Activating Mutations in the Human Glucokinase Gene Revealed by Genetic Selection

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ABSTRACT: We describe the discovery of 11 new activating mutations in the human *glk* gene associated with the disease persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Three of the newly identified substitutions colocalize to a region of the glucokinase polypeptide where a synthetic allosteric activator binds. Of these substitutions, I211F is the most active variant identified to date, with a $k_{cat}/K_{0.5,glucose}$ value (6.6×10^4 M^{-1} s^{-1}) that is 12-fold higher than that of wild-type glucokinase. The stimulatory mutations described herein represent surreptitious genetic determinants of PHHI. They also identify novel features of the glucokinase scaffold that could be targeted during the development of diabetes therapeutics.

Glucokinase catalyzes the ATP-dependent phosphorylation of glucose in the first reaction of glycolysis. This chemical transformation is the rate-limiting step of glucose metabolism in the human liver and pancreas (*1, 2*). As such, glucokinase is a central regulator of whole-body glucose homeostasis (*3*). Disabling mutations in *glk* that impair catalysis result in maturity-onset diabetes of the young (MODY[1]), whereas activating lesions cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (*4*−*6*). These two disease states demonstrate the extent to which glucokinase action must be precisely regulated in vivo. They also emphasize the potential therapeutic value of modulating the activity of this key metabolic enzyme. Indeed, the identification of small-molecule glucokinase activators that bind to a distal allosteric site has drawn considerable interest recently, and at least one glucokinase-targeted activator is undergoing phase I clinical trials as a diabetes therapeutic (*7*−*9*).

Glucokinase activity is allosterically regulated by its sugar substrate, D-glucose. The steady-state velocity of glucose phosphorylation is not hyperbolic but instead displays a sigmoidal response to increasing substrate concentrations (*10, 11*). The mechanistic basis for this cooperativity is unknown, but this feature is particularly intriguing since glucokinase functions exclusively as a monomer (*12*). Several models of kinetic cooperativity in monomeric enzymes have been presented in the literature (*13*−*15*), and past studies have demonstrated that glucokinase cooperativity is linked to slow conformational transitions that occur during the course of catalysis (*16*). To provide additional insight into glucokinase cooperativity, and to uncover previously undiscovered genetic determinants of PHHI, we sought to identify new activating *glk* mutations.

We used a mutagenic DNA polymerase to construct three distinct *glk* libraries, with mutational frequencies ranging from 0.05 to 0.3% and an average size of 5×10^6 members. Amplified products were inserted behind the T7 promoter of pBGM101, and each library was transformed into BM5340(DE3), an auxotrophic strain of *Escherichia coli* K-12 harboring chromosomal deletions in the *glk* gene and the *pts* operon (*17*). As a result of these genetic lesions, BM5340(DE3) cannot survive on glucose minimal medium unless provided with an extrachromosomal source of glucokinase. Genetic selection experiments were performed by plating BM5340(DE3) cells harboring individual *glk* libraries on glucose minimal M9 medium. Using wild-type human glucokinase and three known activating variants (Y214C, V455M, and A456V) as benchmarks for growth, a variety of singly substituted polypeptides with enhanced activities were isolated. Notably, the selected enzymes include five of six known activating variants previously identified in patients suffering from PHHI. Only the G68V activating variant was not identified in this study.

The activating variants were classified into three distinct categories based upon their location within the glucokinase structure (Figure 1) (*18*). The first category involves residues that form direct interactions with the synthetic allosteric activator. These include S64F, S64P, I211F, and the previously identified T65I, Y214C, V455M, and A456V substitutions. The second category involves residues that are located close to the allosteric site but do not make direct contact with the activator. This group includes S69P, D73E, V91L, E216D, the previously identified W99R variant, and two residues, G446S and S453A, located within helix α13. Helix α13 undergoes large structural and packing rearrangements upon glucose binding. The final category of activating substitutions includes N180D and M197V, two residues whose side chains are more than 15 Å from the allosteric site. The discovery of activating substitutions at these distal positions is noteworthy, as they represent a previously undiscovered region of the polypeptide, discrete from the active site, which can modulate enzyme activity.

A competitive growth experiment on glucose minimal medium indicated that the S64P, M197V, I211F, and S453A variants exhibited the highest degree of activation. To determine the values of catalytic constants k_{cat}, $K_{0.5,glucose}$, and

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[1] Abbreviations: MODY, maturity-onset diabetes of the young; PHHI, persistent hyperinsulinemic hypoglycemia of infancy.

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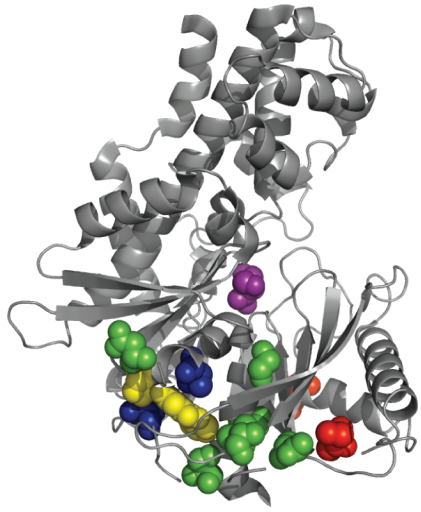

FIGURE 1: Structure of human glucokinase in complex with glucose (magenta) and an allosteric activator (yellow), depicting the location of the activating substitutions. The first category (blue) involves residues that directly contact the activator, including Ser-64 and Ile-211. The second category (green) involves residues located near the allosteric site, including Ser-69, Asp-73, Val-91, Glu-216, Gly-446, and Ser-453. The final category (red) involves residues more than 15 Å from the allosteric site, including Asn-180 and Met-197. Images were created with Pymol and Protein Data Bank entry 1V4S (*18*).

Table 1: Kinetic and Thermodynamic Parameters for Glucose

enzyme	k_{cat} (s^{-1})	$K_{0.5}$ (mM)	$k_{cat}/K_{0.5}$ (M^{-1} s^{-1})	Hill coefficient	K_D (mM)
wild-type	31	6.3	4.9×10^3	1.6	10
S64P	18	1.6	1.1×10^4	1.2	0.12
S453A	27	2.4	1.1×10^4	1.7	0.65
M197V	37	2.5	1.5×10^4	1.6	0.95
I211F	43	0.72	6.0×10^4	1.3	1.9

$K_{m,ATP}$ for these four enzymes, we performed steady-state kinetic assays. As expected, each variant displayed an enhanced $k_{cat}/K_{0.5,glucose}$ value compared with that of wild-type glucokinase (Table 1). Similar to the case for activated glucokinase variants identified from hyperinsulinemic patients, the increase in catalytic efficiency detected in each enzyme partially originates from a decrease in the glucose $K_{0.5}$ value. By comparison, the $K_{m,ATP}$ value for I211F decreased to 130 μM from the wild-type value of 540 μM, and for S453A, the $K_{m,ATP}$ value decreased to 220 μM (Table S2 of the Supporting Information). The value of $K_{m,ATP}$ remained unchanged for the S64P and M197V variants. S64P and I211F have lower Hill coefficients compared with that of the wild-type enzyme, indicating a decrease in kinetic cooperativity. In contrast, S453A and M197V are unique among the activated glucokinase variants described to date, in that their Hill coefficients are unchanged. This observation provides the first evidence that human glucokinase activity can be enhanced without sacrificing cooperativity.

To determine if the most active variants discovered in our study possess an enhanced affinity for glucose, we determined equilibrium dissociation constants for S64P, M197V, I211F, and S453A via fluorescence (Table 1). All variants displayed a decreased $K_{D,glucose}$ value compared to the wild-type value (10 mM). Surprisingly, the $K_{D,glucose}$ value for S64P is 120 μM, an 80-fold decrease over that of the wild-type enzyme. This is the largest increase in glucose binding affinity thus far reported for a single amino acid substitution

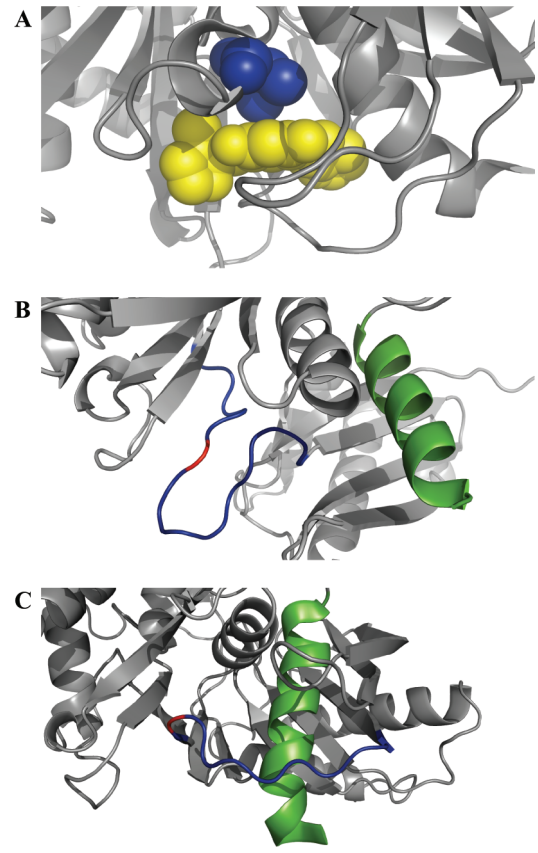

FIGURE 2: (A) Substitution of Ile-211 (blue) with phenylalanine activates human glucokinase by filling the vacancy otherwise occupied by the synthetic activator (yellow). (B) Activating residue Ser-64 (red) is located in a solvent-exposed loop (blue) that connects the large and small domains of human glucokinase. (C) When glucose binds, the connecting loop prevents release of helix α13 (green). Images were created with Pymol and Protein Data Bank entries 1V4S (A and C) and 1V4T (B).

in human glucokinase. The lack of correlation between $K_{D,glucose}$ and $K_{0.5,glucose}$ values for each variant emphasizes the fact that the $K_{0.5,glucose}$ values determined from steady-state measurements are kinetic in origin. It also indicates that individual activating substitutions likely stimulate catalysis via distinct mechanisms, perhaps by altering discrete microscopic rate constants that contribute differentially to the values of $K_{D,glucose}$ and $K_{0.5,glucose}$.

To understand the mechanism of activation afforded by the S64P, M197V, I211F, and S453A alterations, we examined the crystal structure of human glucokinase in complex with glucose and a synthetic allosteric activator. In this structure, the side chain of Ile-211 makes direct contact with the allosteric activator (Figure 2A). Replacement of this residue with the more bulky phenylalanine side chain is expected to fill the pocket otherwise occupied by the activator. Thus, we postulate that the I211F substitution stabilizes the glucose-bound enzyme conformation, which is similar to the proposed mode of action of the allosteric activator (*18, 19*). Replacement of Ser-64 with either proline or phenylalanine also stimulates activity. This residue is located within a loop that connects the large and small domains of the glucokinase polypeptide. In the unliganded enzyme structure, residues in this loop are largely solvent exposed, but in the glucose-bound structure, this loop appears to stiffen. In so doing, the connecting loop promotes a more compact enzyme conformation by preventing release of helix

α13 (Figure 2B,C). Replacement of Ser-64 with either proline or phenylalanine is expected to favor the glucose-bound state by constraining the flexibility and increasing the hydrophobicity of the connecting loop. Consistent with this hypothesis is our discovery that substitution of proline for another loop residue, Ser-69, is also activating. Both S64P and S69P exhibited decreased Hill coefficients compared with that of wild-type glucokinase, suggesting that conformational mobility in this region is essential for kinetic cooperativity.

The mechanistic basis of the M197V and S453A activation is less clear. In both cases, the observed substitutions increase the hydrophobicity at positions 197 and 453. Ser-453 is located in helix α13, which harbors additional activating residues, including Val-455 and Ala-456. In the crystal structure of unliganded human glucokinase, the Met-197 and Ser-453 side chains are solvent-exposed. Upon formation of the glucose-bound complex, both side chains are sequestered into the enzyme's interior. On the basis of this observation, we speculate that substitution of valine at position 197 and alanine at position 453 favors the more compact glucose-bound conformation, in part by destabilizing the unliganded state.

I211F is the most active glucokinase variant identified to date, with a $k_{cat}/K_{0.5,glucose}$ value of 6.6×10^4 M^{-1} s^{-1}. This second-order rate constant is 12-fold higher than the $k_{cat}/K_{0.5,glucose}$ value of the wild-type enzyme. Prior to this study, Y214C was the most active variant described, with a $k_{cat}/K_{0.5,glucose}$ value that is 5-fold higher than that of wild-type glucokinase (*20*). The Y214C lesion causes severe persistent hypoglycemia, leading to mental retardation, epilepsy, and early death. Our results suggest that the failure to observe the more activating I211F substitution in clinical studies of patients suffering from hypoglycemia may result from the fact that this genetic defect is fatal.

This study significantly increases both the number and scope of activating *glk* single-nucleotide polymorphisms. Moreover, these findings predict a variety of new genetic defects that could lead to the emergence of PHHI. This study provides the first evidence of stimulatory single-site substitutions that are not located in the binding site for the synthetic allosteric activator. Since human glucokinase is a prominent target for diabetes therapeutic development, the identification of such residues suggests additional features of the glucokinase scaffold that could be targeted during future drug design efforts. In particular, the discovery of the M197V variant, which enhances glucokinase activity without altering the Hill coefficient, offers hope that small-molecule glucokinase activators that do not sacrifice the regulatory properties conferred by this enzyme's unique cooperativity can be developed.

SUPPORTING INFORMATION AVAILABLE

Experimental methods, mutant growth rates, and kinetic and thermodynamic binding data. This material is available free of charge via the Internet at http://pubs.acs.org.

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