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Quantitative image analysis reveals that phosphorylation of liver-type isozyme of fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase does not affect nuclear translocation of glucokinase in rat primary hepatocytes

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We have developed a new quantification method to measure translocation of glucokinase between nucleus and cytoplasm in primary hepatocytes. The method is robust, reliable and sensitive with the use of a high content fluorescence microscope, which can analyse more than 20,000 hepatocytes under each experimental condition. Frequency distributions of the nuclear and cytoplasmic contents of glucokinase did not exhibit a Gaussian distribution. Moreover, the distributions have large standard deviation values compared with their average values. These results indicate that a large number of cells must be analysed for the accurate quantification. Glucose and sorbitol promoted the translocation of glucokinase from nucleus to cytoplasm. These results show good agreement with previous reports. However, glucagon did not affect the localization of glucokinase. Under the same conditions, liver-type isozyme of fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F6P2K), whose dephosphorylated form has been proposed as a cytoplasmic binding protein with glucokinase, was completely phosphorylated. These results indicate that the phosphorylation and dephosphorylation of F6P2K does not have any appreciable effect on the intracellular localization of glucokinase.

Keywords: fructose-6-phosphate 2-kinase/glucagon/glucokinase/glucose/nuclear translocation.

Abbreviations: AU, arbitrary units; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; F6P2K, fructose-6-phosphate 2-kinase/fructose-2, 6-bisphosphtase; FBS, fetal bovine serum; GKRP, glucokinase regulatory protein; PBS, phosphate-buffered saline.

Glucokinase (EC 2.7.1.1) is one of four hexokinase isozymes catalyzing phosphorylation of glucose and is mainly expressed in liver and pancreatic β cell (*1*, *2*). Under hypoglycemic conditions, hepatic glucokinase is localized to the nucleus (*3*–*5*) and is inhibited by glucokinase regulatory protein (GKRP), which is predominantly present in the nucleus (*6*–*8*). The inhibition by GKRP is thought to avoid creating a futile cycle between glucokinase and glucose 6-phosphatase (*4*–*7*). Under hyperglycemic conditions, glucokinase moves to cytoplasm and catalyzes phosphorylation of glucose to lower the blood glucose level. Therefore, hepatic glucokinase is considered to play a major role in control of blood glucose level by changing its intracellular localization (*4*, *5*, *7*).

Several workers have hypothesized that the cytoplasmic localization of glucokinase may involve binding to dephosphorylated fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F6P2K) (EC 2.7.1.105/EC 3.1.3.46), but not to phosphorylated one (*9*–*12*). However, the binding affinity of glucokinase for F6P2K is much weaker than for GKRP (*13*). Moreover, the hypothesized binding site of F6P2K is buried in the catalytic domain of the enzyme so that it cannot interact with glucokinase (*14*, *15*). Last but not least, there is a fatal defect in a conventional image analysis method to quantify the intracellular glucokinase. By the conventional method, some representative microscope fields comprising ~100 cells are selected for each condition and are analysed with image processing software (*9*, *11*, *16*–*19*). However, the numbers of the examined cells are not enough to estimate the nuclear translocation of glucokinase accurately. Therefore, their hypothesis remains in doubts.

In this study, we have developed a new method using a high content fluorescence microscope to quantify the localization of glucokinase and to analyse more than 20,000 hepatocytes at a time. Under hypoglycemic and hyperglycemic conditions, the amount of glucokinase in the nucleus changed to 134 and 54%, respectively, compared with euglycemic conditions. However, the phosphorylation state of F6P2K did not change under the same glucose conditions. Moreover, glucagon promoted the phosphorylation of F6P2K, but not the nuclear translocation of glucokinase. These results demonstrate that the phosphorylation state of F6P2K does not affect the intracellular localization of glucokinase.

Experimental procedures

Animals

Male Sprague-Dawley rats weighing ~150 g were used. They were starved overnight and anesthetized by inhalation of ether followed

by intraperitoneal injection of pentobarbital at 50 mg/kg body weight. All animals were maintained in accordance with the guidelines of the Review Committee for Animal Experiments of Osaka Medical College.

Preparation of hepatocytes

Hepatocytes were isolated by collagenase perfusion of the rat liver (*20*). The hepatocytes were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetus bovine serum (FBS), 1 μM insulin, 1 μM dexamethasone as well as 30 μg/ml kanamycin and were seeded at a density of 1×10^5 cells/cm^2 in collagen-coated 24-well plates for immunostaining and on 60-mm collagen-coated culture dishes for western blot. They were incubated at 37°C in a 5% CO_2, 95% air-humidified incubator for 2 h for cell attachment. After attachment, the medium was replaced with serum-free DMEM containing 1 μM dexamethasone and 30 μg/ml kanamycin and the cells were cultured for 16–18 h before use for metabolic studies. Unless stated otherwise, the DMEM contained 100 mg/dl glucose.

Immunofluorescence staining and image analysis

Cells cultured in 24-well plates were fixed in 3% paraformaldehyde containing 0.1 M phosphate buffer (pH 7.4) for 15 min and then permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. They were blocked with 3% bovine serum albumin in PBS for 10 min and incubated with primary antibody against glucokinase (SC-7908, Santa Cruz Biotechnology) overnight at 4°C, followed by Alexa 488- conjugated second antibody (A11034, Invitrogen/Molecular Probes) as well as 1 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Each incubation step was followed by three washes with PBS containing 10 mM glycine. Images of stained cells were obtained with a high content fluorescence microscope, ImageXpress$^{MICRO\ TM}$ (Molecular Devices), equipped with DAPI-5060B and FITC-3540B filter sets. The images were analysed by MetaXpress^{TM} software with Cell Scoring Application Module to calculate the sum of pixel values of the immunofluorescence intensity in nucleus and cytoplasm.

Western blot

Cells cultured on 60-mm dishes were washed with PBS and solubilized in 2% SDS containing 1 mM EDTA and 10 mM Tris–HCl (pH 7.4). The cell samples were boiled for 2 min immediately and homogenized by sonication. The phosphorylated rat liver-type F6P2K specific polyclonal antibody was raised against a synthetic peptide (Ser Ser Ser Val Leu Gln Arg Arg Arg Gly phosphorylated Ser Ser Ile Pro Gln) corresponding to the N-terminus of the enzyme and purified with an affinity column. The blots were treated with the primary antibody (0.14 μg/ml) as well as alkaline phosphatase-conjugated secondary antibody (Promega) (1 : 7000) and were developed with Western Blue stabilized substrate for alkaline phosphatase (Promega).

Results

Defect of conventional method for quantification of glucokinase

Hepatocytes were incubated in DMEM containing various concentrations of glucose for 30 min and were stained with anti-glucokinase antibody by immunofluorescence. When the cells were incubated in low-glucose media, glucokinase was predominantly observed in nuclei (Fig. 1A and B). On the other hand, glucokinase was translocated from nucleus to cytoplasm in proportion to the increase in extracellular glucose concentration (Fig. 1C–E). To quantify the intracellular glucokinase by a conventional method, some representative microscope fields comprising ~100 cells are selected for each condition and are analysed with image processing software (*9*, *11*, *16–19*). However, the procedure to select the representative microscope fields may lose quantitative information of the original images, because the sample cell number ~100 is too small to draw any definite conclusions about these phenomena. This can be easily understood from the following reason. Figure 2 shows two enlarged parts of the picture of Fig. 1D. The upper left microscope field in Fig. 2 shows 17 glucokinase positive nuclei and 88 negative nuclei but the upper right one contains 66 positive nuclei and 10 negative nuclei. The opposite conclusions can be drawn from the same photograph depending on the selection of the representative microscope fields. Therefore, the conventional method is not appropriate to measure the amount of glucokinase in nucleus and cytoplasm. It is necessary to develop a robust method for quantification of the intracellular localization of glucokinase without the selection of the so-called representative microscope fields.

New quantification method for intracellular localization of glucokinase

Recently, we have developed a new method for the quantification of intracellular localization of glucokinase using a high content fluorescence microscope. The microscope can capture images of DAPI-stained nuclei (Fig. 3A) and intracellular distribution of immunostained glucokinase (Fig. 3B), simultaneously. The size and resolution of the images were 696×520 pixels and 1.29 μm/pixel, respectively. The fluorescent intensities of DAPI and Alexa-488 of each pixel were digitized and stored separately as 16-bit image depth data. The nuclear and cytoplasmic areas were defined by the pixel intensities of the fluorescence of DAPI and Alexa-488, respectively, above the surrounding backgrounds with the use of the image analysis software equipped with the microscope (Fig. 3C). The segmentation process that defines the nuclear and cytoplasmic areas does not modify the pixel fluorescence-intensity measurements. The software can integrate the fluorescence intensity of Alexa-488 of the pixels belonging to each area to analyse the intracellular distribution of glucokinase. Using the microscope, we quantified the intracellular localization of glucokinase to analyse more than 20,000 cells for each condition. Frequency distribution tables of the glucokinase in nucleus and cytoplasm are shown in Tables I and II. These distribution patterns of glucokinase do not exhibit a Gaussian distribution. The average values of nuclear fluorescence intensity of the cells incubated in the medium containing 0, 50, 100, 200 and 400 mg/dl glucose were 52.3, 51.2, 43.5, 37.4 and 32.8 arbitrary units (AU), respectively, and their cytoplasmic values were 120, 136, 135, 135 and 146 AU, respectively. The values of standard deviation are ~50% of those of the average, indicating that the data are spread out over a large range of values. These results demonstrate that the quantitative analysis of nuclear translocation of glucokinase needs to examine a large number of cells. Therefore, we carried out the same experiment of Tables I and II for three times. The averages with standard deviations of the triplicate experiments are shown in Fig. 4. The nuclear content of glucokinase decreased to 40% when the extracellular glucose level increased from 0 to 400 mg/dl. The decrease in the nuclear content of glucokinase was found to be

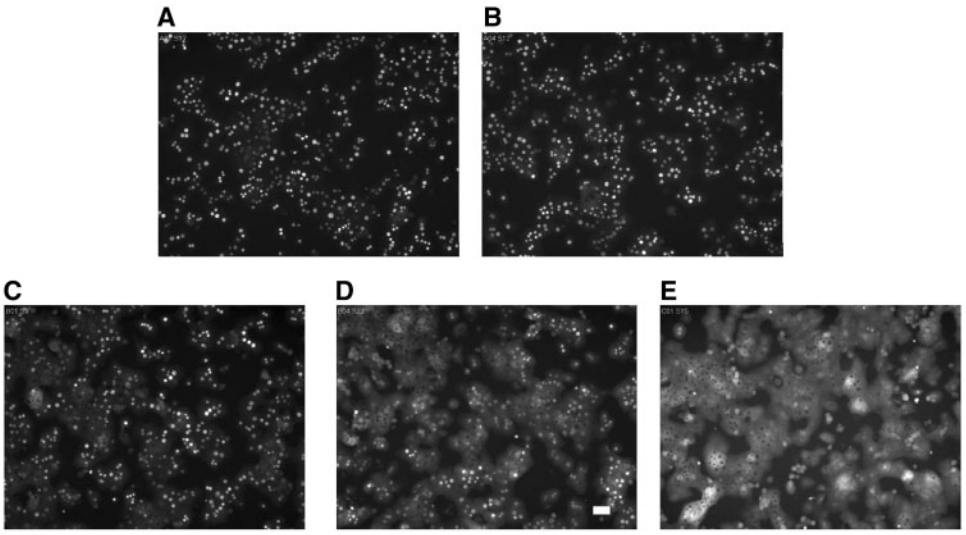

Fig. 1 Glucose-induced translocation of glucokinase in primary hepatocytes. Hepatocytes were incubated for 30 min in DMEM containing 0 mg/dl (A), 50 mg/dl (B), 100 mg/dl (C), 200 mg/dl (D) and 400 mg/dl glucose (E). Glucokinase was visualized by immunocytochemistry. Scale bar, 50 μm.

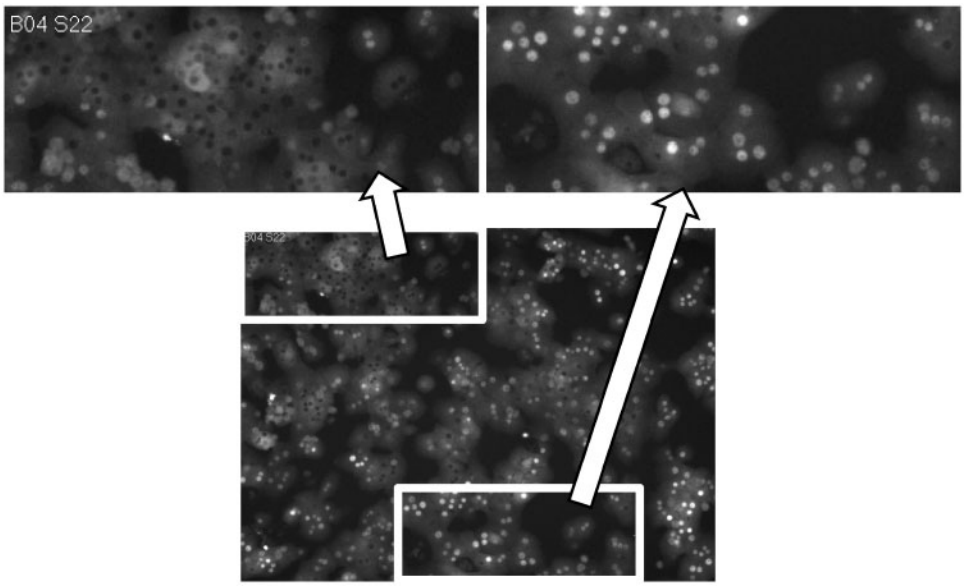

Fig. 2 Defect of conventional method to analyse representative microscopic images. Two areas of the picture of Fig. 1 (D) comprising ~100 hepatocytes were chosen. The upper left photograph shows 17 glucokinase positive nuclei and 88 negative ones but the upper right photograph contains 66 positive ones and 10 negative ones.

accompanied by increase in the cytoplasmic one, so that the sum of the nuclear and cytoplasmic contents of glucokinase remained constant [one-way analysis of variance (ANOVA) test, $P<0.05$]. These results demonstrate that not only is the total content of glucokinase in the cells conserved during the incubation but also the new method with the use of the high content fluorescent microscope provides robust, reliable and sensitive means of quantitative evaluation of the intracellular localization of glucokinase.

No effect of phosphorylation of F6P2K on translocation of glucokinase

Several groups have hypothesized that dephosphorylated liver-type F6P2K but not phosphorylated F6P2K may interact with glucokinase and may retain it in cytoplasm (*9–12*). To test the hypothesis, the phosphorylation of F6P2K and the intracellular translocation of glucokinase were analysed simultaneously by western blot and by immunofluorescence. The glucokinase was translocated to nucleus and cytoplasm under the hypoglycemic and hyperglycemic conditions, respectively (Fig. 4). However, very faint bands were observed under these conditions (Fig. 5B, lanes 1–5). This shows that the amount of phosphorylated F6P2K was negligible compared with that of fully phosphorylated state of F6P2K, which was prepared from hepatocytes treated with glucagon (Fig. 5B, lane 6). The total amount of F6P2K did not change during the incubation in the various concentration of

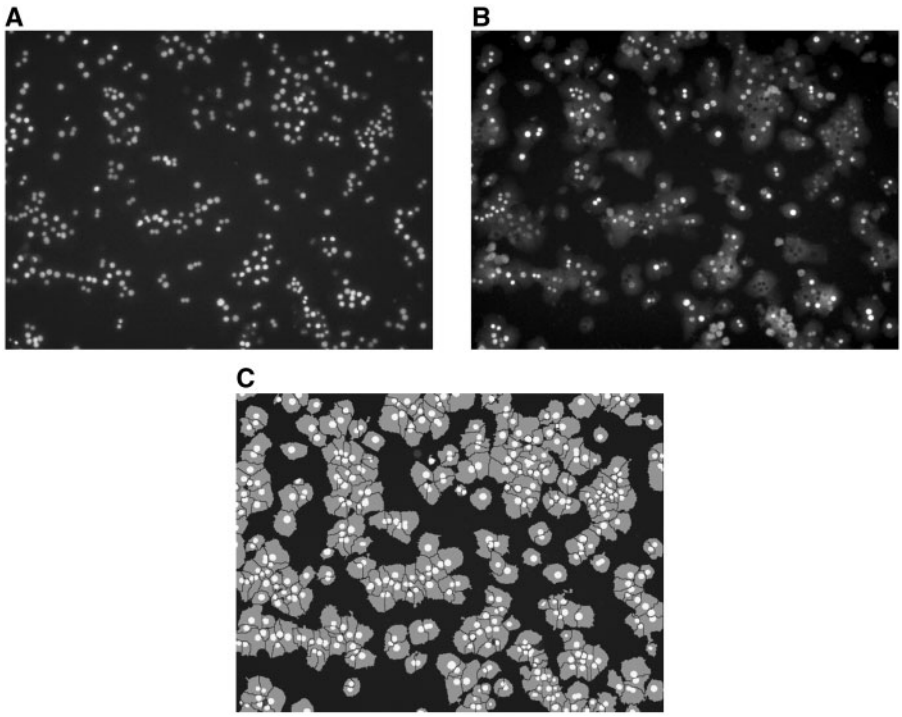

Fig. 3 Segmentation of nuclear and cytoplasmic areas by new image analysis system. Images of DAPI-stained nuclei (A) and immunostained glucokinase (B) of primary hepatocytes were simultaneously captured by the new image analysis system. The size of each image is 696 × 520 pixels and its resolution is 1.29 μm/pixels. The nuclear and cytoplasmic boundaries were defined by MetaXpress™ software automatically. The nuclear and cytoplasmic areas are indicated in white and gray, respectively (C). The amount of glucokinase was estimated by integration of the fluorescence intensity in each area.

Table I. Frequency distribution of glucokinase in nucleus.

Fluorescence intensity (arbitrary units)	Glucose (mg/dl)						
	0	50	100	200	400	Sorbitol[a]	Control[b]
25≥	4,580	3,130	5,424	5,709	7,824	7,604	22,004
50	9,547	9,774	12,741	12,574	12,368	10,636	4,603
75	5,837	4,682	4,356	3,877	3,191	2,750	62
100	2,358	2,367	2,143	869	194	154	8
125	1,562	1,207	722	88	34	42	3
150	647	314	88	14	12	7	2
175	159	67	22	8	5	3	0
200	32	15	7	3	3	0	0
225	13	9	3	0	0	1	0
250	5	3	0	1	1	0	0
>250	1	1	0	0	0	1	0
Total cell number	24,741	21,569	25,506	23,143	23,632	21,198	26,682
Average of intensity	52.3	51.2	43.5	37.4	32.8	32.5	17.9
Standard deviation	31.6	28.3	23.9	18.1	14.7	14.8	7.5

[a]Hepatocytes were incubated for 30 min in DMEM containing 100 mg/dl glucose and 0.1 mM sorbitol.
[b]For the control experiment of cellular autofluorescence, the primary antibody was omitted in the immunostaining procedure.

Table II. Frequency distribution of glucokinase in cytoplasm.

Fluorescence intensity (arbitrary units)	Glucose (mg/dl)						
	0	50	100	200	400	Sorbitol[a]	Control[b]
50≥	3,757	2,553	3,934	3,686	4,151	3,698	23,132
100	7,870	6,107	6,796	6,293	5,668	4,822	2,472
150	6,322	5,414	5,810	5,095	4,535	4,054	676
200	3,283	3,291	3,697	3,256	3,293	2,956	240
250	1,911	1,972	2,407	2,148	2,277	2,099	73
300	942	1,156	1,377	1,306	1,534	1,404	47
350	428	613	754	722	985	919	20
400	135	270	403	354	574	565	8
450	54	118	188	169	299	322	8
500	20	45	85	53	143	157	2
>500	19	30	55	61	173	202	4
Total cell number	24,741	21,569	25,506	23,143	23,632	21,198	26,682
Average of intensity	120	136	135	135	146	150	30
Standard deviation	74.4	83.8	89.9	90.7	107	112	33.5

[a]Hepatocytes were incubated for 30 min in DMEM containing 100 mg/dl glucose and 0.1 mM sorbitol.
[b]For the control experiment of cellular autofluorescence, the primary antibody was omitted in the immunostaining procedure.

glucose (Fig. 5A). These results indicate that the phosphorylation of F6P2K may not be responsible for the intracellular translocation of glucokinase.

To confirm this, hepatocytes were treated with various concentrations (0–100 nM) of glucagon for 30 min. The total amount of F6P2K also did not change during the incubation (Fig. 6A). At this time, the liver-type isozyme of F6P2K was phosphorylated by the addition of glucagon (Fig. 6B). The dose response profile of phosphorylation state of F6P2K by glucagon is consistent with a previous report (*21*). However, the nuclear content of glucokinase was not changed by glucagon (Fig. 7). We cannot detect any significant difference of the intracellular localization of glucokinase using one-way ANOVA ($P < 0.05$). Moreover, the amount of nuclear glucokinase also did not change when the experiment was carried out at 200 mg/dl glucose concentration (data not shown).

These results demonstrate that the phosphorylation state of F6P2K does not affect the intracellular localization of glucokinase in hepatocytes.

Discussion

Intracellular translocation of glucokinase in hepatocytes under various glucose conditions has been studied by many workers. They have measured the subcellular distribution of glucokinase by two conventional methods. In one method, they quantified the release of glucokinase from digitonin-permeabilized hepatocytes (*22–25*). Speaking strictly, the method did not measure the distribution of glucokinase between nucleus and cytoplasm but the distribution of the enzyme between free and bound states in hepatocytes. Moreover, the permeabilization of cell membrane may cause depletion of intracellular components, such as ATP, GTP, importin and exportin, which play an important role in the nuclear-cytoplasmic protein transport. The digitonin release assay has to be conducted under the non-physiological conditions concerning the nuclear protein transport system. In the other method, the images of intracellular distribution of glucokinase, which was visualized by immunocytochemistry or by expression of a fluorescent protein fused to the enzyme, were analysed with the use of image processing software. However, the investigators who use the latter method analyse the images comprising of only a small number of cells (~100 cells) and have shown representative photographs containing 10–20 cells in their papers (*8*, *11*, *16–19*, *26*). The cell number they examined is too small to estimate the translocation of glucokinase accurately, because it is easy to draw wrong conclusions depending on the selection of the microscopic fields comprising ~100 cells (Fig. 2). Therefore, it is necessary to develop a new method to select an unbiased subset of the microscopic fields for the image processing and to analyse the images comprising a large number of cells for the accurate estimation of the localization of glucokinase.

In the present study, we have developed a new image analysis method, which can quantify the intracellular localization of glucokinase to examine ~20,000 cells each condition. Moreover, it can distinguish cytoplasmic and nuclear areas of hepatocytes automatically and integrate fluorescence intensities of each area. Using the method, we elucidate that the frequency distribution patterns of the nuclear and cytoplasmic contents of glucokinase can be fitted to neither the

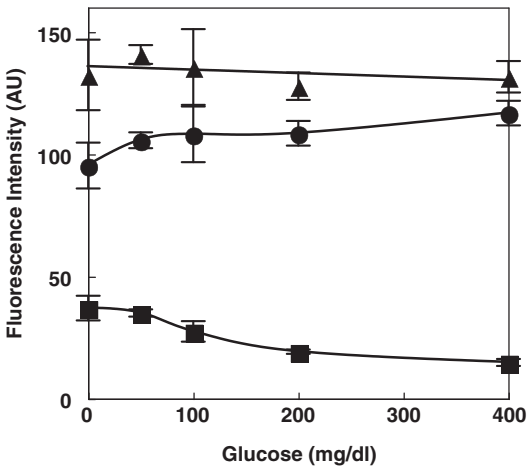

Fig. 4 Changes in nuclear and cytoplasmic contents of glucokinase in primary hepatocytes. Hepatocytes were incubated for 30 min in DMEM containing various concentrations of glucose. Glucokinase was visualized by the immunofluorescence method and the fluorescent intensity was quantified by the ImageXpress^{TM} analysis system. Data are presented as mean values with standard deviation of three independent experiments. Filled square, nuclear glucokinase; filled circle, cytoplasmic glucokinase; filled triangle, total glucokinase.

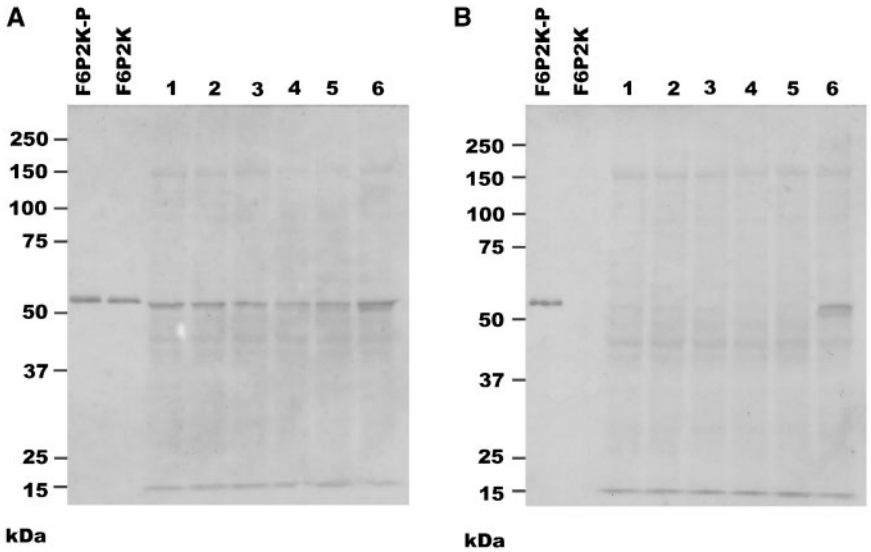

Fig. 5 Effects of glucose on phosphorylation state of F6P2K. Hepatocytes were incubated for 30 min in the medium containing various concentrations of glucose (0 mg/dl, lane 1; 50 mg/dl, lane 2; 100 mg/dl, lane 3; 200 mg/dl, lane 4 and 400 mg/dl, lane 5) as well as 100 nM glucagon (lane 6). The cell lysates were analysed by immunoblotting with anti-F6P2K (A) and anti-phospho F6P2K (B) antibodies. Lanes F6P2K and F6P2K-P contain 5 ng of dephospho and phospho forms of rat liver-type isozyme of F6P2K, respectively.

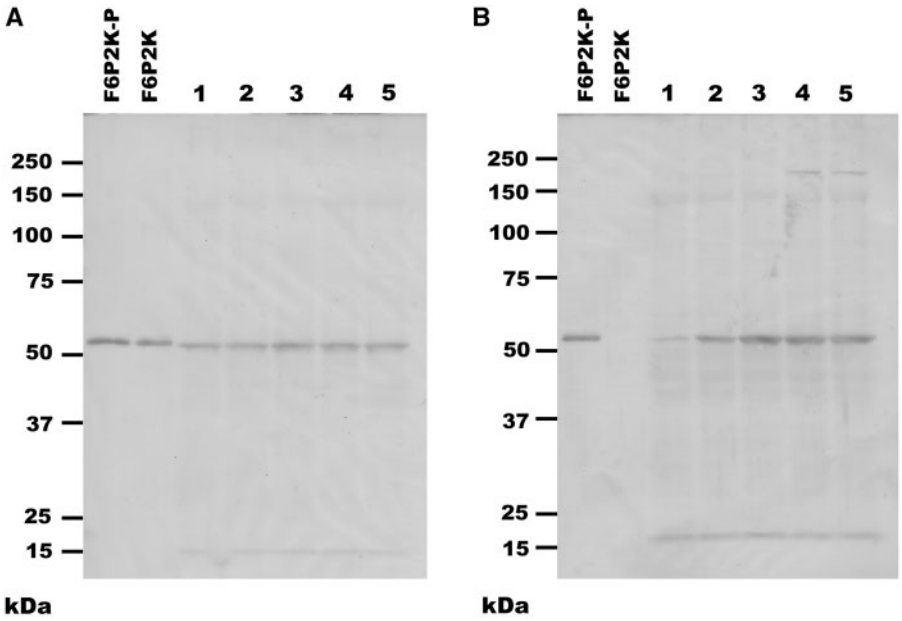

Fig. 6 Effects of glucagon on phosphorylation state of F6P2K. Hepatocytes were incubated for 30 min in DMEM containing various concentration of glucagon (0 nM, lane 1; 0.1 nM, lane 2; 1 nM, lane 3; 10 nM, lane 4; and 100 nM, lane 5). The cell lysates were analysed by immunoblotting with anti-F6P2K (A) and anti-phospho F6P2K (B) antibodies. Lanes F6P2K and F6P2K-P contain 5 ng of dephospho and phospho forms of rat liver-type isozyme of F6P2K, respectively.

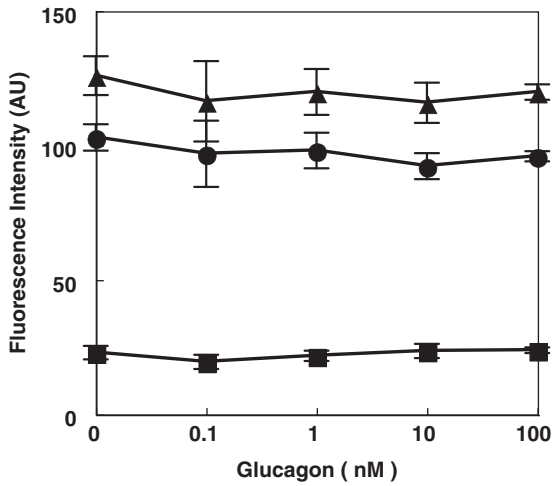

Fig. 7 Effects of glucagon on intracellular localization of glucokinase. Hepatocytes were incubated for 30 min in DMEM containing various concentrations of glucagon. Glucokinase was visualized by the immunofluorescence method and the fluorescent intensity was quantified by ImageXpress^{TM}. Data are presented as mean values with standard deviation of three independent experiments. Filled square, nuclear glucokinase; filled circle, cytoplasmic glucokinase; filled triangle, total glucokinase.

Gaussian distribution nor any other elementary statistical distribution (Table I and II). This implies that the statistical significance cannot be determined by the usual parametric analysis. Moreover, we found that the standard deviations of nuclear and cytoplasmic contents of glucokinase were relatively large (Table I and II). These results indicate that a large number of cells must be analysed to obtained reliable values, because the data are spread out over a large range of values. Our new method is particularly useful when the large number of cells must be examined for accurate quantification.

Several workers have proposed a mechanism that the dephosphorylated form of F6P2K but not the phosphorylated form might act as a cytoplasmic receptor for glucokinase and might retain it in the cytoplasm under hyperglycemic conditions (*9–12*). However, several findings disagree with the hypothesis concerning the interaction between glucokinase and F6P2K. For example, glucokinase cannot access the hypothesized binding site of F6P2K because the site is concealed by the catalytic domain of F6P2K (*14*, *15*). To test their hypothesis, we simultaneously measured the intracellular distribution of glucokinase by our new quantification method and the phosphorylation state of F6P2K by western blot. The nuclear content of glucokinase decreased to 40% under conditions in which the extracellular glucose level increased from 0 to 400 mg/dl (Fig. 4). However, the phosphorylation state of F6P2K did not change under the conditions (Fig. 5). These results indicate that the phosphorylation of F6P2K is not indispensable for the nuclear translocation of glucokinase. Moreover, F6P2K was phosphorylated by the addition of glucagon in a dose dependent manner (Fig. 6), but the intracellular distribution of glucokinase did not change (Fig. 7). These results demonstrate that the phosphorylation of F6P2K do not promote the nuclear translocation of glucokinase. Consequently, our results refute the hypothesis that the dephosphorylation of F6P2K might retain glucokinase in cytoplasm and the phosphorylation of F6P2K might promote the nuclear translocation of the enzyme. The workers presented the wrong hypothesis because they might fail to quantify the intracellular translocation of glucokinase using the conventional method.

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Conflict of interest
None declared.

References

1. Agius, L. (2008) Glucokinase and molecular aspects of liver glycogen metabolism. *Biochem. J.* **414**, 1–18
2. Iynedjian, P.B. (2009) Molecular physiology of mammalian glucokinase. *Cell. Mol. Life Sci.* **66**, 27–42
3. Miwa, I., Mitsuyama, S., Toyoda, Y., Nonogaki, T., Aoki, S., and Okuda, J. (1990) Evidence for the presence of rat liver glucokinase in the nucleus as well as in the cytoplasm. *Biochem. Int.* **22**, 759–767
4. Toyoda, Y., Miwa, I., Kamiya, M., Ogiso, S., Nonogaki, T., Aoki, S., and Okuda, J. (1994) Evidence for glucokinase translocation by glucose in rat hepatocytes. *Biochem. Biophys. Res. Commun.* **204**, 252–256
5. Toyoda, Y., Miwa, I., Kamiya, M., Ogiso, S., Nonogaki, T., Aoki, S., and Okuda, J. (1995) Tissue and subcellular distribution of glucokinase in rat liver and their changes during fasting-refeeding. *Histochem. Cell. Biol.* **103**, 31–38
6. Van Schaftingen, E., Detheux, M., and Veiga da Cunha, M. (1994) Short-term control of glucokinase activity: role of a regulatory protein. *FASEB J.* **8**, 414–419
7. Toyoda, Y., Miwa, I., Satake, S., Anai, M., and Oka, Y. (1995) Nuclear location of the regulatory protein of glucokinase in rat liver and translocation of the regulator to the cytoplasm in response to high glucose. *Biochem. Biophys. Res. Commun.* **215**, 467–473
8. Brown, K.S., Kalinowski, S.S., Megill, J.R., Durham, S.K., and Mookhtiar, K.A. (1997) Glucokinase regulatory protein may interact with glucokinase in the hepatocyte nucleus. *Diabetes* **46**, 179–186
9. Payne, V.A., Arden, C., Wu, C., Lange, A.J., and Agius, L. (2005) Dual role of phosphofructokinase-2/fructose bisphosphatase-2 in regulating the compartmentation and expression of glucokinase in hepatocytes. *Diabetes* **54**, 1949–1957
10. Smith, W.E., Langer, S., Wu, C., Baltrusch, S., and Okar, D.A. (2007) Molecular coordination of hepatic glucose metabolism by the 6-phosphofructo-2-kinase/fructose-2,6- bisphosphatase:glucokinase complex. *Mol. Endocrinol.* **21**, 1478–1487
11. Payne, V.A., Arden, C., Lange, A.J., and Agius, L. (2007) Contributions of glucokinase and phosphofructokinase-2/fructose bisphosphatase-2 to the elevated glycolysis in hepatocytes from Zucker fa/fa rats. *Am. J. Physiol.* **293**, R618–R625
12. Okar, D.A., Lange, A.J., and Wu, C. (2009) Interaction with PFK-/FBP-2 is essential to glucokinase molecular physiology. *Cell. Mol. Life Sci.* **66**, 731–732
13. Baltrusch, S., Lenzen, S., Okar, D.A., Lange, A.J., and Tiedge, M. (2001) Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. *J. Biol. Chem.* **276**, 43915–43923
14. Hasemann, C.A., Istvan, E.S., Uyeda, K., and Deisenhofer, J. (1996) The crystal structure of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase reveals distinct domain homologies. *Structure* **4**, 1017–1029
15. Watanabe, F., Jameson, D.M., and Uyeda, K. (1996) Enzymatic and fluorescence studies of four single-tryptophan mutants of rat testis fructose 6-phosphate,2-kinase:fructose 2,6-bisphosphatase. *Protein Sci.* **5**, 904–913
16. Mukhtar, M.H., Payne, V.A., Arden, C., Harbottle, A., Khan, S., Lange, A.J., and Agius, L. (2008) Inhibition of glucokinase translocation by AMP-activated protein kinase is associated with phosphorylation of both GKRP and 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase. *Am. J. Physiol.* **294**, R766–R774
17. Guigas, B., Bertrand, L., Taleux, N., Foretz, M., Wiernsperger, N., Vertommen, D., Andreelli, F., Viollet, B., and Hue, L. (2006) 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside and Metformin Inhibit Hepatic Glucose Phosphorylation by an AMP-Activated Protein Kinase-Independent Effect on Glucokinase Translocation. *Diabetes* **55**, 865–874
18. Chu, C.A., Fujimoto, Y., Igawa, K., Grimsby, J., Grippo, J.F., Magnuson, M.A., Cherrington, A.D., and Shiota, M. (2004) Rapid translocation of hepatic glucokinase in response to intraduodenal glucose infusion and changes in plasma glucose and insulin in conscious rats. *Am. J. Physiol.* **286**, G627–G634
19. Bosco, D., Meda, P., and Iynedjian, P.B. (2000) Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. *Biochem. J.* **348**, 215–222
20. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. *J. Biochem.* **84**, 937–946
21. Connelly, P.A., Botelho, L.H., Sisk, R.B., and Garrison, J.C. (1987) A study of the mechanism of glucagon-induced protein phosphorylation in isolated rat hepatocytes using (S_p)-cAMPS and (R_p)-cAMPS, the stimulatory and inhibitory diastereomers of adenosine cyclic 3′,5′-phosphorothioate. *J. Biol. Chem.* **262**, 4324–4332
22. Agius, L. and Peak, M. (1993) Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin. *Biochem. J.* **296**, 785–796
23. Agius, L. (1994) Control of glucokinase translocation in rat hepatocytes by sorbitol and the cytosolic redox state. *Biochem. J.* **298**, 237–243
24. Agius, L., Peak, M., Newgard, C.B., Gomez-Foix, A.M., and Guinovart, J.J. (1996) Evidence for a role of glucose-induced translocation of glucokinase in the control of hepatic glycogen synthesis. *J. Biol. Chem.* **271**, 30479–30486
25. Agius, L. and Stubbs, M. (2000) Investigation of the mechanism by which glucose analogues cause translocation of glucokinase in hepatocytes: evidence for two glucose binding sites. *Biochem. J.* **346**, 413–421
26. Mukhtar, M., Stubbs, M., and Agius, L. (1999) Evidence for glucose and sorbitol-induced nuclear export of glucokinase regulatory protein in hepatocytes. *FEBS Lett.* **462**, 453–458