The Role of the Regulatory Protein of Glucokinase in the Glucose Sensory Mechanism of the Hepatocyte*

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Glucokinase has a very high flux control coefficient (greater than unity) on glycogen synthesis from glucose in hepatocytes (Agius et al., J. Biol. Chem. 271, 30479–30486, 1996). Hepatic glucokinase is inhibited by a 68-kDa glucokinase regulatory protein (GKRP) that is expressed in molar excess. To establish the relative control exerted by glucokinase and GKRP, we applied metabolic control analysis to determine the flux control coefficient of GKRP on glucose metabolism in hepatocytes. Adenovirus-mediated overexpression of GKRP (by up to 2-fold above endogenous levels) increased glucokinase binding and inhibited glucose phosphorylation, glycolysis, and glycogen synthesis over a wide range of concentrations of glucose and sorbitol. It decreased the affinity of glucokinase translocation for glucose and increased the control coefficient of glucokinase on glycogen synthesis. GKRP had a negative control coefficient of glycogen synthase that is slightly greater than unity (~1.2) and a control coefficient on glycolysis of ~0.5. The control coefficient of GKRP on glycogen synthesis decreased with increasing glucokinase overexpression (4-fold) at elevated glucose concentration (35 mm), which favors dissociation of glucokinase from GKRP, but not at 7.5 mm glucose. Under the latter conditions, glucokinase and GKRP have large and inverse control coefficients on glycogen synthesis, suggesting that a large component of the positive control coefficient of glucokinase is counterbalanced by the negative coefficient of GKRP. It is concluded that glucokinase and GKRP exert reciprocal control; therefore, mutations in GKRP affecting the expression or function of the protein may impact the phenotype even in the heterozygote state, similar to glucokinase mutations in maturity onset diabetes of the young type 2. Our results show that the mechanism comprising glucokinase and GKRP confers a markedly extended responsiveness and sensitivity to changes in glucose concentration on the hepatocyte.
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hepatic glycogen metabolism and to determine the effects of changes in the ratio of expression of GKRP and glucokinase on the control coefficients of the two proteins and thereby evaluate the contribution of GKRP to the glucose sensory mechanism of the hepatocyte. Our results show that the mechanism comprising glucokinase and GKRP confers a markedly enhanced responsiveness and sensitivity to glucose on the hepatocyte.

MATERIALS AND METHODS

Materials—DEAE-Sephrose, CH-Sepharose 4B, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, sorbitol 6-phosphate, glucose 6-phosphate dehydrogenase from Escherichia coli, all other enzymes were from Sigma. [U-14C]glucose, [2-3H]glucose, and [3-3H]glucose were from New England Nuclear. Sources of other reagents were as described in Ref. 18.

Preparation of Recombinant Adenoviruses—The cDNA encoding rat liver GKRP (a generous gift from Dr. E. Van Schaftingen, University of Brussels, Brussels, Belgium) was cloned into pACC vector and co-transformed with pM17 vector into 293 cells to achieve homologous recombination and production of recombinant adenoaviruses (AdCMV-GKRP) as described previously (19). Recombinant adenoaviruses containing the cDNA for rat liver glucokinase (AdCMV-GKL) (20) was a kind gift from Dr. C. Newgard (University of Texas, Dallas, TX). The adenoaviruses were amplified in 293 cells (19), and aliquots of medium were used. Viral titers were determined that resulted in protein overexpression of between 30% and 2-fold above endogenous levels for GKRP and between 30% and 4-fold above endogenous levels for glucokinase at 18 h after treatment.

Hepatocyte Isolation and Culture—Hepatocytes were isolated by collagenase perfusion of the liver of male Wistar rats (body weight, 180–250 g) fed ad libitum (21). They were suspended in minimal essential medium (MEM) containing 7% (v/v) neonatal calf serum and seeded at a density of 4 × 10⁶ cells/cm² in 24-well plates for metabolic studies and glucokinase determination, in 6-well plates for GKRP determination, or on gelatin-coated coverslips for immunostaining. After cell attachment (2–3 h), the medium was replaced by serum-free MEM containing the adenoaviruses or control medium. After an additional 2 h, the medium was replaced by serum-free MEM containing 10 mM dexamethasone, and the cells were cultured for 16–18 h before use for metabolic studies. Unless stated otherwise, the MEM contained 5 mM glucose.

Immunostaining—Cells cultured on 13-mm coverslips were washed in PBS, fixed in 4% paraformaldehyde in PBS (30 min), and washed in PBS (11). They were incubated with NaBH₄ (1 mg/ml, 10 min) and then permeabilized in 0.5% (v/v) Triton X-100 in PBS and blocked with 1% bovine serum albumin/0.02% Triton X-100/PBS. They were incubated overnight at 4 °C with primary antibody against GKRP (22) washed in PBS and incubated with a Texas Red-conjugated antibody against rabbit IgG (Jackson Immunoresearch) in 0.1% Triton X-100/1% bovine serum albumin for 1 h at room temperature. They were then washed in PBS, water, and ethanol and mounted onto microscope slides with Mostow's glycerol/glycine (22.2:100 v/v) (Aldrich). Images of stained cells were obtained using a Bio-Rad-600 Laser Scanning Confocal Microscope equipped with a 15 mW krypton-argon laser. The 568 nm line was used to excite the Texas Red fluorophore. For each field, a series of 1-μm optical sections were combined to produce a single image.

Metabolic Studies—For metabolic studies (Figs. 4 and 5) and control coefficient determinations (Figs. 6–8), parallel incubations were performed for determination of glucose metabolism and the activities of glucokinase and GKRP. Incubations for determination of glucose phosphorylation ([2-3H]glucose), glycogen synthesis ([U-14C]glucose), and glycogen synthesis ([U-14C]glucose) were for 2.5 h in MEM containing the substrates (25 mM) and all other components as described previously (23). They were then cultured in fresh MEM containing 10 mM dexamethasone and diluted with unsubstituted Sepharose 4B (26). After elution with 1 mM glucose, the glucokinase was purified and concentrated by DEAE-Sephadex chromatography. The preparation had a specific activity of 150–200 units/mg protein (using bovine serum albumin as standard).

Control Coefficients of GKRP and Glucokinase—The control coefficient (CGL) of GKRP or glucokinase were determined for glycogen synthesis or glycolysis. The control coefficient is defined as the fractional change (±, increase; , decrease) in metabolic flux (J) that results from a fractional change in enzyme activity (ε).

\[ CGL = \frac{\delta J}{\delta \epsilon} \]  

(Eq. 1)

where J is the flux through the pathway, and ε is the total activity of GKRP or glucokinase (14, 15). Control coefficients were determined from the slope of log J (glycogen synthesis or glycolysis) against log ε (total activity of GKRP or glucokinase) as described previously (16, 18). For determination of the control coefficient of GKRP, hepatocytes were treated with either 3 or 5 titers of AdCMV-GKRP, and for determination of the control coefficients of glucokinase, 4 titers of AdCMV-GKL were used. In experiments in which the control coefficients of both glucokinase and GKRP were determined, hepatocytes were treated with 3 titers of AdCMV-GKRP, in each case without or with four titers AdCMV-GK.

Results are expressed as the means ± S.E. for the number of cell preparations indicated. Statistical analysis was performed by using the paired t test.
RESULTS

Treatment of Hepatocytes with AdCMV-GKRP Increases the Activity of GKRP—The activity of GKRP in hepatocyte extracts was determined from the inhibition of purified glucokinase in the presence of sorbitol 6-phosphate, which enhances binding of GKRP to glucokinase (7) after fractionation of the hepatocyte extracts on DEAE-Sepharose as described previously (7). GKRP eluted at 75–100 mM KCl in fractions 7–10 (Fig. 1A), and glucokinase activity eluted in fractions 12 and 13 (data not shown). Treatment of hepatocytes with AdCMV-GKRP resulted in an increase in GKRP activity of between 37% and 2-fold above endogenous activity (Fig. 1B), an increase in immunostaining in the nucleus (Fig. 2), and a more intense band by Western blot analysis (data not shown). The endogenous GKRP content of untreated hepatocytes in this study was 0.68 ± 0.15 Units/mg cell protein, and the glucokinase activity was 19.8 ± 3.7 milliunits/mg. Assuming an activity of 0.2 Units/pmol for GKRP (27) and 10 milliunits/pmol for glucokinase, this corresponds to a GKRP/glucokinase molar ratio of 1.8 ± 0.18 (means ± S.E., n = 7) in untreated hepatocytes.

GKRP Overexpression Increases the Binding of Glucokinase in Hepatocytes—In the experiments shown in Fig. 3, glucokinase and GKRP were overexpressed simultaneously by treatment of hepatocytes with either varying titers of AdCMV-GK alone (open symbols) or in the additional presence of 3 titers (50, 100, and 200 μl/ml) of AdCMV-GKRP. Overexpression of GKRP by up to 2-fold above endogenous activity decreased free (Fig. 3A) and increased bound (Fig. 3B) glucokinase, confirming that the overexpressed protein binds glucokinase in the intact hepatocyte.

GKRP Overexpression Inhibits Glucose Phosphorylation, Glycolysis, and Glycogen Synthesis—Binding of glucokinase to GKRP in intact hepatocytes is decreased by elevated glucose concentrations and by precursors of fructose 1-phosphate such as sorbitol (9, 10, 28). Control hepatocytes and cells treated with AdCMV-GKRP with approximately 2-fold overexpression of GKRP (1.5 ± 0.16 versus 0.7 ± 0.1 units/mg) were incubated with varying glucose concentration (Fig. 4). GKRP overexpression caused a decrease in free glucokinase activity, glucose phosphorylation, glycolysis, and glycogen synthesis (Fig. 4, A–D). The concentration of glucose that caused a half-maximal effect on free glucokinase activity was 36 mM in cells treated with AdCMV-GKRP compared with 25 mM for untreated cells (Fig. 4A).

Increased glucokinase binding and inhibition of glucose phosphorylation, glycolysis, and glycogen synthesis by GKRP overexpression were also observed when hepatocytes were incubated with 7.5 mM glucose and varying concentrations of sorbitol (Fig. 5, A–D). Plots of rates of glycolysis or glycogen synthesis against the corresponding free glucokinase activity (Fig. 5, E and F) show that the inhibition of glycolysis and glycogen synthesis can be explained by increased binding of glucokinase.

GKRP Has a Greater Negative Control Coefficient on Glycogen Synthesis than on Glycolysis—The flux control coefficient of an enzyme (or protein) with respect to a particular metabolic pathway is a measure of the fractional response of pathway flux (J) to a fractional change in the concentration of the protein. The control coefficients of GKRP on glycolysis and
Effects of Glucokinase Overexpression on the Control Coefficient of Glucokinase—In the previous paragraph, the flux control coefficient of glucokinase was determined at endogenous levels of GKRP and at overexpressed levels from double logarithmic plots of glycogen synthesis (at 7.5 and 35 mM glucose) against total glucokinase activity. As shown in Fig. 8, the control coefficient of glucokinase was greater at 7.5 mM than at 35 mM glucose, and overexpression of GKRP increased the control coefficient of glucokinase at both 7.5 and 35 mM glucose.

DISCUSSION

The liver has a major role in the control of blood glucose homeostasis. Glucokinase, the main glucose-phosphorylating enzyme in hepatocytes, and its inhibitor, GKRP, together are key components of the glucose sensory mechanism. The objective of this study was to gain a deeper understanding of the advantages conferred on the cell by this complex mechanism of regulation. To this end, we overexpressed GKRP by up to 2-fold above endogenous activity using recombinant adenovirus. This produced a functional protein that localized in the nucleus and inhibited glucokinase activity both in vitro and in the intact cell in vivo. Twofold overexpression of GKRP did not block the translocation of glucokinase by either elevated glucose concentration or sorbitol. However, it decreased the affinity of glucokinase translocation and glucose phosphorylation for glucose. The initial observation that the affinity of glucose phosphoryl-

glycogen synthesis were determined from the slope of double logarithmic plots of rates of metabolic flux against GKRP activity (Fig. 6). These plots were linear for glycogen synthesis but not for glycolysis (Fig. 6), indicating that the control coefficient of GKRP on glycogen synthesis decreases with GKRP overexpression over the range of protein overexpression studied. GKRP had a greater negative control coefficient on glycogen synthesis than on glycolysis (Table I). The control coefficient on glycogen synthesis was slightly greater than unity (–1.2). A control coefficient of unity (negative) signifies that a fractional increase in protein concentration is associated with a proportional decrease in flux. The control coefficient on glycolysis was lower in the presence of sorbitol or elevated glucose (Table I).

Effects of Glucokinase Overexpression on the Control Coefficient of GKRP—In another series of experiments, hepatocytes were treated with combinations of both AdCMV-GK (4 titers to overexpress glucokinase) and AdCMV-GKRP (3 titers to overexpress GKRP) to determine the control coefficient of GKRP at basal glucokinase and at elevated glucokinase activity. The control coefficient of GKRP on glycogen synthesis was calculated for glucokinase activity at 7.5 and 35 mM glucose in the control coefficient of GKRP on glycogen synthesis in cells with endogenous glucokinase activity (Fig. 7 and Table I), with increasing glucokinase activity, the control coefficient of GKRP on glycogen synthesis decreased at 35 mM glucose but not at 7.5 mM glucose (Fig. 7).

FIG. 3. Treatment of hepatocytes with AdCMV-GKRP increases the binding of overexpressed glucokinase. Hepatocytes were treated with different titers of AdCMV-GK in the absence (○) or presence of AdCMV-GKRP (■, 50 μl; ▲, 100 μl; ▼, 200 μl/ml). After 20 h of culture, the free glucokinase activity and bound glucokinase activity were determined by permeabilization of hepatocytes with digitonin. Free activity (A) and bound activity (B) are plotted against total glucokinase activity. Results are representative of five experiments.

FIG. 4. AdCMV-GKRP increases glucokinase binding and inhibits glucose phosphorylation, glycolysis, and glycogen synthesis at varying glucose concentrations. Hepatocytes were either untreated (○) or treated with 200 μl/ml AdCMV-GKRP (■). After 18 h of culture, they were incubated for 3 h in MEM containing the concentrations of glucose indicated and either [2-3H]glucose (glucose phosphorylation), [3-3H]glucose (glycolysis), or [U-14C]glucose (glycogen synthesis). Free glucokinase activity and bound glucokinase activity were determined by permeabilization of hepatocytes with digitonin. Results are the means ± S.E. for three (B) or five (A, C, and D) cultures. *, p < 0.05; **, p < 0.005 AdCMV-GKRP-treated hepatocytes versus respective controls.
ation for glucose in hepatocytes is lower than can be explained by the sigmoidal kinetics of glucokinase (S < 0.5, 8 mM) versus 25 mM) was made by Bontemps et al. (29). The discovery of GKRP by Van Schaftingen (30) provided an explanation for the low affinity of glucose phosphorylation for glucose in intact hepatocytes based on competitive inhibition of glucokinase by GKRP. The present study demonstrates that in cells with 2-fold overexpression of GKRP, the concentration of glucose causing a half-maximal effect on glucokinase translocation was increased by 44% (36 versus 25 mM). This clearly establishes that the affinity of the hepatocyte for glucose depends on the ratio of GKRP:glucokinase and decreases with increasing ratio. Overexpression of GKRP also inhibited glycolysis and glycogen synthesis. This inhibition correlated with the free glucokinase activity, confirming that it can be explained by glucokinase binding to GKRP.

Metabolic control analysis is a very powerful analytical approach for quantitative description and understanding of how control is distributed among the component enzymes of a metabolic pathway in the intact cell, and accordingly of the magnitude of the control exerted by a particular enzyme (14, 15, 31). It defines the quantitative relation between metabolic flux and the activities or concentrations of enzymes that are components of the pathway or compete for substrate (32, 33). In the case of glucokinase, the activity is a function of the total glucokinase concentration and the proportion that is bound to GKRP. The latter is determined by the concentration of GKRP and also by the concentrations of glucose (9) and fructose 1-phosphate (34). It has been proven by the summation theorem that the sum of the flux control coefficients of all the component enzymes in relation to a metabolic pathway must equal unity (33). We have shown previously that glucokinase has a very high control coefficient (greater than unity) on glycogen synthesis from glucose (16), and we suggested two possible explanations for this high control coefficient. First, it could be due to a substrate cycle between glucokinase and glucose 6-phosphatase. This would require a comparable and negative control coefficient of glucose 6-phosphatase. This was found not to be the case because glucose 6-phosphatase has a low control coefficient (< 0.3) on glycogen synthesis (18). Second, it could be due to the compartmentation of glucokinase in association with GKRP. In this study, we were able to test the latter hypothesis directly by measuring the control coefficient of GKRP. The results provide direct support for this hypothesis.

Four findings emerged from the control analysis experiments: 1) the negative control coefficient of GKRP on glycogen synthesis was greater than unity, 2) GKRP overexpression increases the control coefficient of glucokinase on glycogen synthesis, 3) a moderate increase in glucokinase overexpression was associated with a decrease in control coefficient of GKRP at high (35 mM) glucose concentration but not at low (7.5 mM) glucose concentration (taken together, these results suggest that the control coefficients of both glucokinase and GKRP on glycogen synthesis are a function of the ratio of the two proteins), and 4) GKRP has a greater control coefficient on glycogen synthesis than on glycolysis.

A key finding from this study is the high (negative) control coefficient of GKRP on glycogen synthesis. This suggests that the negative control coefficient of GKRP is the major determinant of the high control coefficient of glucokinase in such a way that the high positive control coefficient of glucokinase is coun-
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Hepatocytes treated with varying titers of AdCMV-GKRP were incubated in MEM containing 7.5 mM glucose and either [3-3H]glucose or [U-14C]glycogen for determination of glycolysis and glycogen synthesis, respectively. The control coefficient of GKRP was determined from the initial slope of the double logarithmic plot of rates of glycolysis or glycogen synthesis against GKRP activity. Results are the means ± S.E. for either three (glycolysis) or seven (glycogen synthesis) experiments.

| Substrate       | Glycolysis C^GKRO  | Glycogen synthesis C^GSKRO |
|-----------------|--------------------|----------------------------|
| 7.5 mM glucose  | $-0.50 ± 0.06$     | $-1.2 ± 0.16^c$           |
| 7.5 mM glucose + 200 μM sorbitol | $-0.25 ± 0.06^b$ | $-1.1 ± 0.16^c$           |
| 35 mM glucose   | $-0.27 ± 0.07^b$   | $-1.1 ± 0.16^c$           |

*p < 0.01, glycogen synthesis versus glycolysis.

*p < 0.03, versus corresponding values at 7.5 mM glucose.

The finding that GKRP has a greater control coefficient on concentration of GKRP determines the free glucokinase activity, a moderate increase in GKRP by about 2-fold decreases the free glucokinase and thereby increases its control coefficient and capacity for regulation. The greater decrease in the flux control coefficient of GKRP with increasing glucokinase overexpression at high glucose concentration (35 mM) compared with 7.5 mM glucose is also consistent with the hypothesis that there is an optimal ratio of GKRP:glucokinase that ensures maximum control by the two proteins. Because elevated glucose concentrations favor increased dissociation of glucokinase and GKRP, the more rapid decline in flux control coefficient of GKRP at increasing glucokinase can be explained by the lower affinity of GKRP for glucokinase at high glucose concentrations. The lack of decrease in the flux control coefficient of GKRP with increasing glucokinase overexpression at 7.5 mM glucose can be explained by two factors: 1) the lack of decrease in control coefficient of GKRP on glycogen synthesis at 7.5 mM glucose at increasing GKRP overexpression (linear response in Fig. 6B), and 2) the lower affinity of glucokinase translocation for glucose at elevated GKRP over expression.

Fig. 6. The control coefficient of GKRP on glycolysis and glycogen synthesis. Hepatocytes treated with varying titers of AdCMV-GKRP were incubated in MEM containing 7.5 mM glucose and either [3-3H]glucose or [U-14C]glycogen for determination of glycolysis and glycogen synthesis, respectively. Results are representative experiment of three (A) or seven (B) experiments that are summarized in Table I.

Fig. 7. The control coefficient of GKRP on glycogen synthesis is dependent on the expression of GKRP. Experimental details were as described in the Fig. 7 legend. The control coefficient of glucokinase on glycogen synthesis was determined at either 7.5 (○) or 35 mM (●) glucose from the initial slope of double logarithmic plots of glycogen synthesis versus total glucokinase activity. Results are the means ± S.E. of three to four cultures. *, p < 0.05. **, p < 0.005.
glycogen synthesis than on glycolysis was not surprising because both glucokinase and glucose 6-phosphatase have higher control coefficients on glycogen synthesis compared with glycolysis (18). This is most likely due to differences in the glucose 6-phosphate dependence of the two pathways. The linear inhibition of glycogen synthesis as compared with the nonlinear inhibition of glycolysis with increasing GKRP overexpression may be explained by the absolute dependence of glycogen synthase activation on glucose 6-phosphate produced by glucokinase (35, 36).

In metabolic pathways that are regulated by cooperative feedback inhibition of enzymes, flux control is shifted downstream of the inhibitory metabolite (37). The present study, combined with our previous finding on the control coefficient of glucokinase (16), suggests that a high degree of control of hepatic glycogen synthesis resides before the formation of glucose 6-phosphate. This is consistent with the role of this metabolite in feed-forward activation of glycogen synthase (35, 36).

Based on the above findings, three conclusions are drawn from this study:

1. GKRP has a high control coefficient on glycogen synthesis and glycolysis. Because we have shown that glucokinase also has a very high control coefficient on hepatic glycogen synthesis (16), we conclude that small changes in either protein will have a large metabolic impact. This is the case for glucokinase because impaired hepatic glycogen synthesis has been shown in diabetic MODY-2 subjects who are heterozygous for glucokinase mutations (16), we conclude that small changes in either protein will have a significant impact on glucose utilization by the liver in the heterozygote state, similar to those shown for MODY-2.

2. Another important conclusion from this study is that changes in the molar ratio of GKRP:glucokinase within the physiological range will affect the sensitivity (S_{0.5}) of the liver cell to glucose. Therefore, by changing the ratio between the components of the system, the cell can adapt to different conditions. This seems to be the case in vivo. Fasting and insulin deficiency are associated with a more rapid decay of glucokinase relative to GKRP, with a consequent increase in the GKRP:glucokinase ratio relative to the normal fed state (7). Our results indicate that under these conditions, the affinity of the hepatocyte for glucose is decreased. Conversely, the more rapid increase in glucokinase compared with GKRP during refeeding (7) would be associated with an increase in glycogen synthesis because of the high control coefficient of glucokinase and the increased affinity for glucose, resulting in a markedly amplified response.

3. Our results show in a quantitative way that the mechanism comprising glucokinase and GKRP confers on the hepatocyte a versatile mechanism to adjust glucose phosphorylation far beyond the sensitivity and responsiveness possible by a single sigmoidal enzyme alone. This mechanism represents a step forward in the design of enzymatic systems with increased responsiveness and ability to respond to an extended range of substrate concentrations.

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