in intron 6 from AG → TG; and S1481G→C is a mutation of the splice acceptor site in intron 9 from AG → AC. The mutations that were expressed in E. coli and characterized kinetically (Table 1) include those from French [Gly-175 → Arg, Val-182 → Met, Val-203 → Ala, Thr-228 → Met, Gly-261 → Arg, Glu-279 → amber, Gly-300 → Lys, Glu-300 → Gln, Leu-309 → Pro (refs. 11 and 12; P.F., unpublished data)], Swedish [Glu-256 → Lys (H.L., unpublished data)], British [Gly-299 → Arg (12)], and African-American [Glu-279 → Gln (G.I.B., unpublished data)] patients.
important and substitution with a less hydrophobic amino acid such as Ala may alter the conformation of the active site. Thr-228 is a conserved amino acid in all hexokinases and glucokinases and its mutation was predicted to affect affinity for glucose and/or ATP (13). The Thr-228 → Met mutation resulted in a large decrease in maximal velocity that could not be overcome by increasing the ATP/Mg concentration to 20 mM (data not shown). Glu-256 is predicted to hydrogen bond with glucose and its mutation to Lys resulted in a large decrease in maximal velocity but with no significant change in $K_m$ for glucose. Mutation of Glu-256 to a basic amino acid would remove this hydrogen bond and thus reduce the affinity for glucose and/or interfere with the open/closed equilibrium of the enzyme. In support of this hypothesis, the Gibbs free energy ($\Delta G$) estimated from the relative ratios of $V_{\text{max}}/K_m$ of the Glu-256 → Lys mutation and the native enzyme is about 3.0 kcal/mol (1 kcal = 4.18 KJ), which is consistent with the energy of one hydrogen bond of 3.5–4.5 kcal/mol (26). However, $K_m$ is not simply correlated with binding affinity, especially when a significant conformational change occurs during catalysis, so that the effect of mutation of a binding residue such as Glu-256, or perhaps Thr-228, may be on $V_{\text{max}}$ rather than $K_m$, which is what was observed.

Other mutations affect residues that are conserved between human $\beta$-cell glucokinase and yeast hexokinase B (Val-182, Glu-279, Gly-299, and Glu-300), implying that they are functionally important. For example, Glu-300 is predicted to form a salt bridge with Arg-303, a residue that is also conserved (Fig. 3), and mutation of Glu-300 to Lys or Asn would eliminate this interaction, which may be required for glucose binding. Consistent with this, the Lys and Asn mutants had a lower affinity for glucose (Table 1). Several mutations such as Gly-261 → Arg, Gly-299 → Arg, and Leu-309 → Pro are predicted to alter the structure of the protein, and these mutations are associated with large decreases in $V_{\text{max}}$. Of the missense mutations described in Table 1, all but two, Gly-175 → Arg and Val-182 → Met, involve amino acids located in the active site cleft or in surface loops leading into this cleft. Gly-175 and Val-182 map to a region that is on the surface of the enzyme and far from the active site. However, this region is part of the smaller of the two domains of glucokinase and is predicted to participate in the substrate-induced conformational change that results in cleft

Table 1. Enzymatic properties of native and mutant forms of human $\beta$-cell glucokinase

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$V_{\text{max}}$, units/mg</th>
<th>$K_m$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native liver isoform</td>
<td>80</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>Native $\beta$-cell isoform</td>
<td>20</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Gly-175 → Arg</td>
<td>20</td>
<td>51 ± 5*</td>
</tr>
<tr>
<td>Val-182 → Met</td>
<td>10</td>
<td>49 ± 6*</td>
</tr>
<tr>
<td>Val-203 → Ala†</td>
<td>15</td>
<td>0.5 ± 0.04*</td>
</tr>
<tr>
<td>Thr-228 → Met‡</td>
<td>10</td>
<td>0.4 ± 0.03*</td>
</tr>
<tr>
<td>Glu-256 → Lys§</td>
<td>8</td>
<td>0.25 ± 0.02*</td>
</tr>
<tr>
<td>Gly-261 → Arg</td>
<td>8</td>
<td>≤0.46*</td>
</tr>
<tr>
<td>Glu-279 → amber</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>Glu-279 → Gin</td>
<td>15</td>
<td>55 ± 6*</td>
</tr>
<tr>
<td>Gly-299 → Arg</td>
<td>5</td>
<td>≤0.32*</td>
</tr>
<tr>
<td>Glu-300 → Lys</td>
<td>12</td>
<td>33 ± 3*</td>
</tr>
<tr>
<td>Glu-300 → Gin</td>
<td>20</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Leu-309 → Pro‡</td>
<td>5</td>
<td>≤0.98*</td>
</tr>
</tbody>
</table>

Glucokinase activity was determined as described (17, 18). The values shown are the mean ± SEM of three to five separate expression experiments. The kinetic analysis was done with purified native and mutant enzyme forms. However, the same changes in kinetic properties were observed after partial purification of the enzyme by (NH$_4$)$_2$SO$_4$ fractionation.

* $V_{\text{max}}$ and $K_m$ values that are significantly different from native $\beta$-cell enzyme.
† Residues located in the cleft near glucose.
‡ Residues whose mutation predicts a distortion in glucokinase structure.
§ No active enzyme.
Fig. 3. Comparison of the sequences of human β-cell glucokinase and yeast hexokinase B: Structural alignment of the amino acid sequences of human β-cell glucokinase (Hugk) and yeast hexokinase B (Yshkb) based upon placing insertions and deletions between secondary structural elements in the crystal structure of yeast hexokinase B. The single-letter abbreviations for the amino acids are used. Identical residues are shown in black boxes, and similar residues are in shaded boxes. The 13 α-helices and β-strands are indicated below the sequences. The small arrows indicate the positions at which introns interrupt the sequence.

Our results suggest that even relatively conservative substitutions in this surface loop region may affect this conformational change.

**DISCUSSION**

Although mutations in the glucokinase gene have been identified in nine different exons, the missense mutations appear to be clustered with 10 of these mutations occurring in three exons, exons 5, 7, and 8 (Figs. 1, 3, and 4), which suggests that these exons may encode functionally important domains of the protein. Residues encoded by exons 7 and 8 form much of the surface of the cleft between the two domains, as well as surface loops leading into this cleft. However, only one of the mutations in this region is of a residue (Glu-256) directly implicated in the binding of glucose. The others are predicted to be in the cleft (Thr-228, Gly-261), in a region leading to the
per se on β-cell glucose sensing and/or hepatic glucose metabolism (13).

Glucokinase mutations may be the most common genetic cause of NIDDM identified to date, especially among young patients with NIDDM diagnosed before age 25. Studies of the effects of these mutations on glucokinase activity in vitro have led to the identification of key amino acid residues involved in determining glucokinase activity. The identification and functional characterization of further glucokinase mutations should lead to the identification of other key residues. These studies together with glucokinase will provide new insight into the molecular mechanism responsible for the efficient transfer of phosphate from ATP to glucose and may lead to therapeutic approaches for treating NIDDM resulting from mutations in this important glycolytic enzyme.

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