

Co-localization of glucokinase with actin filaments

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Abstract A portion of glucokinase appeared to be co-localized with actin filaments in the cytoplasm of cultured rat hepatocytes incubated with 25 mM glucose. When liver- or islet-type glucokinase was transiently expressed in COS-7 cells, the expressed glucokinase was also co-localized with actin filaments in the cytoplasm of these transfected cells. Although co-localization of glucokinase with actin filaments was not clearly demonstrated in the pancreatic β -cell line MIN6, islet glucokinase was found to be present in both the nucleus and the cytoplasm, though predominantly in the nucleus. These findings suggest that subcellular localization of glucokinase, including co-localization with actin filaments, may have an important physiological role in metabolic regulation.

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Key words: Liver glucokinase; Islet glucokinase; Actin filament

1. Introduction

Glucokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) plays a key role in the regulation of glucose homeostasis by catalyzing the first step in glycolysis [1]. Expression of this enzyme is limited to hepatocytes, pancreatic β -cells, and some neuroendocrine cells [2]. Furthermore, the regulation of glucokinase differs among these tissues.

We previously reported that liver glucokinase was translocated from the nucleus to the cytoplasm in response to a high concentration of glucose [3]. In addition, studies on digitonin-permeabilized rat hepatocytes demonstrated that, in cells equilibrated with 5 mM glucose as the sole carbohydrate substrate, glucokinase was present predominantly in a bound state involving a Mg^{2+} -dependent mechanism and that some substrates, particularly sorbitol and fructose, cause translocation of glucokinase from the Mg^{2+} -dependent binding site [4,5]. With respect to glucokinase localization in pancreatic β -cells, a recent study showed that glucokinase has a perinuclear cap-like distribution in normal rats and a diffuse distribution throughout the cytoplasm in acutely hyperglycemic rats [6]. These results suggest that the subcellular distribution of glucokinase plays an important role in glucose metabolism.

The present study was designed to immunohistochemically define the subcellular distributions of liver and islet glucokinase. We first observed that the immunofluorescent staining of the enzyme had a mesh-like appearance in the cytoplasm of cultured rat hepatocytes incubated with 25 mM glucose for 30

min. This observation led us to investigate the possibility that glucokinase is co-localized with cytoskeletal elements in rat hepatocytes. Herein, we present evidence that glucokinase is co-localized with actin filaments in rat hepatocytes and COS-7 cell transfectants expressing liver- or islet-type glucokinase. We further examined the subcellular distribution of endogenous glucokinase in the murine insulinoma cell line MIN6.

2. Materials and methods

2.1. Cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) containing 5 mM glucose and supplemented with 10% heat-inactivated fetal bovine serum (FBS) in humidified 5% CO_2 , 95% air at 37°C.

Hepatocytes were isolated by collagenase perfusion of livers from male Wistar rats fed standard rat chow ad libitum according to the method of Tanaka et al. [7]. The rat hepatocytes were suspended in Minimum Essential Medium (MEM; GIBCO BRL, USA) supplemented with 5 mM glucose and 10% heat-inactivated FBS, and plated onto 12-mm round coverslips in 50-mm plastic Petri dishes. The hepatocytes were maintained in MEM containing 5 mM glucose and supplemented with 10% heat-inactivated FBS in humidified 5% CO_2 , 95% air at 37°C. Unattached cells were removed, after 4 h, by replacing serum-free MEM containing 10 nM dexamethasone. The culture was then continued for a further 12 h. Histochemical analysis was performed after incubation of hepatocytes with 25 mM glucose at 37°C for 30 min.

Pancreatic β -cell line MIN6 cells [8] were maintained in DMEM containing 25 mM glucose and supplemented with 15% heat-inactivated FBS in humidified 5% CO_2 , 95% air at 37°C.

2.2. Overexpression of liver and islet glucokinase in COS-7 cells

Rat liver glucokinase cDNA [9] or rat islet glucokinase cDNA [10,11] was subcloned under the chicken β -actin promoter of the expression vector pCXN [12]. The resulting expression vector was transfected into COS-7 cells using Lipofectamine (GIBCO BRL, USA) following the manufacturer's instructions. The pCXN vector alone was transfected into COS-7 cells in parallel experiments to prepare the control cells. The cells were fixed for histochemical analysis 42 h after transfection.

2.3. Assay of glucokinase and hexokinase activities in COS-7 cell transfectants

Enzyme activities were assayed fluorometrically by a modification of the method of Trus et al. [13]. COS-7 cell transfectants were harvested with trypsin-EDTA, washed twice with phosphate-buffered saline (PBS) to remove glucose, and homogenized with seven strokes in a homogenizing buffer (20 mM K_2HPO_4 , 110 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.4) using a Potter-Elvehjem glass-Teflon homogenizer. The glucose phosphorylating activity of the total cell homogenate was measured. An aliquot of the homogenate was removed to determine protein concentrations. The enzyme assays were performed using reaction mixtures (300 μ l) containing 50 mM Hepes-NaOH buffer (pH 7.6), 100 mM KCl, 8 mM $MgCl_2$, 0.5 mM NAD, 5 mM ATP, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin (BSA), 1 U/ml glucose-6-phosphate dehydro-

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genase from *Leuconostoc mesenteroides*, 0.5 or 100 mM glucose, and sample solution. The reaction mixture was incubated at 37°C for 90 min. The reaction was then stopped by addition of 150 µl of 500 mM sodium phosphate buffer (pH 8.0) containing 0.9 mM sodium dodecylsulfate. The hexokinase activity was measured at a glucose concentration of 0.5 mM. The glucokinase activity was estimated by subtracting the activity observed at 0.5 mM glucose from that observed at 100 mM glucose. The protein concentration was determined by the Bradford method with a kit from Bio-Rad (Richmond, CA), using BSA as a standard.

2.4. Histochemical analysis

For analysis of subcellular distributions of glucokinase and actin, the cells were grown on 12-mm round coverslips in 50-mm plastic Petri dishes to approximately 80% confluence, washed once with PBS, fixed in PBS containing 4% paraformaldehyde for 15 min, and permeabilized with PBS containing 0.2% Triton X-100 for 15 min. The cells were washed once with PBS containing 0.2% gelatin and incubated with affinity-purified anti-glucokinase IgG [3] at 1:100 dilution in PBS containing 0.2% gelatin for 1 h at room temperature for the transfected COS-7 cells, or overnight at 4°C for cultured hepatocytes and MIN6 cells. After three washings with PBS containing 0.2% gelatin, the cells were incubated with a mixture of FITC-conjugated swine anti-rabbit IgG (DAKO, Denmark) at 1:100 dilution in PBS containing 0.2% gelatin and 0.5 µg/ml TRITC-labeled phalloidin (Sigma, USA) in PBS containing 0.2% gelatin for 1 h at room temperature, washed 3 times with PBS containing 0.2% gelatin, mounted in 1,4-diazobicyclo-2,2,2-octane/glycerol/PBS, and examined by fluorescence microscopy. Appropriate filters were used to obtain two-color fluorescence with FITC and TRITC in the same cell population.

For analysis of the subcellular glucokinase distribution in MIN6 cells by the immunoperoxidase staining method, the cells were grown on 12-mm round coverslips in 50-mm plastic Petri dishes to approximately 60% confluence, washed once with PBS, fixed in PBS containing 3% paraformaldehyde for 15 min, permeabilized with PBS containing 0.2% Triton X-100 for 15 min, washed once with PBS, and incubated with PBS containing 0.3% (w/v) H₂O₂ for 30 min to block endogenous peroxidase activity. The cells were then washed once with PBS, and incubated with normal goat serum for 20 min at room temperature, with affinity-purified anti-glucokinase IgG [3] at 1:100 dilution in 5% normal goat serum overnight at 4°C, with biotin-labeled goat serum against rabbit IgG (Seikagaku Kogyo, Japan) for 1 h at 20°C, and then with streptavidin-peroxidase (Seikagaku Kogyo, Japan) for 1 h at 20°C. Each incubation was followed by three washings with PBS. The cells were then incubated with 0.05% (w/v) 3,3'-diaminobenzidine-4HCl and 0.01% (w/v) H₂O₂ for 15 min at room temperature for staining, washed with distilled water, mounted in PBS/glycerol, and examined by light microscopy.

In control experiments for histochemical studies, the cells were treated in the same fashion except that the anti-glucokinase IgG was replaced by non-immune rabbit IgG, pre-immune rabbit IgG, or anti-glucokinase rabbit IgG preabsorbed with rat liver glucokinase.

The cells were also incubated directly with the second antibody, i.e. without a preceding incubation with anti-glucokinase IgG.

3. Results

Glucokinase immunofluorescence staining with affinity-purified anti-glucokinase IgG was present in both the nucleus and the cytoplasm of cultured rat hepatocytes incubated with 25 mM glucose for 30 min (Fig. 1A). It was noteworthy that a portion of the glucokinase staining exhibited a mesh-like distribution in the cytoplasm. The specificity of glucokinase immunostaining was demonstrated by the following results: (1) no staining was observed when non-immune or pre-immune IgG was used instead of anti-glucokinase IgG; (2) immunoabsorption of anti-glucokinase IgG with purified rat liver glucokinase completely prevented immunostaining of glucokinase; (3) no staining was observed when the cells were incubated with the second antibody without preceding incubation with anti-glucokinase antibody (data not shown). These observations indicate that the immunostaining is specific for glucokinase. When double labeling of cultured rat hepatocytes using anti-glucokinase antibody and TRITC-labeled phalloidin was performed, a portion of the glucokinase staining appeared to coincide with actin staining in the same area of the cytoplasm (Fig. 1A,B).

Glucose phosphorylating activities in COS-7 cells expressing liver- or islet-type glucokinase are shown in Table 1. The DNA constructs for the expressions of liver- and islet-type glucokinase were transiently transfected into COS-7 cells. The total homogenates of cells transfected with the pCXN vector alone (control) possessed only high-affinity glucose phosphorylating activity, consistent with that of hexokinase. The total homogenates of cells transfected with the pCXN vector containing either liver or islet glucokinase cDNA exhibited not only high-affinity but also low-affinity glucose phosphorylating activities, indicating that glucokinase was indeed expressed in the transfectants.

COS-7 cell transfectants expressing liver- or islet-type glucokinase were examined by double staining using anti-glucokinase antibody and TRITC-labeled phalloidin. The control cells transfected with pCXN alone did not exhibit glucokinase staining (data not shown). Abundant glucokinase staining was recognized in both the nucleus and the cytoplasm of the trans-

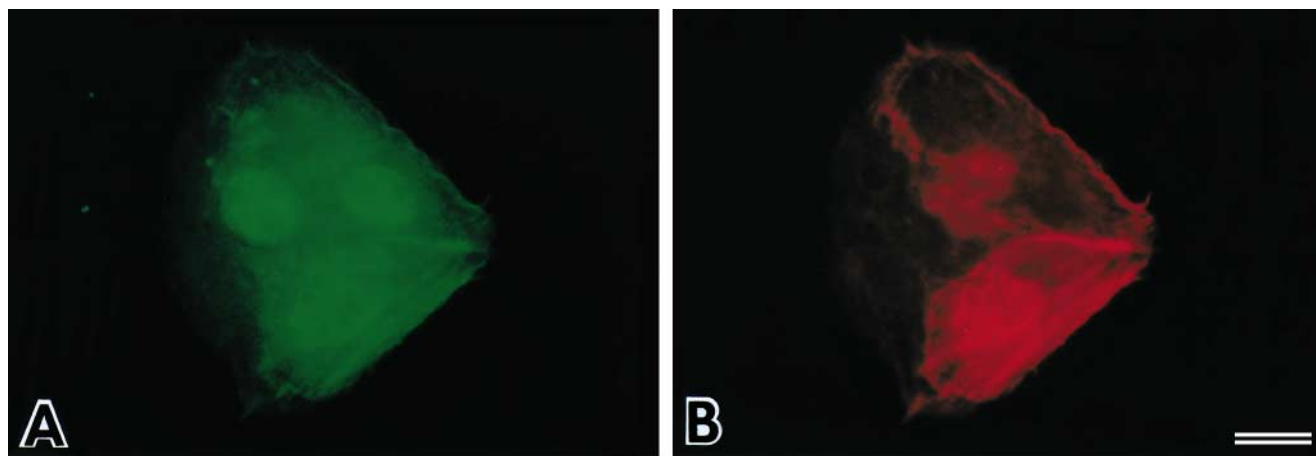


Fig. 1. Double fluorescent labeling of cultured rat hepatocytes with anti-glucokinase antibody and TRITC-labeled phalloidin. The same population of cultured rat hepatocytes was double stained with anti-glucokinase antibody (A) and TRITC-labeled phalloidin (B). Bar = 10 µm.

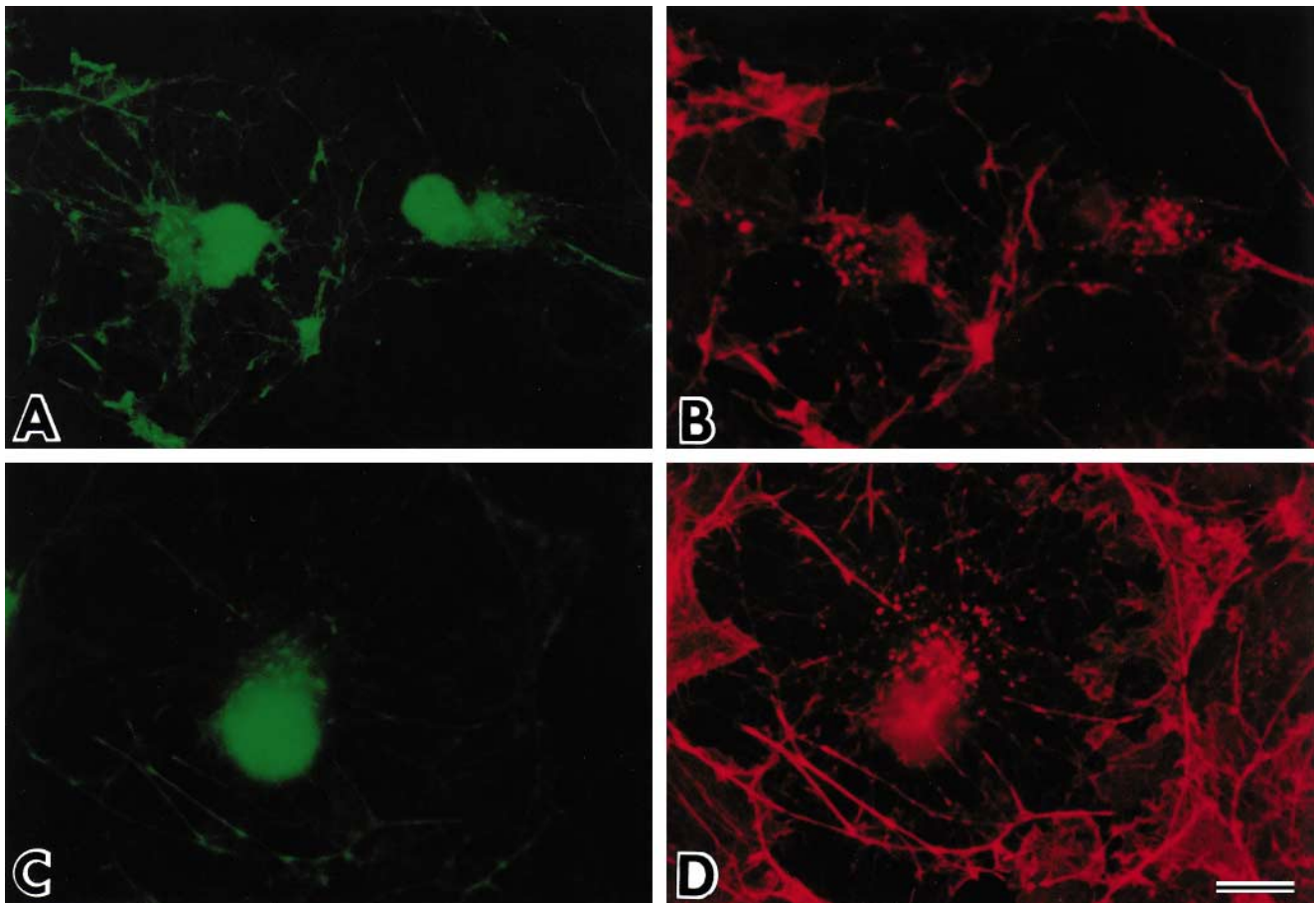


Fig. 2. Double fluorescent labeling of COS-7 cells expressing either liver- or islet-type glucokinase using anti-glucokinase antibody and TRITC-labeled phalloidin. COS-7 cells were transfected with expression vector pCXN containing the cDNA encoding rat liver glucokinase or rat islet glucokinase. The same population of liver-type glucokinase expressing COS-7 cells was double stained with anti-glucokinase antibody (A) and TRITC-labeled phalloidin (B). The same population of islet-type glucokinase expressing COS-7 cells was double stained with anti-glucokinase antibody (C) and TRITC-labeled phalloidin (D). Bar=20 μ m.

fectants expressing liver-type glucokinase (Fig. 2A) and islet-type glucokinase (Fig. 2C). In the same area of the cytoplasm in transfected cells, the staining of the expressed glucokinase was co-localized with that of actin filaments (Fig. 2A–D), with a subcellular distribution similar to that of endogenous glucokinase in cultured rat hepatocytes (Fig. 1A,B).

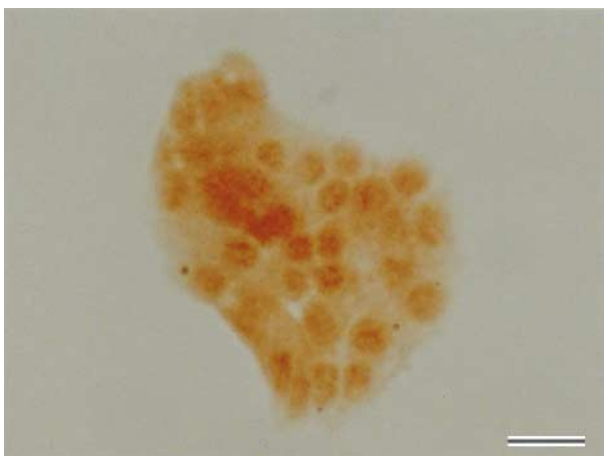


Fig. 3. Immunohistochemical distribution of glucokinase in the pancreatic β -cell line MIN6. The brown color indicates positive staining for glucokinase. Bar = 10 μ m.

We then attempted to examine the subcellular distribution of glucokinase, especially the apparent co-localization with actin filaments, in the pancreatic β -cell line MIN6 by double staining using anti-glucokinase antibody and TRITC-labeled phalloidin. However, we were unable to obtain clear results reflecting the subcellular distribution of glucokinase. This was due mainly to the low sensitivity for glucokinase staining as the amount of glucokinase is relatively low in MIN6 cells compared with cultured rat hepatocytes and COS-7 cell transfectants. To increase this sensitivity and thereby enhance detection, we employed an immunoperoxidase staining method, demonstrating that glucokinase immunoreactivity was present in both the nucleus and the cytoplasm of MIN6 cells (Fig. 3), and that the signal was far stronger in the nucleus than in the cytoplasm. These signals were not observed when MIN6 cells were first incubated with non-immune rabbit IgG, pre-immune rabbit IgG, or anti-glucokinase rabbit IgG preincubated with rat liver glucokinase (data not shown).

4. Discussion

Several glycolytic enzymes have been demonstrated to be associated with cytoskeletal elements such as actin and tubulin, and the activities of these enzymes differ between bound and soluble states [14]. It has therefore been proposed that the associations of some glycolytic enzymes with the cytoskeleton

Table 1
Glucokinase and hexokinase activities in homogenates of COS-7 cells expressing liver- or islet-type glucokinase

Expression vector	Glucokinase (nmol/min/mg protein)	Hexokinase (nmol/min/mg protein)
pCXN (control)	not detected	18.3 ± 1.2
pCXN-liver glucokinase	4.7 ± 0.8	21.5 ± 2.7
pCXN-islet glucokinase	4.2 ± 1.1	20.3 ± 2.6

The expression vector pCXN containing liver glucokinase cDNA (pCXN-liver glucokinase) or islet glucokinase cDNA (pCXN-islet glucokinase) was transiently transfected into COS-7 cells. The pCXN vector alone was transfected into COS-7 cells in parallel experiments to prepare the control cells. Glucose phosphorylating activities in transfectant homogenates were determined 42 h after transfection. Glucokinase and hexokinase activities were determined as described in Section 2.

Data are means ± SEM of three experiments.

have functional significance, perhaps involving a localized source of glycolytically derived energy for energy-dependent and cytoskeletal affiliated intracellular functions [14]. In the present study, we demonstrated that a portion of endogenous liver glucokinase is co-localized with actin filaments in the cytoplasm of cultured rat hepatocytes incubated with 25 mM glucose for 30 min. In addition, exogenous liver-type glucokinase expressed in COS-7 cells also showed partial co-localization with actin filaments in the cytoplasm. These results suggest that the co-localization of liver glucokinase with actin filaments has functional significance; the metabolic control which occurs via glucokinase may be due, at least in part, to the physical association of glucokinase with actin filaments in hepatocytes.

It has been reported that cytochalasin D and phalloidin, which interfere with microfilament structure, increase glucokinase release during permeabilization of cultured rat hepatocytes with digitonin [4]. Although these results suggest that changes in microfilament conformation affect glucokinase binding, no morphological evidence was provided [4]. In the present study, co-localization of glucokinase with actin filaments was directly demonstrated in cultured hepatocytes employing a histochemical technique. Taken together with our results, the previous observation of increased glucokinase release by cytochalasin D and phalloidin may be attributable to disruption of the association between glucokinase and actin filaments.

Another interesting observation is that cultured hepatocytes, COS-7 cells expressing liver- or islet-type glucokinase, and MIN6 cells exhibited strong nuclear glucokinase staining. It is not clear whether this nuclear staining indicates an association of the enzyme with the nuclear membrane or localization within the nucleus, because glucokinase immunoreactivity was not analyzed in thin sections or with confocal laser scanning microscopy in these cells. However, considering that liver glucokinase has been identified as a nuclear protein by immunohistochemical studies using paraffin-embedded sections prepared from rat liver [3,15], it is more likely that the nuclear stainings observed in cultured hepatocytes and COS-7 cells expressing liver-type glucokinase come from glucokinase localized within the nucleus.

We previously reported that in situ perfusion of the liver with 20 mM glucose for 10 min caused a marked decrease in nuclear immunoreactivity and an increase in cytoplasmic immunoreactivity, and that insulin (10 nmol/l) potentiated this glucose effect [3]. Similarly, incubation of cultured rat hepatocytes with high concentrations of glucose (10–40 mM) for 30 min induced translocation of glucokinase from the nucleus to the cytoplasm [16]. Therefore, it has been hypothesized that the localization of glucokinase in nuclei minimizes the futile

substrate cycle between glucose and glucose 6-phosphate (G/G-6-P cycle) and that translocation of glucokinase between the nucleus and the cytoplasm may operate as a means of regulating glucose metabolism. In the present study, incubation of COS-7 cells expressing liver-type glucokinase with different concentrations of glucose (5, 10 and 20 mM) for 30 min produced no apparent translocation of glucokinase from the nucleus to the cytoplasm in response to an increase in the glucose concentration (data not shown). Therefore, glucokinase translocation may be unique to the liver, and may have functional significance in hepatic glucose metabolism.

As to the mechanism governing the specific subcellular localization of glucokinase, it is noteworthy that liver-type and islet-type glucokinases have been shown to exert essentially the same stimulatory effect on glucose metabolism when over-expressed in primary cultured rat hepatocytes [17]. This observation suggests a possibility that these expressed glucokinases are localized to the same compartment in primary cultured rat hepatocytes, and thus that the amino-terminal domain of glucokinase, which differs between the liver-type and islet-type, is not involved in the specific subcellular targeting of glucokinase. Further studies are needed to clarify the mechanism governing the specific localization of glucokinase.

Glucose-induced translocation of glucokinase has also been reported in rat pancreatic β -cells [6], although the subcellular localization is somewhat different from our observation in MIN6 cells. We were unable to obtain clear immunofluorescent staining data to identify whether the endogenous islet glucokinase is associated with cytoskeletal elements in the pancreatic β -cell line MIN6 because of the weak signal. However, the exogenous islet-type glucokinase was co-localized with actin filaments in COS-7 cells expressing islet-type glucokinase, suggesting the possibility that endogenous islet glucokinase is co-localized with actin filaments in pancreatic β -cells as well. Overexpression of glucokinase in isolated islets reportedly has minimal effects on glucose metabolism and insulin release [18], suggesting that the number of putative glucokinase binding sites which upon activation engage in metabolic signaling is limited in islet cells. Our observations suggest that a glucokinase binding site in pancreatic β -cells resides in the nucleus and/or on actin filaments. The possible co-localization with cytoskeletal elements and location-dependent enhancement of glucokinase activity in the glucose-sensing machinery of β -cells merit further investigation.

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