Molecular basis for the role of glucokinase regulatory protein as the allosteric switch for glucokinase

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Glucokinase (GK), a hexokinase isozyme, is a monomeric allosteric enzyme and mainly expressed in hepatocytes and pancreatic \(\beta\)-cells (1, 2). Through its unique kinetic character, GK plays a central role in blood glucose homeostasis by converting glucose to glucose-6-phosphate (G6P), enhancing glycogen synthesis, in hepatocytes (3), and sensing glucose for insulin secretion in pancreatic \(\beta\)-cells (4). Defects and mutations in GK are directly associated with type 2 diabetes and maturity-onset diabetes of the young type 2 (5, 6). Thus, GK has been an important molecular target for studying blood glucose homeostasis and developing antidiabetes drugs (5, 8). The crystal structure of human GK (hGK) revealed that GK undergoes a slow and energetically unfavorable structural rearrangement of the small domain in response to glucose during the transition from a super-open conformation into an open conformation (9). As a result, GK exhibits a sigmoidal activity curve with respect to glucose concentration as a monomeric allosteric enzyme (9).

Unlike pancreatic \(\beta\)-cells, blood glucose control by the hepatocytes is more complicated. Hepatocytes remove high amount of exogenous glucose after a meal in a fast and efficient manner (3). At the same time, hepatocytes also produce endogenous glucose to blood stream to maintain blood glucose level in a fasting state (3, 10). GK must be fully active for fast glucose clearance after a meal (3), whereas it should be turned off during a fasting state to prevent futile cycling of endogenous glucose to G6P (10). Therefore, GK in hepatocytes is presumed to be regulated in a different way from that in pancreatic \(\beta\)-cells. In hepatocytes, GK is regulated by GK regulatory protein (GKRP) that is located mainly in hepatocytes with an excess ratio to GK (11, 12). GKRP allosterically regulates the activity and subcellular localization of GK (13, 14). GKRP inhibits and sequesters GK into the nucleus of hepatocytes and releases GK into the cytoplasm in response to glucose concentration (13–15). GK is also indirectly regulated by allosteric effectors of GKRP, such as fructose-1-phosphate (F1P) and fructose-6-phosphate (F6P) (11, 16). F1P is derived from fructose and sorbitol in a meal, and F6P is an intermediate product of glycolysis (16). Recent studies have characterized the biophysical features regarding the interaction between GK and GKRP and a modulation through the effectors (17, 18). Despite the intensive studies on GK and GKRP, the molecular basis for the allosteric regulation mechanism of GK by GKRP remains poorly understood because of the lack of structural information of the GK/GKRP complex.

Here, to demonstrate the molecular mechanism for the allosteric regulation of GK by GKRP and effectors, we determined the crystal structure of a Xenopus laevis GK and GKRP complex in the presence of F6P. Structural analysis of the complex and mutational studies with human GK and GKRP revealed that GKRP interacts with GK mainly through hydrophobic interaction and inhibits GK activity through a single ion pair between GKRP and the small domain of GK. We also show the molecular mechanism by which F1P and F6P modulate the activity of GK through molecular dynamics simulations and mutational analysis. Importantly, GKRP was revealed to release GK in a sigmoidal manner in response to glucose concentration by restricting a structural rearrangement of the GK small domain through a single ion pair, acting as an allosteric switch for GK.

Results

Determination of Crystal Structure of GK/GKRP Complex. We determined the crystal structure of a X. laevis GK/GKRP (xGK/xGKRP) complex in the presence of F6P at 2.9 Å (Fig. 1 and Table S1). Our attempts to crystallize a hGK/human GKRP (hGKRP) complex have been unsuccessful. The crystals contain two xGK/xGKRP complexes in an asymmetric unit, and share 82% and 58% sequence identity with hGK and hGKRP, respectively (Fig. S1). The overall structure of the xGK/xGKRP complex reveals that xGKRP is bound to the super-open conformation of xGK (Fig. 1B), sharing an rmsd of 1.7 Å for 406 C\(_{\alpha}\) atoms for the super-open conformation of apo-hGK [Protein Data Bank (PDB) ID code 1V4T] with an approximately 11° rotation of a small domain of xGK toward a large domain (Fig. S2). The structure of xGKRP comprises two sugar isomerase (SIS) superfamily domains and a C-terminal extended all-helical motif (Fig. L4). The sugar-isomerase domains show typical \(\alpha\beta\alpha\) folds as reported elsewhere (19, 20) (Fig. S3). We identified the electron density of F6P as a linear keto form, as previously suggested (16), in SIS domain 1, where SIS domain 2 and the C-terminal motif meet together (Fig. 1A and C). A stretch of four residues including Ser179, Ser258, Glu347, and Lys513 of xGKRP

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participate in the binding of F6P through hydrogen bonding (Fig. 1C). A previous study on rat GKRP also revealed that Ser179 and Lys513 interact with F6P (21).

**Analysis of Interface Between GK and GKRP.** Based on the crystal structure of the xGK/xGKRP complex, we analyzed the interface between GK and GKRP. The xGK/xGKRP complex shows that a wedge-shaped structure is formed by two loops (L1 and L2), two helices (α8 and α14), and one strand (β10) in SIS domain 2 of xGKRP, anchoring to the allosteric cleft in the hinge region of xGK (Fig. 2A). The interface between xGK and xGKRP is predominantly hydrophobic, with a total burial area of 1,913 Å². The edge of the wedge formed by two loops, L1 and L2, of xGKRP creates multiple van der Waals contacts with the hydrophobic surface in the cleft formed by a strand (S1), loops L1 and L2 from the large domain, loop L3 from the small domain, and connecting region II of the xGK, generating major hydrophobic interactions (Fig. 2A). A hydrophobic surface is composed of Leu40, Lys49, Leu51, Pro52, Tyr54, Arg56, Lys136, Met231, Leu236, Val237, Glu238, and Met244 from the large domain, and Asp191, Val192, and Val193 from the small domain of xGK (Fig. 2A and B). This hydrophobic surface interacts with Ala440, Gly441, and Tyr443 of loop L1 and Pro461, Ile462, Leu463, and Phe464 of loop L2 in xGKRP (Fig. 2A and B). It is interesting to note that most of the hydrophobic surface of xGK is located in the large domain. To assess the importance and universality of the interactions in a GK/GKRP complex, we performed a mutational analysis for hGKRP.

![Fig. 1. Overall structure of xGK/xGKRP complex.](image)

![Fig. 2. Interface of xGK/xGKRP complex.](image)
We mutated Leu463 and Phe465 in hGKRP to Ala, which are equivalent to Ile462 and Phe464 in xGKRP and share approximately one fourth of the hydrophobic contacts (481 Å²). In gel filtration analysis, the resulting hGKRP_L463A/F465A mutant did not form a complex with hGK (Fig. S4), which indicates that hydrophobic interactions are crucial for the binding of GKRPs to GK.

The interface is further stabilized by conserved two ion pairs and three hydrogen bonds showing a charge complementarity to the wedge of xGKRPs (Fig. 2A and Fig. S5). Glu238 of loop L4 in the large domain and Arg179 of d in the small domain of xGK form ion pairs with Arg300 and Asp412 of xGKRPs, respectively (Fig. 2A and B). Ile462 of xGK makes backbone-to-hubbone hydrogen bonds with Leu236 and Glu238 of loop L4 in xGK. Lys136 of L3 in xGK makes a hydrogen bond with a backbone carbonyl group of Leu463 in xGKRPs (Fig. 2B). Mutation in Lys136 of xGK, which is equivalent to Lys143 in rat GK, was shown to decrease the inhibition exerted by GKRPs in the study of rat GK (22). It is noteworthy that a single ion pair is the main interaction between xGKRP and the small domain of xGK. The mutations of Arg301 (Arg300 in xGKRP) or Asp413 (Asp412 in xGKRPs) in hGKRP to Ala display comparable binding affinities to hGK as WT hGKRP does (Table 1).

Table 1. *K₆₅* values of hGK for WT hGKRP and its mutants in the presence and absence of F6P (200 μM) or F1P (100 μM) by ITC

<table>
<thead>
<tr>
<th>Type</th>
<th><em>K₆₅</em> nM</th>
</tr>
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<tbody>
<tr>
<td>WT hGKRP</td>
<td>230 ± 26</td>
</tr>
<tr>
<td>WT hGKRP + F6P</td>
<td>95 ± 25</td>
</tr>
<tr>
<td>WT hGKRP + F1P</td>
<td>ND</td>
</tr>
<tr>
<td>hGKRP_L463A/F465A</td>
<td>ND</td>
</tr>
<tr>
<td>hGKRP_L463A</td>
<td>375 ± 97</td>
</tr>
<tr>
<td>hGKRP_L463A + F6P</td>
<td>235 ± 34</td>
</tr>
<tr>
<td>hGKRP_L463A + F1P</td>
<td>207 ± 27</td>
</tr>
<tr>
<td>hGKRP_L463A/F465A + F1P</td>
<td>194 ± 21</td>
</tr>
<tr>
<td>hGKRP_L463A/F465A + F6P</td>
<td>225 ± 50</td>
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Furthermore, the resulting hGKRP_L463A/F465A mutant, Leu463 is the key residue of hydrophobic interaction in the GK/GKRP complex. Thus, F1P perturbs the binding of GK to GKRPs and disrupts the complex by altering hydrophobic interaction between GK and GKRPs. It is noteworthy that F1P is likely to be more efficient than glucose or GKA in releasing GK from GKRPs. Although F1P, glucose, and GKA bind to distant sites from the interface of the GK/GKRP complex (9), F1P induces direct disruption of the interface to release GK from GKRPs, whereas glucose and GKA induce slow and energetically unfavorable rearrangement of the small domain (9, 17). The superimposition of F6P-bound hGKRP onto apo-hGKRP revealed that the interaction between the hydroxyl group of C1 in F6P and Glu348 of a10 in SIS domain 2 shifts a10 downward (Fig. S8 A and B) and rotates the helices a11 to a13 and a b-sheet, b6 to b10, of SIS domain 2 toward F6P (Fig. S8 A and B), thereby moving L1 and L2 of the wedge close to a14, enhancing the binding of GK compared with apo-hGKRP (Fig. S8C). Thus, His351 and Glu348 are likely to be the key residues in the allosteric regulation of hGKRP by its effectors. To verify the modulation mechanism described earlier, we mutated Glu348 and His351 in hGKRP to Asp and Pro, respectively. The structural consistency between hGKRP_E348A/H351P and WT hGKRP were confirmed based on CD analysis and binding of F6P to hGKRP_E348A/H351P by isothermal titration calorimetry (ITC; Fig. S9 and Table S2).

Table 2. The binding affinity of hGKRP_E348A/H351P and WT hGKRP were confirmed based on CD analysis and binding of F6P to hGKRP_E348A/H351P by isothermal titration calorimetry (ITC; Fig. S9 and Table S2). The binding affinity of hGKRP_E348A/H351P and WT hGKRP were confirmed based on CD analysis and binding of F6P to hGKRP_E348A/H351P by isothermal titration calorimetry (ITC; Fig. S9 and Table S2). The binding affinity of hGKRP_E348A/H351P and WT hGKRP were confirmed based on CD analysis and binding of F6P to hGKRP_E348A/H351P by isothermal titration calorimetry (ITC; Fig. S9 and Table S2). The binding affinity of hGKRP_E348A/H351P and WT hGKRP were confirmed based on CD analysis and binding of F6P to hGKRP_E348A/H351P by isothermal titration calorimetry (ITC; Fig. S9 and Table S2).
a key role in the interaction with GK upon the binding of F6P and F1P to hGKRP, acting as a pivotal residue inducing the structural change of hGKRP. On the contrary, His-351, which plays a critical role in the interaction with GK, is not conserved in xGKRP, and consequently xGKRP does not respond to the occupation of its allosteric site by F1P and F6P.

**Implications for Physiological Role of GKR.** GKR is known to be located mainly in hepatocytes with an excess ratio to GK, regulating the allosteric GK in response to glucose concentration (12, 14, 15). To get some insight into the physiological implications for the role of GKR, we attempted to investigate the regulatory features of GKR. To this end, we first examined the dissociation of GK from GKR in response to glucose, and determined the binding affinity of hGKR and hGKR(D413A) for hGK at varying glucose concentrations by using ITC (Fig. 5A and Fig. S10). As shown in Fig. 5A, the $K_d$ of hGKR for hGK displays a sigmoidal curve with respect to glucose concentration as presumed, showing an inflection point at approximately 20 mM glucose. On the contrary, the binding affinity of hGKR(D413A) for hGK greatly decreased even at 5 mM glucose. This result indicates that the ion pair between GKR and the GK small domain is a key for generating a sigmoidal-type release of GK from GKR in response to glucose concentration. To investigate the effect of GKR on the kinetic behavior of GK, we measured the hGK activity with increasing ratios of hGKR to hGK (Fig. 5B). The activity curve of hGK for a fourfold excess of hGKR became more sigmoidal, resulting in a higher Hill coefficient (2.8) than with hGK alone (1.8; Table S3). The increased sigmoidicity of the GK activity curve is coincident with previous in vivo reports showing the free GK activity or detritiation of [2-3H]glucose of hepatocytes in response to glucose (25). Thus, the regulation of GK by GKR in a sigmoidal pattern also seems to be valid in vivo. Interestingly, the hGK activity curve in the presence of hGKR(D413A) or WT hGKR and 25 mM KCl showed a decreased sigmoidity compared with that with only WT hGKR (Fig. 5C and Table S3). This result demonstrates that the increased sigmoidicity of the GK activity curve in the...
presence of GKRP is attributed to the ion pair between GKRP and the GK small domain. KCl seems to perturb the ion pair between GKRP and the GK small domain. Thus, previous studies describing GKRP as a classical competitive inhibitor (11, 26) are likely to be misled by addition of KCl. Our results imply that the release of GK from GKRP in a sigmoidal manner in response to glucose concentration is crucial for blood glucose control by the liver.

Discussion

We have demonstrated the molecular mechanism for the allosteric regulation of GK by GKRP and effectors based on the structure of the GK/GKRP complex. Our structural analysis revealed that GKRP binds to a super-open conformation of GK mainly through hydrophobic interaction, inhibiting the GK activity by locking a small domain of GK. We also showed how allosteric effectors of GK such as F1P and F6P indirectly modulate the GK activity in negative and positive way, respectively. More importantly, GKRP was revealed to release GK in a sigmoidal manner in response to glucose concentration by restricting a structural rearrangement of the GK small domain via a single ion pair. This study elucidates the molecular mechanism by which an allosteric protein regulates a monomeric allosteric enzyme. The GK/GKRP system exemplifies a unique case showing a cascade regulation mechanism of an allosteric enzyme by an allosteric regulatory protein and its effectors. Our results demonstrate the role for GKRP as an allosteric switch (27) that turns GK “on” and “off” in response to glucose concentration, providing crucial insight into the understanding of blood glucose homeostasis and the development of new antidiabetes drugs.

The sigmoidal nature driven by GKRP in regulating GK can be characterized into two distinct features. One is the release of GK from GKRP in a sigmoidal manner with respect to glucose concentration, leading to an amplification of the GK activity at high glucose levels. The other is an increased sigmoidicity of the GK activity curve, resulting in a turning-off of GK at low glucose concentration. This sigmoidal feature results from the binding of GKRP to a super-open conformation of GK and consequent restriction of a structural rearrangement of GK through a single ion pair. Those two features in regulation of GK by GKRP seem to be essential for two major functions of the liver, glucose clearance and glucose production in blood glucose homeostasis (3, 10). As GK itself displays a sigmoidal activity curve with respect to glucose concentration, leading to an amplification of the GK activity in the cytosol, which is crucial for fast glucose clearance.

At low glucose concentration, GK remains almost turned off in the range of 5 to 10 mM of glucose by the action of GKRP (Fig. 5B). Thus, the negative effector such as F1P is likely to be essential for glucose clearance in this range of glucose. Accordingly, precursors of F1P, such as fructose or sorbitol (16), are likely to be important for glucose clearance by liver after a meal. Indeed, supply of fructose together with glucose was shown to increase glycogen synthesis by liver in the rat and dog (32, 33). Although fructose can be efficient to relieve hyperglycemia in diabetes, the use of fructose as a substituent for glucose for diabetes patients has raised the controversy (34, 35). F1P is effective for releasing GK from GKRP, but a high amount or long-term supply of fructose might result in unexpected loss of GK as a result of proteasomal degradation of free GK in cytosol (29), which can cause the hyperglycemia to be more serious.

Allosteric activators of GK (i.e., GKAs) have been of great significance as antidiabetes drugs, and a number of diverse GKAs have been reported (5, 7–9). However, GKAs may cause serious side effects, as they change a sigmoidal activity curve of GK into a hyperbolic one (8, 9), and consequently alter the sensing of blood glucose level by GK in pancreatic β-cells, resulting in hyperinsulinemic hypoglycemia. As an approach to avoid the side effect, moderate or hepatocyte-specific GKAs are under development (36–38). With the same rationale and based on our result, GKRP can be a target for the development of new antidiabetes drugs that activate GK in hepatocytes. As GKRP is predominantly located in hepatocytes, the GKRP-targeting drugs can activate GK specifically in hepatocytes, thus preventing possible hypoglycemia. The binding site of allosteric effectors in GKRP and the interface of the GK/GKRP complex can be most promising drug targets.

Materials and Methods

The genes encoding GK and GKRP from X. laevis and humans were expressed in *Escherichia coli* and purified by Ni affinity, anion exchange,
and gel-filtration chromatography. The crystals of Xenopus GK/GKRP complex were obtained by the hanging-drop vapor diffusion method, and the structure was solved by using the MR method. The binding affinities of GK for GKRP mutants were measured by ITC. The activities of GK in the presence and absence of GKRP and allosteric effectors were determined by a G6P dehydrogenase-coupled assay. Detailed materials and methods are described in SI Materials and Methods.


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