Differences in regulatory properties between human and rat glucokinase regulatory protein

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The inhibition of glucokinase by rat and *Xenopus* GKRPs (glucokinase regulatory protein) is well documented. We report a comparison of the effects of human and rat GKRPs on glucokinase activity. Human GKRP is a more potent inhibitor of glucokinase than rat GKRP in the absence of fructose 6-phosphate or sorbitol 6-phosphate, and has a higher affinity for these ligands. However, human and rat GKRPs have similar affinities for fructose 1-phosphate and chloride. Residues that are not conserved between the rodent and human proteins affect both the affinity for fructose 6-phosphate and sorbitol 6-phosphate and the inhibitory potency of GKRP on glucokinase in the absence of these ligands.

Key words: diabetes, glucokinase regulatory protein, hexokinase IV, human glucokinase, liver.

INTRODUCTION

Glucokinase (hexokinase IV) is the main glucose phosphorylating enzyme expressed in the insulin secretory cells of the pancreas [1] and in the liver of most mammals [2]. It is also expressed in the hypothalamus [2]. In hepatocytes of mammals and lower vertebrates, glucokinase is regulated by a 68 kDa regulatory protein, GKRP (glucokinase regulatory protein), that inhibits glucokinase competitively with respect to glucose [3,4]. GKRP is also expressed in the hypothalamus [5]. The effects of rat GKRP on the kinetics of rat or human glucokinase have been extensively characterized using either GKRP purified from rat liver [3,4,6–9] or the recombinant protein [10,11]. Inhibition of glucokinase by rat GKRP *in vitro* is markedly potentiated by fructose 6-*P* (fructose 6-phosphate) or its analogue sorbitol 6-*P* (sorbitol 6-phosphate) [8], and counteracted by fructose 1-*P* (fructose 1-phosphate) [4]. Based on GKRP mutants that have altered affinity for fructose 6-*P* and fructose 1-*P*, these two ligands are thought to bind to the same site [11].

GKRP is also expressed in the livers of toad, turtle and *Xenopus laevis* [4]. GKRP from the last-mentioned species shares 57 % identity with rat liver GKRP [12]. It inhibits glucokinase competitively with respect to glucose, but is totally insensitive to both fructose 6-*P* and fructose 1-*P* [4,12].

Human GKRP shares 88 % identity with rat GKRP [13]. However, its properties with respect to the inhibition of glucokinase have not been reported. Because inactivating or activating mutations in the glucokinase gene cause diabetes or hypoglycaemia respectively [14], the GKRP gene is considered to be a candidate gene for involvement in Type II diabetes [15] or insulin resistance [16]. Accordingly, various mutations in the human GKRP gene have been identified [15,16]. Some, but not all, of these are in conserved residues [16].

We report in the present study that human GKRP has a higher affinity for fructose 6-*P* and sorbitol 6-*P*, and is also a more potent inhibitor of glucokinase in the absence of these ligands, compared with rat GKRP. This indicates that residues that are not conserved between human and rat GKRPs affect the inhibitory properties of human GKRP.

MATERIALS AND METHODS

Materials

Fructose 6-*P*, fructose 1-*P*, sorbitol 6-*P* (barium salt) and glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) were from Sigma, and phosphoglucoisomerase was from Roche.

Expression and purification of recombinant proteins

Human pancreatic and rat liver glucokinases were expressed in *Escherichia coli* with an N-terminal 6His tag. Purification of the expressed products from bacterial cell lysates was achieved using Ni^{2+}-nitrilotriacetate chromatography. The final protein was estimated as > 90 % pure by SDS/PAGE, with a final yield of 1 mg/g of cell paste. Human and rat GKRPs were expressed in *E. coli* with a C-terminal FLAG tag. Accumulation levels remained very low (detectable in whole cell lysates by Western blot only) despite attempts to manipulate fermentation conditions to maximize expression of soluble protein. The product was purified from bacterial cell lysates by chromatography on DEAE-Sepharose followed by M2 anti-FLAG antibody conjugated to agarose. The final products were over 90 % pure as estimated by SDS/PAGE (Tris/glycine 8–16 % gel; Novex), and were obtained at final yields of ~ 0.1 mg/g of cell paste for rat GKRP and < 0.1 mg/g of cell paste for human GKRP.

Assay of GKRP

The effect of GKRP on glucokinase activity was determined using the glucose-6-phosphate dehydrogenase coupled assay at 37 °C by a modification of the method described in [9]. The final reaction mixture contained 25 mM KCl, 25 mM Hepes, 1 mM ATP, 2 mM $MgCl_2$, 0.5 mM NAD^+, 1 mM dithiothreitol, 4 units/ml glucose-6-phosphate dehydrogenase, 0.25 mg/ml BSA, 5 mM glucose, pH 7.1, and 10 m-units/ml human pancreatic glucokinase, unless otherwise indicated. One unit of GKRP was determined as the amount that causes 50 % inhibition of glucokinase activity (10 m-units/ml) assayed in the presence of 5 mM glucose

Abbreviations used: GKRP, glucokinase regulatory protein; fructose 1-*P*, fructose 1-phosphate; fructose 6-*P*, fructose 6-phosphate; sorbitol 6-*P*, sorbitol 6-phosphate.

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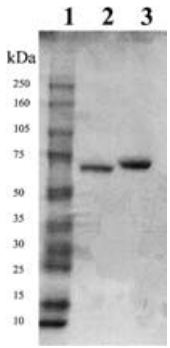

Figure 1 SDS/PAGE of purified recombinant GKRPs stained with Coomassie Blue

Lane 1, molecular mass markers (kDa); lane 2, human GKRP (2 μg); lane 3, rat GKRP (4 μg).

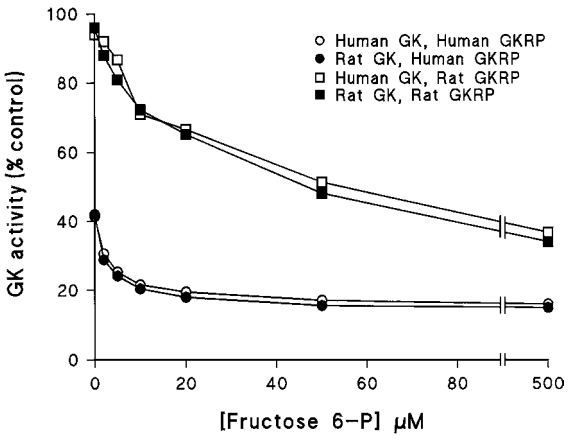

Figure 2 Inhibition of human pancreatic and rat liver glucokinases by human and rat GKRPs

Human pancreatic glucokinase (GK) (○, □) and rat liver glucokinase (●, ■) were assayed at an activity of 10 m-units/ml in the absence (100 %) or presence of 1.5 μg/ml human GKRP (○, ●) or 1.5 μg/ml rat GKRP (□, ■) and the concentrations of fructose 6-*P* indicated. Glucokinase (GK) activity is expressed as a percentage of the respective control in the absence of GKRP. There was no effect of fructose 6-*P* on glucokinase activity in the absence of GKRP. Results are representative of three experiments.

and 50 μM sorbitol 6-*P* under standard assay conditions. The activity of GKRP both in the absence and in the presence of sorbitol 6-*P* was determined from Dixon plots (reciprocal of glucokinase activity against GKRP concentration), which were linear as described previously [3]. Phosphoglucoisomerase activity was assayed in the presence of 100 mM KCl, 25 mM Hepes, 0.5 mM NAD^+, 1 mM fructose 6-*P* and glucose-6-phosphate dehydrogenase (4 units/ml) at 37 °C. One m-unit is defined as the amount catalysing the conversion of 1 nmol of substrate per min under assay conditions.

Protein was measured by the Bio-Rad reagent using BSA as standard. Statistical analysis was by the unpaired *t*-test.

RESULTS

Purity of GKRP preparations

Purified recombinant human and rat GKRPs were 90 % pure, as judged by densitometry analysis of SDS/polyacrylamide gels stained by Coomassie Blue (Figure 1). We determined the activity of phosphoglucoisomerase in the preparations of recombinant proteins, because this enzyme may interfere with the determination of the effects of fructose 6-*P* on the inhibition of glucokinase by GKRP. There was no detectable phosphoglucoisomerase activity in the glucokinase preparations. However, there was a low activity of phosphoglucoisomerase in the GKRP preparations (0.2 m-units/μg of rat GKRP and 0.4 m-units/μg of human GKRP). This phosphoglucoisomerase activity causes a small amount of interference in assays containing high concentrations (> 50 μM) of fructose 6-*P*. In assays with fructose 6-*P* (Figures 2 and 6), absorbance changes were corrected for appropriate blanks with fructose 6-*P* lacking glucose. For all other assays we used sorbitol 6-*P*, which is a more potent analogue than fructose 6-*P*, for determining the maximum inhibition of glucokinase by GKRP (as in [4,9]).

Inhibition of glucokinase by GKRP in the absence of fructose 6-*P* or sorbitol 6-*P*

Previous work by Veiga-da-Cunha et al. [9] showed that rat GKRP inhibits human pancreatic glucokinase to a similar extent as with rat liver glucokinase. This was confirmed in the present study (Figure 2). Likewise, human GKRP caused similar inhibition of human pancreatic and rat liver glucokinases (Figure 2). However, human GKRP differed from rat GKRP in that it caused a marked inhibition of glucokinase in the absence of fructose 6-*P* (Figure 2).

One unit of rat GKRP has been defined as the activity that inhibits glucokinase by 50 % in 1 ml in the presence of fructose 6-*P* [3] or sorbitol 6-*P* [4] under defined standard assay conditions. Studies on rat GKRP purified to homogeneity [8] and on recombinant rat GKRP [10] showed that, in the absence of fructose 6-*P* or sorbitol 6-*P*, there is a small inhibition of glucokinase corresponding to approx. 7 % of that in the presence of fructose 6-*P*. Accordingly, 50 % inhibition by GKRP in the absence of fructose 6-*P* or sorbitol 6-*P* occurred at 15–17 units of GKRP. In the present study the activity of rat GKRP determined from Dixon plots in the presence of sorbitol 6-*P* was 647 units/mg of protein (Figure 3). In the absence of sorbitol 6-*P* or fructose 6-*P*, 50 % inhibition occurred at a 17-fold higher concentration of rat GKRP, in agreement with previous findings on recombinant [10] or purified [9] rat GKRP. For human GKRP the activity in the presence of sorbitol 6-*P* was 1350 units/mg of protein, whereas, in the absence of sorbitol 6-*P* or fructose 6-*P*, 50 % inhibition of glucokinase occurred at 5.9 units of human GKRP. Accordingly, human GKRP has a lower dependence on the presence of sorbitol 6-*P* (or fructose 6-*P*) than rat GKRP, with an activity ratio (in the presence compared with the absence of sorbitol 6-*P*) of 5.5 ± 0.5 for human GKRP (means $\pm$ S.E.M.; $n = 13$) and 17.9 ± 1.1 for rat GKRP ($n = 9$; $P < 0.001$ for human compared with rat GKRP).

The difference in activity ratio (+/− sorbitol 6-*P*) between human and rat GKRPs could not be explained by contaminating activity of phosphoglucoisomerase, because addition of this enzyme at activities up to 5-fold higher than were present in the preparation of human GRKP to rat or human GKRP had no effect on the activity ratio. Similarly, increasing the activity of the coupling enzyme glucose-6-phosphate dehydrogenase 3-fold did not affect the difference in activity ratios between the human and rat proteins. Incubation of human or rat GKRP for 3 h at 25 °C caused a small (10 %) decrease in activity (units/mg of protein), but did not affect the activity ratio (+/− sorbitol 6-*P*) of either the human or the rat protein, suggesting that the difference is not due to stability of the protein.

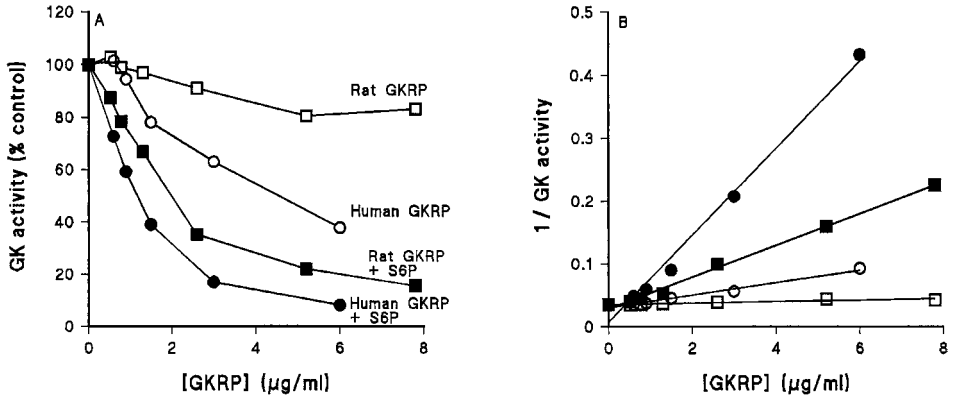

Figure 3 Effect of the concentration of human or rat GKRP on human glucokinase activity

Glucokinase (GK) activity (10 m-units/ml) was measured in the absence (○, □) or presence (●, ■) of 50 μM sorbitol 6-*P* (S6P) and the concentrations of human (○, ●) GKRP or rat (□, ■) GKRP indicated. (**A**) Glucokinase activity (% of control without GKRP); (**B**) reciprocal of glucokinase activity. Results are representative of nine (rat GKRP) or 13 (human GKRP) experiments.

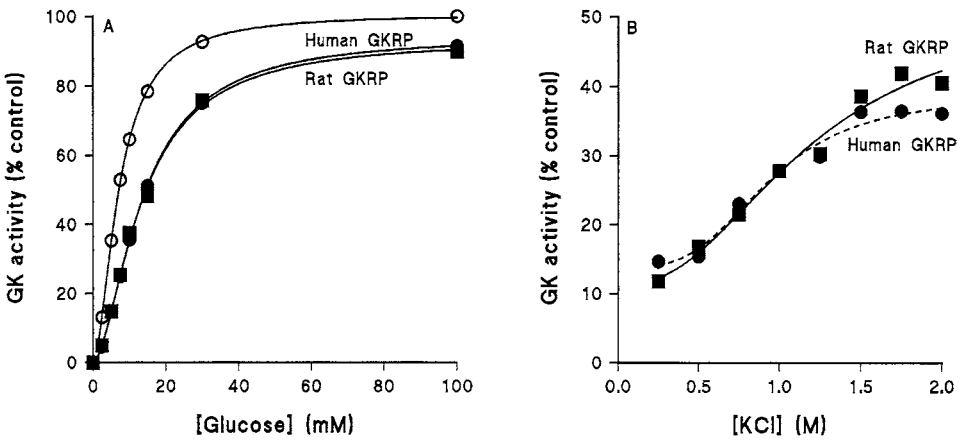

Figure 4 Effects of human and rat GKRPs on the activity of glucokinase at various concentrations of glucose or KCl

Glucokinase (GK) activity (15 m-units/ml) was measured in the presence of 50 μM sorbitol 6-*P* and the concentrations of glucose (left-hand panel) or KCl (right-hand panel) indicated, in the absence (○) or presence of human GKRP (●; **A**, 0.5 μg/ml; **B**, 2 μg/ml) or rat GKRP (■; **A**, 0.8 μg/ml; **B**, 3 μg/ml). Results are representative of three experiments.

Competitive inhibition with respect to glucose, and counteraction by chloride

When the effects of human and rat GKRPs were compared at various glucose concentrations in the presence of sorbitol 6-*P*, human GKRP caused a similar increase in the $S_{0.5}$ (concentration that causes half-maximal activity) for glucose, with a negligible change in V_{max}, to rat GKRP (Figure 4A). Similarly, when the effects of GKRP were tested at increasing KCl concentrations in the presence of sorbitol 6-*P*, human GKRP showed similar partial reversal of the inhibition by KCl as rat GKRP (Figure 4B), in agreement with previous findings for rat GKRP [4].

Effects of fructose 1-*P*, sorbitol 6-*P* and fructose 6-*P*

The inhibition of glucokinase by rat GKRP in the presence of fructose 6-*P* or sorbitol 6-*P* is reversed by fructose 1-*P* [4]. It was confirmed in the present study that fructose 1-*P* counteracts the inhibition of gluco-kinase by rat GKRP in the presence of sorbitol 6-*P*. In addition, it is shown that fructose 1-*P* also counteracted the inactivation of glucokinase caused by rat and human GKRPs in the absence of sorbitol 6-*P* (Figure 5). It is noteworthy that, in

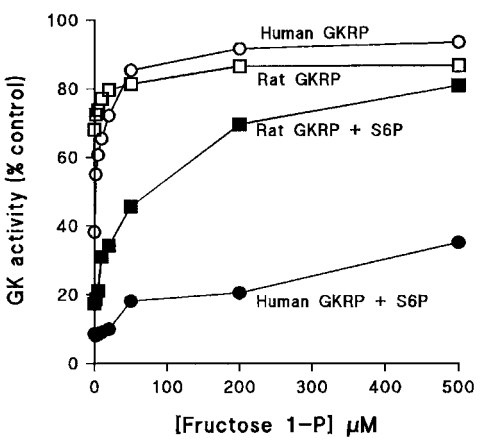

Figure 5 Effect of fructose 1-*P* concentration on the inhibition of glucokinase activity by human and rat GKRPs

Glucokinase (GK) activity (10 m-units/ml) was measured in the absence (○, □) or presence (●, ■) of 50 μM sorbitol 6-*P* (S6P) and either 2.5 μg/ml human GKRP (○, ●) or 4.8 μg/ml rat GKRP (□, ■). Results are expressed as a percentage of the activity in the absence of GKRP, and are representative of three experiments.

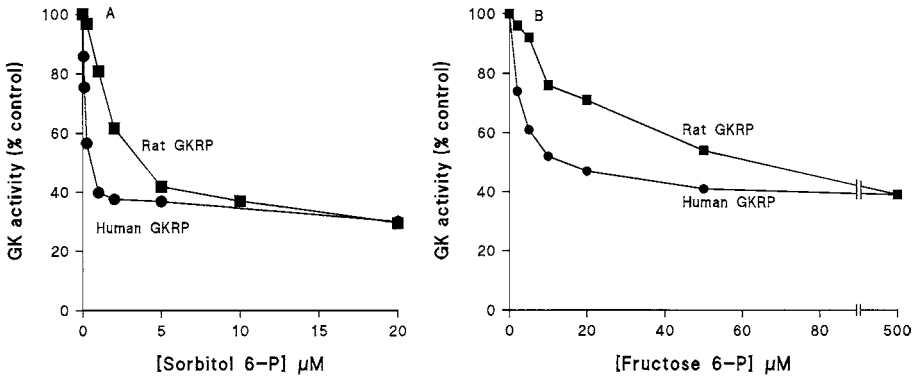

Figure 6 Effect of sorbitol 6-*P* or fructose 6-*P* concentration on the inhibition of glucokinase by human and rat GKRPs

Glucokinase (GK) activity (9 m-units/ml) was measured in the presence of human GKRP (●; **A**, 2 μg/ml; **B**; 1.5 μg/ml) or rat GKRP (■; **A**, 4 μg/ml; **B**, 1.5 μg/ml) and the concentrations of sorbitol 6-*P* (left-hand panel) and fructose 6-*P* (right-hand panel) shown. Values are expressed as a percentage of the activity in the presence of GKRP and the absence of sorbitol 6-*P* or fructose 6-*P*, and are representative of eight (sorbitol 6-*P*) and seven (fructose 6-*P*) experiments.

the presence of fructose 1-*P*, the activity of glucokinase was not higher than in the absence of GKRP, indicating that the effect of fructose 1-*P* can be explained by reversal of inhibition by GKRP. The concentration of fructose 1-*P* that caused half-maximal activation (or reversal of inhibition) in the absence of sorbitol 6-*P* was similar (10 μM) for human and rat GKRPs. In the presence of 50 μM sorbitol 6-*P* the concentration of fructose 1-*P* that caused half-maximal activation was greater for human than for rat GKRP. This can be explained by the higher affinity of human compared with rat GKRP for sorbitol 6-*P* and fructose 6-*P* (Figure 6A).

The affinities of human and rat GKRPs for sorbitol 6-*P* or fructose 6-*P* were determined at concentrations of GKRP between 2 and 4 units/ml. Human GKRP had a 5–10-fold higher affinity for sorbitol 6-*P* [IC_{50}: human GKRP, 0.2 ± 0.05 μM ($n = 11$); rat GKRP, 2.5 ± 0.4 μM ($n = 9$); means $\pm$ S.E.M., $P < 0.001$] and fructose 6-*P* [IC_{50}: human GKRP, 4.4 ± 0.5 μM ($n = 5$); rat GKRP, 19.3 ± 1.5 μM ($n = 4$); $P < 0.01$] than did rat GKRP (Figure 6B).

DISCUSSION

The kinetic properties of GKRPs from rat [3,4,6–9] and *Xenopus* [4,12] have been described. Inhibition of glucokinase by purified rat GKRP or recombinant rat GKRP is largely dependent on the presence of fructose 6-*P*, with inhibition in the absence of fructose 6-*P* corresponding to approx. 7% [8,10]. In contrast, inhibition by *Xenopus* GKRP, which shares 57% identity with rat GKRP, is independent of fructose 6-*P* and fructose 1-*P* [12]. Similarly, GKRPs from toad and turtle also inhibit glucokinase independently of fructose 6-*P* [4]. Despite this difference between GKRPs from rat and lower vertebrates, the inhibition of glucokinase by GKRPs from lower vertebrates is also competitive with respect to glucose and shows a similar reversal by KCl to that by rat GKRP [4].

Human GKRP shares 88% identity with rat GKRP [13]. We show in the present study that inhibition of glucokinase by human GKRP shows two differences from that by rat GKRP. First, in the absence of fructose 6-*P* or sorbitol 6-*P*, human GKRP causes a greater inhibition of glucokinase than rat GKRP. Secondly, human GKRP has a higher affinity for fructose 6-*P* and sorbitol 6-*P* than rat GKRP. A key finding from the present study is that not only does fructose 1-*P* reverse the inhibition of glucokinase by GKRP in the presence of fructose 6-*P* or sorbitol 6-*P*, as expected from previous work [3,4,7,8], but it also totally counteracts the inhibition of glucokinase by human GKRP in the absence of fructose 6-*P* or sorbitol 6-*P*. This reinforces the central role of fructose 1-*P* as a regulator/activator of glucokinase in liver by causing dissociation of glucokinase from GKRP [6], and is consistent with the evidence for improved glucose tolerance following fructose administration in humans [17–19].

It has been proposed that fructose 6-*P* and fructose 1-*P* bind to two different conformations of GKRP, only one of which forms a heterodimer with glucokinase [6]. The present findings suggest that residues that are not conserved between human and rat GKRPs favour the conformation that forms a heterodimer with glucokinase. This may be of interest with respect to mutations of the human GKRP gene in residues that are not conserved between the human and rat proteins.

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