Shuttling of Glucokinase between the Nucleus and the Cytoplasm in Primary Cultures of Rat Hepatocytes: Possible Involvement in the Regulation of the Glucose Metabolism

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Summary. Glucokinase (GK) is believed to play a key role in the control of the hepatic glucose metabolism. To address the mechanism of the regulation of glucose metabolism through GK action, we immunohistochemically studied changes in GK distribution in primary cultures of rat hepatocytes. In hepatocyte monolayers incubated in 5 mM glucose, GK staining by the immunoperoxidase method was observed predominantly in the nucleus. When cultured hepatocytes were incubated for 30 min in various concentrations (5-45 mM) of glucose, there was an appreciable decrease in nuclear GK immunoreactivity, even at 10 mM compared with that at 5 mM. After the shift of glucose concentration from 5 mM to 25 mM, the GK distribution changed time-dependently over 1 h. A time-dependent change in GK distribution was also observed when the glucose concentration was shifted from 25 mM to 5 mM. Reversal of GK distribution in response to the change in glucose concentration from 5 to 25 mM and vice versa was shown to repeatedly occur. Lower concentrations (0.05-5 mM) of fructose, which is known to stimulate glucose phosphorylation by GK, in combination with 5 mM glucose, induced the translocation of GK from the nucleus to the cytoplasm. Mannose (20 mM), a substrate of GK, and sorbitol (1 mM), a stimulator of glucose phosphorylation by GK, induced the translocation of GK from the nucleus to the cytoplasm in the presence of 5 mM glucose. L-glucose, galactose, 3-O-methylglucose, and 2-deoxyglucose at 20 mM each did not affect the GK distribution observed in the presence of 5 mM glucose. The results suggest that GK is present mainly in the nucleus under conditions where GK action is not much needed, whereas the enzyme exists mainly in the cytoplasm under conditions where it must function extensively. Our findings indicate that the shuttling of GK between the nucleus and the cytoplasm is essential for the regulation of the glucose metabolism in the liver.

Glucokinase (GK), one of the hexokinase isozymes (types I-IV), is often called hexokinase IV or high-Km hexokinase. GK (52 kDa) is distinguished from other hexokinase isozymes (100 kDa) not only by molecular weight but also by a number of other features including its low affinity for glucose and lack of inhibition by glucose 6-phosphate (Weinhouse, 1976). This enzyme has been identified only in glucose-sensing cells such as hepatocytes, pancreatic islet cells, and some neuroendocrine cells (Jetton et al., 1994). In the liver, GK is considered to play a key role in the control of whole-body glucose homeostasis (Matschinsky, 1990; Printz et al., 1993; Barzilai et al., 1996; Ferre et al., 1996). The mechanism of regulation of the glucose metabolism through GK action, however, has not yet been fully elucidated. It was reported that long chain acyl-CoAs inhibit GK competitively with respect to glucose below the critical micelle concentration (Tippett and Neet, 1982). Some investigators, however, raised questions about the purpose of this inhibition, and suggested that the concentration of free long chain acyl-CoAs is probably too low in the cytosol to modulate the GK activity (van Schaftingen et al., 1994). GK, purified from rat liver, was phosphorylated in vitro by protein kinase A, and its Km increased and Vmax decreased upon phosphorylation in vivo has not been demonstrated yet. Recently, rat liver was reported to contain a regulatory protein which, in the presence of fructose 6-phosphate, inhibits GK competitively with respect to glucose (van Schaftingen, 1989). Abundant evidence has proved that this protein functions as a regulator of GK activity in hepatocytes (van Schaftingen et al., 1994).

We previously reported that GK is immunohistochemically detected predominantly in the nucleus of
Parenchymal cells of the adult rat liver (Miwa et al., 1990; Toyoda et al., 1994, 1995a, b), and that the enzyme is translocated from the nucleus to the cytoplasm when the liver is perfused with 20 mM glucose, but not when 5 mM glucose is used (Toyoda et al., 1995b). We also suggested that the translocation of glucokinase may play an important role in the hepatic glucose metabolism (Toyoda et al., 1994). In the present study, we show that GK shuttles between the nucleus and the cytoplasm in hepatocytes, and that this shuttling of GK is linked to the modulation of the activity of this enzyme. Preliminary reports of the present study have been published (Toyoda et al., 1996a, b).

MATERIALS AND METHODS

Antibody
Antiserum against homogeneous rat liver GK was raised in rabbits as described previously (Miwa et al., 1990). Monospecific antibodies against GK were affinity-purified as described previously (Toyoda et al., 1995a).

Hepatocyte isolation and culture
Hepatocytes were isolated according to the method by Tanaka and co-workers by collagenase perfusion of the liver of male Wistar rats (180-220 g) fed ad libitum (Tanaka et al., 1978). The cells were suspended in a Minimum Essential Medium (MEM) containing 5% heat-inactivated fetal bovine serum, inoculated in 6-well plates (area of well 15.6 cm²) at a cell density of 6.4 × 10⁴ cells/cm², and incubated at 37°C in a humidified atmosphere equilibrated with 5% CO₂ in air. After cell attachment (4 h), the medium was replaced with serum-free MEM containing 10 nM dexamethasone, and the cells were cultured further for 16 h. Hepatocyte monolayers were incubated in MEM supplemented with sugars for the indicated time intervals before fixation. Unless stated otherwise, the standard medium (MEM) contained 5 mM glucose.

Immunoperoxidase staining
The incubation medium was aspirated, and the monolayers were washed once with 2 ml of 150 mM NaCl at 20°C. Fixation medium (4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4) was then added, and the cells were fixed for 40 min at 4°C. After the fixation, the monolayers were washed twice with phosphate-buffered saline (PBS) and permeabilized with 0.5%(v/v) Triton X-100 for 40 min at 20°C. Permeabilized monolayers were washed three times with PBS and, in order to block non-specific staining, consecutively incubated at 20°C with an avidin solution (Seikagaku, Tokyo, Japan) for 10 min, with a biotin solution (Seikagaku) for 10 min, and with a solution (serum/milk solution) containing 5%(v/v) normal goat serum and 5% skimmed milk in PBS for 90 min. Then they were incubated consecutively with anti-GK IgG (2 μg/ml in serum/milk solution) overnight at 4°C, with biotin-labeled goat serum against rabbit IgG (Seikagaku) for 1 h at 20°C, and with streptavidin-peroxidase (Seikagaku) for 1 h at 20°C. Each incubation was followed by 7 washings with PBS. The hepatocytes were stained by incubating them for 15 min at 20°C in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.05% 3,3’-diaminobenzidine-4HCl and 0.05% (w/v) H₂O₂. The stained cells were washed well with distilled water, mounted on glycerol, and examined by light microscopy.

Immunofluorescence staining
After permeabilization of the monolayers and washing with PBS were performed as described above, the monolayers were incubated with serum/milk solution for 90 min at 20°C to block non-specific staining. They were then incubated consecutively with anti-GK IgG (diluted 1: 5 in serum/milk solution) for 6 h at 20°C and with FluoroLink-Cy2-labeled goat serum against rabbit IgG (Amersham, Bucks, UK) (10 μg/ml in serum/milk solution) for 1 h at 20°C. Each incubation was followed by 7 washings with PBS. The stained cells were mounted on glycerol. Cell fluorescence was analyzed with a confocal imaging system MRC-1024 (Bio-Rad, Richmond, CA) mounted on an Axioplan microscope (Carl Zeiss, Jena, Germany) equipped with a Carl Zeiss 63× objective (PlanApo; numerical aperture, 1.4). A krypton/argon laser tuned to produce a 488-nm wavelength beam was used for Cy2 excitation. The signals were recorded by optical sections of a 0.5 μm thickness.

RESULTS

Specificity of immunoperoxidase staining
In hepatocyte monolayers incubated in MEM for up to 2 h, GK immunoreactivity was detected predominantly in the nucleus (Fig. 1A). Immunoabsorption of anti-GK IgG with purified rat liver GK completely prevented the immunostaining of GK (Fig. 1B). In addition, no staining was observed when non-immune or pre-immune IgG was used instead of anti-GK IgG (data not shown). The results, considered together with our previous data (Miwa et al., 1990; Toyoda et
**Fig. 1.** Immunohistochemical staining of GK in primary cultures of rat hepatocytes. Cells were incubated for 30 min in MEM containing 5 mM glucose and then stained for GK with anti-GK IgG (A) or with anti-GK IgG preabsorbed with rat liver GK (B) by the immunoperoxidase method. Dark stain indicates the presence of GK immunoreactivity. Bar=25 μm

**Fig. 2.** Time course of GK translocation from the nucleus to the cytoplasm. Cultured hepatocytes were incubated for 30 min in MEM containing 5 mM glucose, and then the medium was shifted to MEM containing 25 mM glucose. At indicated times after the shift of the medium, cells were stained for GK with the immunoperoxidase method. Bar=25 μm

**Fig. 3.** Time course of GK translocation from the cytoplasm to the nucleus. Cultured hepatocytes were incubated for 30 min in MEM containing 25 mM glucose, and then the medium was changed to MEM containing 5 mM glucose. At the indicated times after the shift of glucose concentration, the cells were stained for GK by the immunoperoxidase method. Bar=25 μm
al., 1994, 1995a), indicate that the immunostaining is specific for GK.

**Time course of GK translocation from the nucleus to the cytoplasm**

Figure 2 shows the time-dependent change in GK distribution observed following the shift of the glucose concentration of the medium from 5 mM to 25 mM. At 1 min after the shift, there was no appreciable change in GK distribution. At 5 min, however, the nuclear immunoreactivity was clearly decreased. At 10 min and thereafter, dense staining for GK was observed in the cytoplasm.

**Time course of GK translocation from the cytoplasm to the nucleus**

Hepatocyte monolayers were incubated with 25 mM glucose for 30 min, and then the medium glucose concentration was shifted to 5 mM to follow the change in GK distribution. The translocation of GK from the cytoplasm to the nucleus took place time-dependently over 1 h as shown in Figure 3. As early as 5 min after the shift, appreciable GK staining was observed in the nucleus. There was no change in GK distribution during at least another hour after the initial 1-h incubation (data not shown).

**Effect of glucose concentration on GK distribution**

We incubated hepatocyte monolayers for 30 min with various concentrations of glucose. As shown in Figure 4, even at 10 mM, there was a decrease in the nuclear GK immunoreactivity and an increase in the cytoplasmic immunoreactivity. The translocation of GK from the nucleus to the cytoplasm was demonstrated to be glucose concentration dependent.

**Reversal of GK distribution by changing glucose concentration**

We examined whether the translocation of GK between the nucleus and the cytoplasm is reversible. When hepatocyte monolayers were incubated for 30 min after the change in medium glucose concentration from 5 mM to 25 mM, GK was obviously translocated from the nucleus to the cytoplasm (Fig. 5A, B). When the monolayers were further incubated with 5 mM glucose for 60 min, GK staining returned to the nucleus from the cytoplasm (Fig. 5B, C). This phenomena was repeatable as shown in Figure 5C–F, indicating that GK shuttles between the nucleus and the cytoplasm in response to the change in glucose concentration.

**Effect of fructose concentration on GK distribution**

It has been reported that the presence of lower concentrations of fructose increases glucose utilization in hepatocytes, and that higher concentrations of fructose do not affect glucose utilization or rather exert a negative effect on it (Clark et al., 1979; van Schaftingen and Vandercammen, 1989; Fillat et al., 1993; Agius and Peak, 1993). We therefore examined the effect of various concentrations of fructose on GK distribution. Hepatocytes were in-
cubated for 30 min in MEM containing 5 mM glucose plus the indicated concentrations of fructose, and then the cells were analyzed for GK distribution. Even at 0.05 mM, fructose decreased the GK immuno-reactivity in the nucleus (Fig. 6). At lower concentrations such as 0.1 and 1 mM, the nuclear GK immuno-reactivity was markedly decreased and seemed to be rather weaker than the cytoplasmic GK immuno-reactivity. The GK distribution observed in the presence of higher fructose concentrations (20 and 40 mM) was not so different from that observed in the absence of fructose.

Effects of various sugars on GK distribution

We determined the effect of various sugars on GK distribution. Hepatocyte monolayers were incubated for 30 min in MEM containing 5 mM glucose plus selected sugars before fixation of the cells. The sugars in the upper panels in Figure 7 are known to cause GK to function: i.e., glucose and mannose are good substrates of GK (WEINHOUSE, 1976), and both fructose (1 mM) and sorbitol (1 mM) stimulate glucose phosphorylation by GK (van Schaftingen and VANDERCAMMEN, 1989). The sugars, except for 2-deoxy-glucose, in the lower panels in Figure 7 are neither substrates of GK nor stimulate glucose phosphorylation. 2-Deoxyglucose is a substrate of GK, but the Km value is as high as 48 mM (GROSSMAN et al., 1974) or 95 mM (PARRY and WALKER, 1966). The sugars in the upper panels caused the translocation of GK from the nucleus to the cytoplasm, whereas those in the lower panels little affected the distribution of GK.

GK distribution examined by confocal laser scanning microscopy

We examined GK distribution by confocal laser scanning microscopy. The nuclear GK, but not the cytoplasmic GK, was clearly stained by the immunoperoxidase method. Bar=25 µm.
Fig. 6. Effect of fructose concentrations on GK distribution. Primary cultures of hepatocytes were incubated for 30 min in MEM containing the indicated concentrations of fructose as well as 5 mM glucose and then stained for GK by the immunoperoxidase method. Bar = 25 μm

Fig. 7. Effect of various sugars on GK distribution. Cultured hepatocytes were incubated for 30 min in MEM containing the indicated concentrations of various sugars as well as 5 mM glucose and then stained for GK by the immunoperoxidase method. Bar = 25 μm
DISCUSSION

There are variety of proteins that shuttle between the nucleus and the cytoplasm: they include nucleolin (Borer, 1989) and hnRNP A1 (Pinol-Roma and Dreyfuss, 1992) as mediators of nucleocytoplasmic transport of RNA; B3 and B4 (Mandell and Feldherr, 1990) as members of the heat-shock protein family; glucocorticoid receptor (Madan and De Franco, 1993); and cAMP-dependent protein kinase (Meinkoth et al., 1990). To our knowledge, only one paper (Singh and Green, 1993) has referred to the shuttling of a glycolytic enzyme: Glyceraldehyde-3-phosphate dehydrogenase was reported to be present in both the cytoplasm and the nucleus of HeLa cells, which raised the possibility that the enzyme may shuttle between nuclear and cytoplasmic compartments and may be involved in RNA nuclear export. That paper, however, did not show any evidence for the shuttling of the enzyme. Our present study is the first to demonstrate that a glycolytic enzyme shuttles between the nucleus and the cytoplasm.

We reported previously that the regulatory protein of GK was immunohistochemically stained predominantly in the nucleus of hepatocytes of liver sections obtained from normal rats (Toyoda et al., 1995c). We also reported that the regulatory protein was appreciably translocated from the nucleus to the cytoplasm when the liver was perfused with 20 mM glucose for 10 min. Recently, however, we found that the regulator was hardly exported to the cytoplasm when cultured hepatocytes were exposed to 20 mM glucose for up to 30 min (manuscript in preparation). Although the difference between the results obtained with a perfused liver and with cultured hepatocytes remains a challenging problem, we believe that it is most likely that, unlike GK, not much regulatory protein is translocated even in the presence of high glucose concentrations. The present data, taken together with the results on the regulatory protein of GK, strongly suggest that GK is present mainly in the nucleus as an inactive form, i.e., as a complex with the regulatory protein, under conditions where the functioning of GK is not much needed, whereas the enzyme is present mainly in the cytoplasm as an active free form when its activity is required. The latter conditions include the presence of high levels of substrates (i.e., glucose and mannose) of GK and the coexistence of glucose with low levels of fructose or sorbitol (Fig. 7). The inhibition of GK by the regulatory protein was released by an increase in the concentration of the substrate glucose (Vandercammen and van Schaftingen, 1990), suggesting that the substrate of GK dissociates the GK-regulator complex. This should be the basis for the translocation of GK to the cytoplasm by the presence of high concentrations of glucose and mannose. The positive effect of fructose and sorbitol on glucose phosphorylation by GK is explainable by the conversion of sorbitol to...
fructose, of fructose to fructose 1-phosphate, and by the subsequent action of the phosphate to dissociate the GK-regulator complex (van Schafingen and Vandercammen, 1989; Davies et al., 1990) followed by the translocation of GK to the cytoplasm.

Agius and Peak showed that, in rat hepatocytes cultured with 5 mM glucose, GK was present predominantly in a bound state when the hepatocytes were permeabilized with digitonin in the presence of Mg, and that metabolic changes induced by incubation of hepatocytes with high glucose (15–35 mM) or with fructose (50 μM) caused a translocation of GK from its Mg-dependent binding site to an alternative site (Agius and Peak, 1993). Their results and ours are compatible provided that the Mg-dependent binding site for GK is in the nucleus and the alternative site is in the cytoplasm.

In animal cells, the bidirectional transport of proteins between the nucleus and the cytoplasm occurs through nuclear pore complexes (Dworetzky and Feldherr, 1988). The complexes are large supramolecular structures that allow the diffusion of proteins (<40–60 kDa) between the two compartments. For large proteins and some small proteins, a nuclear localization signal, typically a short basic sequence motif, is required for nuclear import (Jans, 1995; Hicks and Raikhel, 1995; Jans and Hübner, 1996). We have preliminarily examined whether rat liver GK has any nuclear localization signal(s). However, we have not yet succeeded in identifying such signals. Whether chaperone-like proteins participate in the transport of GK through the nuclear pore also remains to be studied.

Some investigators (Newgard et al., 1990; Lachall et al., 1993) reported the possibility that GK interacts with the high-Km glucose transporter (GLUT-2) in the plasma membrane of pancreatic B cells. If such a complex is formed, it may serve as an efficient system for the regulation of the glucose metabolism. In the present study, GK staining was observed at the plasma membrane when GK distribution was examined by confocal laser scanning microscopy (Fig. 8). We previously reported that GK was stained at the plasma membrane of hepatocytes.

![Fig. 9. Schematic diagram illustrating the possible role of GK shuttling between the nucleus and the cytoplasm in hepatic glucose metabolism.](image-url)
in sections of rat liver excised at 7.5-10 h after the start of refeeding (TOYODA et al., 1995a). Our present and previous results suggest the existence of the interaction of GK with GLUT-2 in hepatocytes. This will be an interesting issue for future study.

The GK immunoreactivity in the nucleus seemed to be weaker than that in the cytoplasm under certain conditions, e.g. in the presence of low concentrations of fructose together with 5 mM glucose (Fig. 6). One simple view explaining this phenomena is that at least some portion of GK in the cytoplasm binds to cytoskeletal proteins and/or other proteins. If this is the case, it is likely that GK, dissociated from the regulatory protein, moves from the nucleus to the cytoplasm and is present at higher concentrations in the cytoplasm than in the nucleus because of the trapping of GK by association with some macromolecules. This hypothetical view is highly probable since several glycolytic enzymes, including phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase, have been reported to be present in association with the cytoskeleton in various cells (MASTERS et al., 1987; KNULL and WALSH, 1992).

Our proposed conclusion based on the present study is illustrated in Figure 9. In the postabsorptive state, the major part of total GK forms a complex with its regulator in the nucleus, so glucose phosphorylation happens very slowly, if at all. On the other hand, in the postprandial state, the complex is dissociated by the action of fructose 1-phosphate and high glucose concentration, and a free form of GK is translocated to the cytoplasm, enabling the rapid phosphorylation of glucose to take place. This scheme implies that the shuttling of GK between the nucleus and the cytoplasm is essential for the regulation of the glucose metabolism in the liver.

REFERENCES


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