RESEARCH COMMUNICATION

The regulatory protein of glucokinase binds to the hepatocyte matrix, but, unlike glucokinase, does not translocate during substrate stimulation

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The kinetic properties of hepatic glucokinase (hexokinase IV) are modulated by binding to a regulatory protein. This study shows that, in hepatocytes incubated with 5 mM glucose as sole carbohydrate substrate, both glucokinase and its regulatory protein bind to the cell matrix by a Mg^{2+}-dependent mechanism. After incubation with an elevated [glucose] or with fructose, glucokinase, but not its regulatory protein, translocates from the Mg^{2+}-dependent binding site. It is suggested that the regulatory protein acts as a receptor for anchoring glucokinase to the hepatocyte matrix and inhibiting its activity in metabolically quiescent conditions.

INTRODUCTION

Hexokinase IV or D, commonly known as glucokinase, is one of four hexokinase isoenzymes present in mammalian tissues [1]. It is expressed in liver and in the insulin-secreting cells of the pancreas and differs from the other isoenzymes in its lower affinity for glucose and sigmoidal kinetics, its lower molecular mass (50 kDa rather than 100 kDa) [1,2], and in binding to a 68 kDa regulatory protein, which decreases its affinity for glucose [3–6]. Binding of glucokinase to this protein is modulated by fructose 6-phosphate and fructose 1-phosphate [7]. Hepatic glucokinase is conventionally assayed in 100000 *g* supernatants and has been assumed to be present in the free state in the cytoplasm [1,2]. However, studies on digitonin-permeabilized hepatocytes have shown that glucokinase binds to the cell matrix by a Mg^{2+}-dependent mechanism [8]. The proportion of enzyme that is bound to the hepatocyte matrix depends on the substrates with which hepatocytes are incubated before addition of the permeabilization medium. Maximum binding occurs at low [glucose] (< 5 mM), and minimum binding occurs at high [glucose] or at low concentrations of fructose or sorbitol [9,10].

The present study demonstrates that the regulatory protein binds to the hepatocyte matrix with similar ionic binding characteristics to those for glucokinase, but, unlike glucokinase, does not translocate from the binding site during incubation with an elevated [glucose] or with fructose. These findings support the hypothesis that the regulatory protein acts as a receptor protein to anchor glucokinase to the hepatocyte matrix in metabolically quiescent conditions, and that metabolic conditions involving an increase in glucose metabolism lead to translocation of glucokinase, but not of its regulatory protein.

MATERIALS AND METHODS

Hepatocyte isolation and culture

Hepatocytes were isolated by collagenase perfusion of the liver of male Wistar rats fed *ad libitum* and were cultured in monolayer in 24-well plates in Minimum Essential Medium. After cell attachment (~ 4 h), the hepatocyte monolayers were cultured for 16–20 h in serum-free Minimum Essential Medium containing 5 mM glucose and 10 nM dexamethasone [11].

Permeabilization of hepatocytes with digitonin

During the 30 min before permeabilization of hepatocyte monolayers with digitonin, the cells were incubated in medium containing 5 mM glucose (basic Minimum Essential Medium) or in medium containing 5 mM glucose + 2 mM fructose or 40 mM glucose. The hepatocyte monolayers were then washed once with 150 mM NaCl and incubated with permeabilization medium, which contained either 300 mM sucrose, 3 mM Hepes, 2 mM dithiothreitol and 0.075 mg/ml digitonin, pH 7.2, and the concentrations of Mg^{2+} indicated, or 150 mM KCl, 3 mM Hepes, 2 mM dithiothreitol and 0.075 mg/ml digitonin. Incubations with permeabilization medium (300 μl/well) were 6 min at 20 °C under static conditions [8]. On termination of this incubation, the plate was swirled gently and the digitonin eluate aspirated. Glucokinase activity was determined in a 60 μl sample of the digitonin eluate, and the remaining eluate was mixed with an equal volume of denaturing medium, containing 4% SDS, 10% sucrose, 10 mM dithiothreitol and 0.8 mg/ml Bromophenol Blue. The residual cell matrix was extracted in 2% SDS/10% sucrose/10 mM dithiothreitol containing 0.8 mg/ml Bromophenol Blue. The glucokinase regulatory protein was determined in these SDS extracts. Glucokinase and the regulatory protein were also determined in cells not exposed to digitonin.

Western blotting of the glucokinase regulatory protein

The regulatory protein was determined in the SDS extracts by Western blotting using a rabbit antibody against rat liver regulatory protein [12]. After size fractionation of the extracts by SDS/PAGE (10% polyacrylamide), proteins were transferred to ECL Hybond N membranes, blocked in 150 mM NaCl/0.1% Tween 20 containing 5% non-fat powdered milk (Marvel) and exposed for 16 h at 4 °C to polyclonal antibody to the regulatory protein (1:1000 in 150 mM NaCl/1% BSA/0.1% Tween 20). After washing in PBS, membranes were incubated at 37 °C for 1 h with horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma; 1:1000 in 150 mM NaCl/1% BSA/0.1% Tween 20). Membranes were washed in PBS and developed by the enhanced chemiluminescence (ECL) method (Amersham International), followed by brief exposure to ECL X-ray film. Bands were

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scanned by laser densitometry and the intensity was determined with HSI gel scan.

Determination of glucokinase activity

Glucokinase activity in the digitonin eluate and total cell extract was determined as described [8], and the activity in the digitonin eluate is expressed as a percentage of total activity [8]. Total glucokinase activity was between 10 and 13 m-units/mg of protein.

RESULTS AND DISCUSSION

We have shown previously that, when hepatocytes that have been incubated with a low glucose concentration (5 mM) are permeabilized with low concentrations of digitonin (0.05 mg/ml), maximum glucokinase release occurs in 300 mM sucrose without Mg^{2+} and minimum release occurs in the presence of 5 mM Mg^{2+}, with intermediate release in 150 mM KCl [8]. Release of phosphoglucoisomerase and phosphoglucomutase is independent of the ionic composition of the permeabilization medium [8]. Because the activity of glucokinase released in the presence of Mg^{2+} at 0.05 mg/ml digitonin is at the lower detection limits of the assay, we used a higher [digitonin] (0.075 mg/ml) in this study, which results in greater enzyme release. Figure 1 shows a

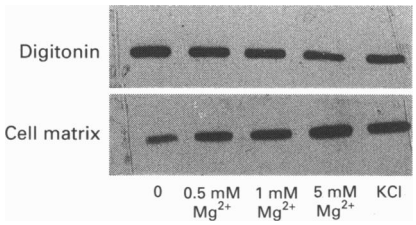

Figure 1 Western blot for determination of the regulatory protein in digitonin eluates and residual cell matrix

Hepatocytes were permeabilized as described in Table 1. The regulatory protein was determined by Western blotting as described in the Materials and methods section in the digitonin eluate (top row) and residual cell matrix (bottom row).

Table 1 Effects of Mg^{2+} and KCl on binding of glucokinase and its regulatory protein to permeabilized hepatocytes

After a 30 min preincubation in basic medium, hepatocytes were permeabilized in medium containing 3 mM Hepes, 2 mM dithiothreitol, 0.075 mg/ml digitonin, pH 7.2, and either 300 mM sucrose and the concentrations of Mg^{2+} indicated, or 150 mM KCl instead of sucrose. The activity of glucokinase released in the permeabilization medium is expressed as a percentage of total activity, and the amount of regulatory protein released is expressed as a percentage of the total. Values are means ± S.E.M. for six experiments for the glucokinase data and means of duplicate wells for two separate experiments for the regulatory-protein data.

Permeabilization medium	Glucokinase release (%)	Regulatory-protein release (%)	
		Expt. 1	Expt. 2
300 mM sucrose	89 ± 2	91	85
+ 0.5 mM $MgCl_2$	64 ± 4	78	64
+ 1.0 mM $MgCl_2$	41 ± 3	44	44
+ 5.0 mM $MgCl_2$	23 ± 3	33	22
150 mM KCl	57 ± 4	34	39

Table 2 Effects of fructose and glucose on binding of glucokinase and the regulatory protein to the hepatocyte matrix

Hepatocytes monolayers were preincubated for 30 min either in basic culture medium containing 5 mM glucose (control) or in medium containing either 2 mM fructose or 40 mM glucose. The monolayers were then washed and incubated in permeabilization medium containing 300 mM sucrose, 5 mM $MgCl_2$, 3 mM Hepes, 2 mM dithiothreitol and 0.075 mg/ml digitonin, pH 7.2, as described in the Materials and methods section. The activity of glucokinase and the amount of the regulatory protein that were released in the digitonin eluate are expressed as a percentage of total activity or total amount of protein, respectively. Values are means ± S.E.M. for six experiments: $^*P < 0.005$ relative to control.

Substrate incubation	Glucokinase release (%)	Regulatory-protein release (%)
Control	23 ± 3	18 ± 3
2 mM fructose	57 ± 3*	18 ± 2
40 mM glucose	68 ± 5*	20 ± 4

Western blot for determination of the regulatory protein in the digitonin eluates and residual cell matrix after permeabilization with 300 mM sucrose with increasing [$MgCl_2$] (0–5 mM) or with 150 mM KCl, and Table 1 shows the proportional release of glucokinase (determined enzymically) and the regulatory protein (determined by Western blotting) after permeabilization in these media. Binding of glucokinase and of the regulatory protein to the hepatocyte matrix show a similar Mg^{2+}-dependency, although release of glucokinase was somewhat higher than release of the regulatory protein during permeabilization in KCl (57% versus 39–45%). This could be due to the fact that KCl causes dissociation of the glucokinase–regulatory-protein complex [5].

Table 2 shows the effects of preincubation of hepatocytes with fructose (2 mM) or glucose (40 mM) on the release of glucokinase and the regulatory protein during permeabilization in the presence of 5 mM Mg^{2+}. It is noteworthy that half-maximal stimulation of enzyme release occurs at 50 μM fructose and 10–15 mM glucose [8]. However, high substrate concentrations were used in the present study to achieve maximum effect. Whereas the release of glucokinase was markedly increased after incubation with either fructose or 40 mM glucose, the release of the regulatory protein was unaffected, indicating that these substrates cause translocation of glucokinase, but not of its regulatory protein.

We postulate that, when intact hepatocytes are exposed to medium containing 5 mM glucose, both glucokinase and its regulatory protein are present in the hepatocyte in a bound state, and that the regulatory protein is bound to the cell matrix by a mechanism that is stabilized by the presence of Mg^{2+} (0.5–5 mM). When hepatocytes are permeabilized in medium without Mg^{2+}, this mechanism is disrupted, with the consequent release of the regulatory protein. We postulate that the regulatory protein acts as a receptor protein for anchoring glucokinase to the hepatocyte matrix. When hepatocytes are incubated in medium containing either an elevated glucose concentration (10–40 mM) or fructose (50 μM–1 mM), glucokinase dissociates from its regulatory protein and translocates to a different intracellular location, while the regulatory protein remains attached to the hepatocyte matrix. We postulate that fructose causes translocation of glucokinase by increasing the concentration of fructose 1-phosphate [13], which causes dissociation of glucokinase from its regulatory protein [4], and that an elevated glucose concentration causes translocation also by dissociation of glucokinase from its regulatory protein, because binding of the regulatory protein to glucokinase is competitive with glucose [5].

We cannot completely exclude the possibility that glucokinase

and its regulatory protein bind to the hepatocyte matrix separately by a similar Mg^{2+}-dependent mechanism. Translocation induced by glucose could then be due to a conformational change in glucokinase [14]. However, the effect of fructose would be more difficult to reconcile with the latter explanation, since the conformation of glucokinase is affected only by glucokinase substrates that show co-operative kinetics, and furthermore glucokinase has a very low affinity for fructose [1] and is insensitive to fructose 1-phosphate [3]. In view of the similarity in the Mg^{2+}-dependency of binding of glucokinase and the regulatory protein, and the finding that both glucose and fructose cause translocation of glucokinase, we consider the hypothesis that the regulatory protein acts as the receptor protein for glucokinase to be the more plausible explanation for these results.

Histochemical analysis has shown that, in livers from fasted rats perfused with 5 mM glucose, glucokinase is localized in the nucleus or perinuclear region [15] and, after perfusion with an elevated [glucose], which causes translocation from the Mg^{2+}-dependent binding site, glucokinase diffuses to a delocalized distribution in the cytoplasm. Whether this represents a true 'free' state in solution or weak binding to subcellular structures in the cell periphery is currently not established. It seems likely therefore that the binding site of glucokinase, and presumably also of its regulatory protein, as determined during permeabilization in the presence of Mg^{2+}, is in the perinuclear region. It is of interest that hexokinase III is also localized in the perinuclear region [16], but there is no reported evidence that it translocates in response to stimuli.

Since binding of glucokinase to its regulatory protein is associated with a decrease in affinity of the enzyme for glucose [4], the bound enzyme (in the presence of Mg^{2+}) represents an inactive state and the translocated enzyme a more active state. This model is analogous to the binding properties of certain glycolytic enzymes that are known to partition between 'bound' and 'free' states. Several glycolytic enzymes bind to the microfilament and microtubule cytoskeleton either directly or indirectly by binding to other glycolytic enzymes that bind to filamentous actin (for review see [17]). In several instances, including aldolase [18] and glyceraldehyde-3-phosphate dehydrogenase [19], binding to cytoskeletal proteins is associated with a decrease in enzyme activity. This is therefore analogous to the bound state of glucokinase, which is in association with its inhibitory regulatory protein.

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