Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization

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Conditional expression of the glucokinase regulatory protein in insulinoma cells, under control of the reverse tetracycline-dependent transactivator, was used to investigate whether expression of this protein *de novo* would alter the intracellular distribution of glucokinase. The regulatory protein, which was undetectable in the basal state, could be induced by doxycycline to levels comparable to those of liver and was detected mostly in the nucleus. Concomitantly, glucokinase accumulated in the nucleus. Human embryonic kidney cells were transiently transfected to express glucokinase and the regulatory protein, either separately or together. Each protein localized predominantly to the cytoplasm when expressed alone. On co-expression, however,

both proteins localized virtually entirely to the nucleus. The enzymic activity of glucokinase was not required for promoting nuclear import of the two proteins, as shown with a glucose-phosphorylation-deficient mutant. Finally, in embryonic kidney cells expressing the regulatory protein alone, treatment with leptomycin B resulted in a partial redistribution of the protein from the cytoplasm to the nucleus, suggesting that this protein can shuttle between the two compartments.

Key words: glucose metabolism, hepatocyte, liver, nuclear import/export, pancreatic β -cell.

INTRODUCTION

Glucokinase, a member of the hexokinase family (hexokinase IV) [1], is the regulatory enzyme that endows liver cells and endocrine cells of the islets of Langerhans with the capacity to increase or decrease their rates of glucose utilization when the plasma glucose concentration rises or falls respectively [2,3]. Glucokinase thus serves the function of a glucose sensor, allowing autonomous cellular responses to changes in circulating glucose levels. In liver, the activity of glucokinase is negatively regulated by protein–protein interaction with the so-called glucokinase regulatory protein [4]. Binding of the regulatory protein to glucokinase, which is competitive with glucose, is favoured when the regulatory protein is liganded to fructose 6-phosphate. Conversely, the dissociation of the two proteins and the release of active glucokinase are favoured when the ligand site of the regulator is occupied by fructose 1-phosphate [5]. By this device, glucokinase activity is increased after the ingestion of carbohydrates (containing fructose) because the intestinal absorption of fructose leads to an increase in the ratio of the hepatic concentrations of fructose 1-phosphate to fructose 6-phosphate [6]. Early findings suggested that islets might contain a glucokinase regulatory protein similar to that of liver [7] but no proof for the presence of this protein in islet cells has been provided.

A higher level of complexity in metabolic regulation arises from the ability of hepatic glucokinase to translocate between discrete subcellular compartments. Glucokinase was shown to be localized predominantly in the cell nucleus in the livers of fasting rats, and to move to the cytoplasm after brief perfusion of the liver with a perfusate containing 20 mM glucose [8]. Using isolated hepatocytes, Agius and Peak showed that the fraction of total glucokinase released in soluble form after cell permeabilization with low concentrations of digitonin increased after

incubation of the cells with high concentrations of glucose or fructose [9]. Subsequently, glucokinase was found to be predominantly nuclear during culture in low-glucose medium and to be exported to the cytoplasm after the addition of fructose or glucose to the medium [10,11]. Under both conditions in isolated hepatocytes, the regulatory protein remained mostly localized to nuclei. These findings prompted the hypothesis that the regulatory protein might serve to trap glucokinase in the nucleus when there is no metabolic need for the enzyme. Export of glucokinase to the cytoplasm would occur at times of need, i.e. when the blood glucose concentration rises or when fructose is ingested.

In the present study we tested the role of the regulatory protein as a putative nuclear anchor for glucokinase by ectopic expression of these proteins in two distinct cell types. The first consisted of insulinoma cells, which contain endogenous glucokinase but no regulatory protein. Controlled expression of the regulatory protein was achieved in these cells by using a two-component tetracycline-dependent induction system. The second cell system, the 293T human embryonic kidney cell line, spontaneously expressed neither glucokinase nor the regulatory protein. Forced expression of the proteins, separately or together, was accomplished in these cells by transient transfection.

EXPERIMENTAL

Plasmids

The entire coding region of the rat liver glucokinase regulatory protein was obtained as an approx. 2 kbp DNA fragment by *EcoRI* digestion of plasmid RPR-pcDNA1 (kindly provided by Maria Veiga-da-Cunha and Emile Van Schaftingen). This fragment was subcloned downstream of the tetra-

Abbreviations used: NES, nuclear export signal; NLS, nuclear localization signal.

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cycline operator–cytomegalovirus minimal promoter in plasmid PUHD10-3 [12] to generate plasmid PUHD-GCKR. The same DNA fragment was cloned in the vector pcDNA3.1/Zeo(+), resulting in a plasmid, termed pGCKR, that was used in the transient transfection of 293T cells.

An expression plasmid for rat liver glucokinase, termed pGCK, was constructed by inserting a *Hind*III–*Bam*HI fragment from plasmid pB-GK2 [13], containing a full-length cDNA, into the pcDNA3.1/Zeo(+) vector. The sequence encoding Myc-tagged glucokinase was produced by the following steps. A DNA fragment from codon 2 to the *Aoc*I site at codon 45 of the rat liver glucokinase sequence, with a *Bam*HI site added at the 5' end, was synthesized by PCR and verified by sequencing. This fragment was ligated to the *Aoc*I–*Eco*RI fragment extending from codon 45 to the 3' end of the rat liver glucokinase cDNA of plasmid pB-GK2. An oligonucleotide coding for the amino acid sequence MASEQKLISEEDLGS (bold letters denote the Myc epitope), with a *Hind*III site added at the 5' end and with a 3' *Bam*HI site, was ligated to the *Bam*HI–*Eco*RI-modified glucokinase fragment. The entire *Hind*III–*Eco*RI DNA fragment was finally cloned in the pcDNA3.1/Zeo(+) vector to generate plasmid pM-GCK encoding Myc-tagged glucokinase. Plasmid pM-GCKm1 with a mis-sense N → D mutation at codon 204 was obtained by site-directed mutagenesis *in vitro* with a T4 DNA polymerase-based system (GeneEditor; Promega). The mutation was verified by sequencing.

Cell culture and transfection

The previously described INS-r3 cell line [3] is an insulinoma cell line with stably integrated DNA coding for the reverse tetracycline-dependent transactivator of Gossen et al. [14]. These cells were secondarily co-transfected by published techniques [3] with plasmid PUHD-GCKR, plus a plasmid conferring hygromycin B resistance. Colonies of secondary transfectants were isolated with cloning rings. One clonal cell line termed INS-GCKR18 was selected for this study. Maintenance culture of INS-r3 cells was in the modified RPMI 1640 medium described previously [15], with the addition of 150 µg/ml G418. The medium for INS-GCKR18 was further supplemented with 100 µg/ml of hygromycin B. For induction experiments, cells in six-well culture dishes were supplied with fresh culture medium containing 5 mM glucose 40 h after seeding; doxycycline in amounts specified in the figure legends was added to cells 3 h after the change of medium. For immunofluorescence experiments, the cells were attached to polyornithine-coated glass coverslips. The cells were harvested for study 42 h after the addition of doxycycline.

Human embryonic kidney cells of the 293T line were cultured in Dulbecco's modified Eagle's medium containing 5.5 mM glucose and 1 mM sodium pyruvate. For immunofluorescence experiments, cells were seeded on polyornithine-coated glass coverslips in six-well culture dishes. Transfection of cells was done 24 h after seeding, by using a calcium phosphate/DNA co-precipitation method (Profection Mammalian Transfection System; Promega). The total amount of DNA in the transfection co-precipitate was usually 3–5 µg, divided between individual plasmids as specified in the figure legends. After the addition of the transfection co-precipitate, cells were left without medium change until harvest, usually 22 h after the start of transfection.

Immunoblotting

For the preparation of total protein extracts and immunoblotting of the glucokinase regulatory protein, cells in six-well dishes were washed twice with 2 ml of ice-cold PBS and scraped with a

rubber spatula into 0.2 ml of lysis buffer containing 25 mM Tris/phosphate, pH 7.8, 2 mM 1,2-diaminocyclohexane-tetraacetic acid, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM dithiothreitol and 250 µM PMSF. The lysates were transferred to Microfuge tubes, vortex-mixed vigorously for 2 min, sonicated four times for 2 s on ice and centrifuged at 4 °C and 12000 g for 1 min. The supernatants were assayed for protein concentration by the Bradford assay (Bio-Rad). Predetermined amounts of protein were resolved by SDS/PAGE in 10% (w/v) polyacrylamide separation gels, transferred to nitrocellulose membranes and immunoblotted essentially as described previously [16]. Primary antibodies were a rabbit antiserum against the rat liver glucokinase regulatory protein (a gift from Emile Van Schaftingen), used at 1:1500 dilution. Secondary antibodies were affinity-purified goat antibodies against rabbit immunoglobulins conjugated with peroxidase. Revelation was by enhanced chemiluminescence (SuperSignal Chemiluminescent Substrate; Pierce).

For assay and immunoblotting of glucokinase, cells from individual wells of six-well dishes were scraped into 0.8 ml of ice-cold PBS, pelleted by centrifugation and suspended at 4 °C in 55 µl of hypotonic buffer containing 22 mM Tris/HCl, pH 7.5, 5.5 mM EDTA, 4.4 mM MgCl₂, 2.2 mM dithiothreitol, 22 mM glucose and 160 µM PMSF. After 10 min, NaCl was added to a final concentration of 100 mM. The cell suspension was vortex-mixed vigorously for 2 min and subjected to three cycles of freezing in liquid N₂ and thawing. The lysate was centrifuged for 30 min at 4 °C and 12000 g. The supernatant was assayed for protein content and used for SDS/PAGE. Transfer to membrane and immunoblotting were as described above, with a previously characterized [16] sheep antiserum against rat liver glucokinase at 1:2000 dilution. Secondary antibodies were rabbit antibodies against sheep immunoglobulins conjugated with peroxidase; revelation was by enhanced chemiluminescence.

Hexokinase assay

Enzyme activity was measured fluorimetrically by a glucose 6-phosphate dehydrogenase coupled assay as described previously [17]. The enzyme assay was run at 0.5 mM and 100 mM glucose. The rate of product formation at 0.5 mM glucose was used to estimate 'low-*K_m*' hexokinase activity; the difference in rates observed at 0.5 and 100 mM glucose provided 'high-*K_m*' hexokinase activity.

Immunofluorescence

Cells on glass coverslips were washed in ice-cold PBS and fixed for 30 min in phosphate-buffered 4% (w/v) paraformaldehyde. Fixed cells were washed with PBS and incubated for 10 min with PBS containing 10 mM NH₄Cl. Next, the cells were permeabilized by incubation in 90% (v/v) ethanol for 5 min, washed with PBS and incubated with PBS containing 0.5% BSA for 30 min. Primary antibodies diluted with PBS containing 0.5% BSA were left to react with the cells for 2–3 h at room temperature. The cells were washed thoroughly and subsequently exposed to fluorescently labelled secondary antibodies diluted with PBS containing 0.5% BSA for 60–90 min at room temperature. The cells were finally washed with PBS and counterstained with PBS containing 0.02% Evans Blue when specified. For immunolocalization of the glucokinase regulatory protein, crude rabbit antiserum at a dilution of 1:300 was used. Immunofluorescence of glucokinase was performed with anti-glucokinase antibodies from sheep that were affinity-purified as described [18]. Epitope-tagged glucokinase was detected with a monoclonal antibody against the Myc tag.

Microscopic examination was performed with a standard fluorescence photomicroscope (Zeiss Axiophot) or with a laser confocal microscope (Zeiss model LSM 410). For each experimental condition, at least 100 cells were analysed by eye for the intracellular distribution of the fluorescence signal. All results of immunolocalization obtained by standard microscopy were verified by confocal microscopy. Control experiments to test the specificity of the immunofluorescence procedures included the absence of signal from cells transfected with vectors devoid of cDNA insert, and the absence of cross-reactivity of the antibodies against glucokinase with the glucokinase regulatory protein. The immunodetection of glucokinase in nucleus or cytoplasm was not affected by counterstaining of insulinoma or embryonic kidney cells with Evans Blue.

Confocal images were acquired with appropriate excitation wavelengths and emission filters for the detection of FITC fluorescence (specific immunolocalization) and Evans Blue fluorescence (non-specific cell staining). Images from the Axiophot microscope were obtained with appropriate sets of excitation and emission filters for the detection of rhodamine or FITC fluorescence and captured with a high-sensitivity Zeiss Photonic Coolview colour camera. Greyscale figures were generated with Adobe Photoshop 5.0 software and printed with a Kodak digital printer.

RESULTS

Quantification of glucokinase regulatory protein and glucokinase in engineered insulinoma cells

The level of expression of the glucokinase regulatory protein was assessed in INS-GCKR18 cells by immunoblot analysis. This analysis did not reveal any endogenous glucokinase regulatory protein in INS-GCKR18 cells during culture in the absence of doxycycline. The lack of regulatory protein was also noted in the parental INS-r3 cells that were cultured with or without doxycycline (Figure 1A). In addition, the regulatory protein remained undetectable in INS-r3 and INS-GCKR18 cells in the absence of doxycycline, even with protein inputs that were 50-fold those permitting easy detection in extracts from hepatocytes (results not shown). These cells therefore contained insignificant amounts of regulatory protein (less than 2% of the amount present in hepatocytes). Culture of the INS-GCKR18 cells with increasing doses of doxycycline resulted in a dose-dependent accumulation of a single immunoreactive protein with the same electrophoretic mobility as the regulatory protein seen in hepatocytes. At the highest concentration of doxycycline, the amount of immunoreactive regulatory protein in INS-GCKR18 cells was estimated to be 30–40% of that in hepatocytes (Figure 1A).

Endogenous glucokinase was readily discerned in INS-GCKR18 as well as parental INS-r3 cells. The enzyme amount was not affected by culture with doxycycline and was approx. 40% of that in cultured hepatocytes (Figure 1B).

Subcellular localization of glucokinase regulatory protein and glucokinase in INS-GCKR18 cells

Having established the virtual absence of glucokinase regulatory protein from INS-GCKR18 cells, and the ability of the cells to accumulate this protein under doxycycline induction, we analysed the cellular localization of the doxycycline-induced protein by indirect immunofluorescence. As expected from the results in Figure 1(A), the regulatory protein was undetectable in INS-GCKR18 cells cultured without doxycycline (Figure 2A) and also in INS-r3 cells cultured without or with doxycycline (results not shown). In contrast, the regulatory protein became readily

Figure 1 Levels of glucokinase regulatory protein (A) and glucokinase (B) in engineered insulinoma cells and hepatocytes

Primary rat hepatocytes maintained in cell culture for 5 h (hep) were used to provide protein samples as reference standards for the quantification of the glucokinase regulatory protein (GCKR) and glucokinase (GCK) in insulinoma cells. Protein samples from INS-GCKR18 cells (GCKR18) and INS-r3 (r3) were prepared after 42 h of culture in doxycycline-free medium (—) or in the presence of doxycycline at the indicated concentrations (dox). Samples were subjected to SDS/PAGE and Western transfer, followed by immunoblotting with a rabbit antiserum against the glucokinase regulatory protein from rat liver (A) or a sheep antiserum against glucokinase from rat liver (B). Primary antibodies were detected with peroxidase conjugates of antibodies against rabbit and sheep immunoglobulins respectively. Blots were revealed by enhanced chemiluminescence. Images of X-ray films were captured with a CCD camera. The positions of molecular mass markers separated in side lanes are indicated (in kDa) at the right.

discernible in approx. 70% of INS-GCKR18 cells incubated for 42 h with a maximal concentration of doxycycline (Figure 2B). The intracellular localization was predominantly nuclear in all expressing cells. A cytoplasmic signal, in addition to nuclear immunofluorescence, was noted only in a minority of very strongly expressing cells.

Glucokinase was undetectable by immunofluorescence in untreated GCKR18 cells (Figure 2C). However, after culture in presence of doxycycline, the enzyme became apparent in more than 35% of the cells, and it was localized exclusively to the nucleus (Figure 2D). Treatment with doxycycline did not produce the appearance of nuclear glucokinase immunofluorescence in parental INS-r3 cells (results not shown). Moreover, treatment

Figure 2 Immunolocalization of glucokinase regulatory protein and glucokinase in INS-GCKR18 cells and rat hepatocytes

Insulinoma INS-GCKR18 cells (**A–D**) were cultured on polyornithine-coated glass coverslips in the absence (**A, C**) or presence (**B, D**) of 0.6 μ g/ml doxycycline for 42 h (**E, F**) Rat hepatocytes in primary culture for 22 h in medium containing 5 mM glucose were incubated for 60 min in the presence of 0.5 mM fructose (**E**) or were left in basal medium without addition (**F**). Cells were processed for indirect immunofluorescence and counterstained with Evans Blue as described in the Experimental section. Indirect immunofluorescence was performed with rabbit antiserum against the glucokinase regulatory protein (**A, B**) or affinity-purified sheep antibodies against glucokinase (**C, D; E, F**). Detection of the primary antibodies was with FITC conjugates of antibodies against rabbit and sheep immunoglobulins respectively. Confocal images were acquired as described in the Experimental section and converted to greyscales with Adobe Photoshop. The bright red fluorescence of cell bodies due to staining with Evans Blue was attenuated and appears as a dark grey background in all panels. Specific FITC labelling shows as white. Scale bar, 15 μ m.

with doxycycline did not cause a net increase in the amount of glucokinase in INS-GCKR18 cells (Figure 1B). A plausible interpretation for the nuclear accumulation of glucokinase seen in doxycycline-treated INS-GCKR18 cells was therefore that it reflected the translocation of glucokinase from the cytoplasmic compartment (where the enzyme would presumably escape detection) to the nucleus (where it would become readily detectable). This interpretation is supported by the fact that glucokinase was not detected by the present immunofluorescence procedure in hepatocytes challenged with fructose for 60 min (Figure 2E), a condition that has been reported to elicit the export of glucokinase from the nucleus to the cytoplasm [10]. In contrast, glucokinase was readily detected in the nucleus of hepatocytes cultured in medium without fructose (Figure 2F).

Expression and cellular localization of glucokinase regulatory protein and glucokinase in transfected 293T cells

The glucokinase regulatory protein was ectopically expressed in 293T cells after transient transfection. As shown by immunoblot analysis, the specific level of regulatory protein relative to total cell protein in 293T cells 42 h after transfection was approximately equivalent to 30 % of the level present in rat hepatocytes (Figure 3A). No immunoreactive protein was seen in cells transfected with vector devoid of cDNA insert.

Transfection of vectors encoding glucokinase, glucokinase tagged with a Myc epitope, or a mutant form of Myc-tagged glucokinase (see below), resulted in massive accumulation of the corresponding proteins. All three proteins accumulated to very similar levels. The specific level of glucokinase, relative to total soluble cellular protein, in 293T cells transfected under standard conditions was approx. 50-fold that in cultured hepatocytes (Figure 3B). The amount of glucokinase in cells could be fine-tuned over a very broad range by varying the amount of expression plasmid used for transfection (Figure 3C). Cells transfected with the 'empty' vector did not exhibit any glucokinase-immunoreactive protein.

The glucokinase regulatory protein and glucokinase were immunolocalized in 293T cells after transfection with plasmids coding for either protein. To achieve a stoichiometric relationship between the two proteins approaching that of liver cells, glucokinase expression plasmids encoding Myc-tagged or untagged glucokinases were transfected at a 20–40-fold lower input than the regulatory protein plasmid. When expressed singly, the regulatory protein displayed a predominantly cytoplasmic localization (Figures 4A and 4E). Similarly, glucokinase was mostly cytoplasmic or diffuse throughout the cell when expressed alone (Figure 4B, Myc-tagged glucokinase; Figure 4F, untagged glucokinase). In contrast, the regulatory protein expressed together with Myc-tagged or untagged glucokinase accumulated predominantly in nuclei (Figures 4C and 4G respectively). In the same cells, glucokinase, with (Figure 4D) or without tag (Figure 4H), localized predominantly to the nuclei.

The intracellular distribution of the two proteins was assessed by visual analysis of at least 100 cells in each of the transfection conditions shown in Figure 4. The cells were scored into three qualitative classes with respect to the relative intensities of the immunofluorescence signal in cytoplasm (C) and nucleus (N): predominantly cytoplasmic (C > N), evenly distributed between cytoplasm and nucleus (C = N), and predominantly nuclear (N > C). Scores for the regulatory protein alone, or on co-expression with glucokinase, were as follows: C > N, 61 % on single expression, compared with 22 % on co-expression; C = N, 38 % on single expression, compared with 18 % on co-expression; N > C, 1 % on single expression, compared with 60 % on co-expression. A similar shift of the regulatory protein towards the nuclear compartment occurred with Myc-tagged glucokinase. Glucokinase, transfected singly or together with the regulatory protein, was localized as follows: C > N, 62 % on single expression, compared with 13 % on co-expression; C = N, 38 % on single expression, compared with 18 % on co-expression; N > C, 0 % on single expression, compared with 69 % on co-expression. Essentially identical results were obtained with Myc-tagged glucokinase.

Catalytic activity of glucokinase is not required for nuclear translocation

Metabolic changes associated with the enzymic activity of glucokinase could conceivably have a role in the nuclear accumulation of the glucokinase regulatory protein and gluco-

Figure 3 Expression of glucokinase regulatory protein (A) and glucokinase (B, C) in transiently transfected 293T cells

Protein samples from cultured rat hepatocytes (Hepa) were used as reference standards for comparison with samples from transfected 293T cells. Transfection of 293T cells was done as described in the Experimental section with plasmids encoding the following proteins: glucokinase regulatory protein (GCKR), glucokinase (GCK), Myc-tagged glucokinase (M-GCK) and Myc-tagged N204D glucokinase (M-GCKm1). A vector without cDNA insert (vect) was used as control. The amounts of plasmids transfected were 3.6 µg in (A) and 5 µg in (B). For the experiment in (C) the amounts of plasmid encoding glucokinase indicated in the figure were complemented with empty vector so that the total amount of plasmid transfected was 5 µg in all transfections. Electrophoresis, transfer to nitrocellulose and immunoblotting were performed as described in the legend to Figure 1. The protein input (µg prot) is indicated above each lane. The positions of molecular mass markers are indicated (in kDa) at the right.

kinase. This issue was addressed by using a mutant glucokinase harbouring a mis-sense mutation known to inactivate the enzyme, the N204D mutation [19]. Residue Asn-204 of glucokinase is thought to reside in the active site of the enzyme and to participate in glucose binding [19,20]. The N204D mutation was introduced by site-directed mutagenesis in Myc-tagged glucokinase. 'Wild-

Figure 4 Immunolocalization of glucokinase regulatory protein and glucokinase expressed alone or together in transfected 293T cells

(A, E) Cells expressing the glucokinase regulatory protein alone. (B, F) Cells expressing Myc-tagged glucokinase (B) or untagged glucokinase alone (F). (C, D) Cells expressing the regulatory protein together with Myc-tagged glucokinase. (G, H) Cells expressing the regulatory protein together with untagged glucokinase. In all cases, transfections were performed with 4 µg of plasmid coding for the glucokinase regulatory protein and 0.2 µg of plasmid coding for glucokinase. The cells on coated glass coverslips were processed for immunofluorescence 22 h after transfection. Antisera against the regulatory protein and glucokinase were the same as in Figure 3; Myc-tagged glucokinase was revealed with monoclonal antibodies against the Myc tag. For detection of the regulatory protein (A, E, C, G), rhodamine-labelled antibodies against rabbit immunoglobulins were used. For the detection of Myc-tagged glucokinase (B, D) or glucokinase (F, H), FITC-labelled antibodies against mouse or sheep immunoglobulins respectively were used. Images were acquired with a Axiophot photomicroscope fitted with a Photonic Coolview colour camera and converted to greyscales with Photoshop software as described in the Experimental section. Scale bar, 10 µm.

type' and mutant Myc-tagged glucokinases were expressed in 293T cells; hexokinase activity was assayed in cell lysates. The data in Table 1 (the three columns at the left) show that 'high- K_m ' hexokinase activity was markedly increased in 293T cells transfected with the Myc-tagged wild-type glucokinase plasmid (M-GCK), in comparison with transfection with the empty vector. In contrast, 'high- K_m ' activity was unchanged in cells transfected with the N204D mutant (M-GCKm1), indicating that this mutant had lost all hexokinase activity. As shown previously, the levels of expression of the N204D mutant and wild-type glucokinase were similar (Figure 3B). In addition,

Table 1 Nuclear translocation of glucokinase regulatory protein co-expressed with wild-type or 'kinase-dead' glucokinases in 293T cells

For the measurement of hexokinase activity, transfection was done with 4 μ g of the plasmids indicated in the column at the left. 'Vector' denotes plasmid pcDNA3.1/Zeo(+) without insert. Soluble protein extracts were prepared at 24 h after transfection. The hexokinase assay was performed as mentioned in the Experimental section. Results are means $\pm$ S.E.M. for samples from three separate transfections. For immunolocalization of the glucokinase regulatory protein, cells on polyornithine-coated glass coverslips were co-transfected with 2 μ g of plasmid pGCKR plus 2 μ g of the plasmid specified in the column at the left. The cells were processed for immunofluorescence 24 h after transfection. The experiment was performed twice. A total of at least 100 cells for each experimental condition were analysed under the microscope and assigned to one of three qualitative categories as indicated. Scores in the two experiments varied by less than 20% and are shown as means of two independent values.

Plasmid	Hexokinase activity (m-units/mg of protein)		Localization of regulatory protein (% of cells)		
	'Low- K_m '	'High- K_m '	C > N	C = N	N > C
Vector	29 $\pm$ 5	12 $\pm$ 2	49	49.5	1.5
pM-GCK	53 $\pm$ 4	986 $\pm$ 216	0	17.5	82.5
pM-GCKm1	26 $\pm$ 4	15 $\pm$ 3	5	15	80

Table 2 Effect of leptomycin B on the intracellular distribution of glucokinase regulatory protein in 293T cells

Cells on polyornithine-coated glass coverslips in six-well culture dishes were transfected with 2 μ g of plasmid encoding the glucokinase regulatory protein. Cells were harvested for immunolocalization of the regulatory protein at 22 h after transfection, following culture in the presence of 40 nM leptomycin B for the specified durations before harvest. The solvent DMSO was added to cells cultured without drug. The experiment was performed twice and at least 100 cells for each experimental condition were scored in each experiment. Results were similar for the two experiments and are shown as means of two independent values.

Drug	Duration of action (min)	Localization of regulatory protein (% of cells)		
		C > N	C = N	N > C
None	—	59	36	5
Leptomycin B	75	36	41	23
Leptomycin B	240	29	30	41

Myc-tagged glucokinase was enzymically as active as untagged glucokinase (results not shown).

The intracellular localization of the glucokinase regulatory protein was investigated in 293T cells after co-transfection with plasmids coding for the wild-type and mutant glucokinases (Table 1, the three columns at the right). In cells co-transfected with the regulatory protein plasmid and an empty vector, the regulatory protein was localized predominantly in the cytoplasm or diffusely throughout the cell. As expected, nuclear translocation of the regulatory protein occurred during co-expression with enzymically active glucokinase. Translocation of the regulatory protein was equally effective in presence of 'hexokinase-dead' glucokinase.

Translocation of glucokinase regulatory protein into nuclei in 293T cells treated with leptomycin B

Leptomycin B inhibits the exportin Crm1 [21] and thereby blocks the export of proteins containing nuclear export signal (NES) from the nucleus [22,23]. The intracellular localization of the glucokinase regulatory protein in singly transfected 293T cells was investigated during treatment of the cells with leptomycin B. As shown in Table 2, a shift of the regulatory protein towards the nuclear compartment occurred during treatment with lepto-

mycin. The effect was noticeable 75 min after addition of the drug and became more pronounced after 4 h.

DISCUSSION

The engineered INS-GCKR18 insulinoma cells provided a useful system for examining the role of the glucokinase regulatory protein as a determinant for the intracellular localization of glucokinase. Under basal culture conditions, INS-GCKR18 cells expressed glucokinase in the absence of the glucokinase regulatory protein. However, the regulatory protein could be acutely induced by the addition of doxycycline to the medium. The newly induced regulatory protein localized predominantly to the cell nucleus. The induction of the regulatory protein was accompanied by the appearance of glucokinase in the nucleus, whereas glucokinase was undetectable in the nucleus of uninduced cells. Because there was no net increase in glucokinase during induction, the findings suggested that the enzyme was redistributed from an extranuclear to an intranuclear location. However, the actual translocation of glucokinase from cytoplasmic sites to the nucleus could not be detected because glucokinase escaped detection in uninduced cells. The failure of the present immunofluorescence procedure to detect glucokinase in uninduced INS-GCKR18 cells, at what we presume to be a cytoplasmic location, might result from a relatively low level of enzyme, the loss of freely diffusible antigen during fixing or antigen-antibody reactions, or other technical limitations. This interpretation is supported by the fact that glucokinase was barely detectable in hepatocytes cultured with fructose, a condition in which glucokinase was found by others to be mostly cytoplasmic [10]. In contrast, glucokinase was readily identified in the nuclei of hepatocytes cultured in basal medium, in agreement with published reports [10,11]. The findings in INS-GCKR18 cells, in conjunction with the parallel observations in hepatocytes, strongly suggest that induction of the glucokinase regulatory protein *de novo* in insulinoma cells caused a translocation of endogenous glucokinase from cytoplasm to nucleus.

Interestingly, glucokinase does not seem to localize to the nucleus in normal islet β -cells [24–26], a finding in agreement with the reported absence of glucokinase regulatory protein from this cell type. Whether a binding protein distinct from the liver-type regulatory protein [27] might contribute to the cytoplasmic retention of glucokinase in the β -cell should be investigated further.

In human embryonic kidney 293T cells, glucokinase localized primarily to the cytoplasm when expressed alone, and was translocated to the nucleus when expressed together with the regulatory protein. Similarly, the glucokinase regulatory protein localized predominantly to the cytoplasm or diffusely throughout the cell in the absence of glucokinase, and was mostly translocated to the nucleus in the presence of glucokinase. Thus glucokinase and the regulatory protein showed a mutual dependence for import and residence in the nucleus. The simplest explanation for these findings is that a physical association of the two proteins is necessary for their translocation through the nuclear pore complex and their retention in the nucleus. Consistent with this hypothesis was the observation that when glucokinase was expressed in vast excess over the glucokinase regulatory protein (taking the ratio of the two proteins found in hepatocytes as benchmark), glucokinase remained mostly cytoplasmic (results not shown). The enzymic activity of glucokinase was dispensable for causing translocation, as shown by the effectiveness of the 'kinase-dead' N204D mutant of glucokinase. This argues against the possibility that translocation might be a consequence of a metabolic change associated with glucokinase expression. Although N204D glucokinase is known to have a decreased affinity for the regulatory protein [20], its ability to elicit nuclear import does not contradict the protein-protein interaction hypothesis because the mutant was purposefully overexpressed to very high levels in these experiments.

Many proteins contain both nuclear localization signals (NLSs) and NES, and shuttle between cytoplasm and nucleus [28]. The results of the experiments with leptomycin B are consistent with the idea that the glucokinase regulatory protein might be a shuttling protein. By blocking the function of a putative NES in the regulatory protein, leptomycin B would tilt the balance between export and import in favour of import, resulting in a redistribution of the regulatory protein towards the nucleus. The inactivation or masking of a putative NES during the formation of a complex with glucokinase in the cytoplasm might similarly account for nuclear translocation of the complex. Such a mechanism has been described in molecular details for the complex between the homeodomain proteins Extradenticle and Homothorax in *Drosophila* [29]. A related mechanism has been reported for nuclear import of the protein complex between the clock components Period and Timeless in *Drosophila*, although in this case complex formation results in the masking of a cytoplasmic retention domain, rather than of NES, in Period [30]. To establish a similar model for mammalian glucokinase and its regulatory protein would require the identification in one or both proteins of functional NESs, NLSs or other intracellular localization domains. A first step in this direction has been made by Shiota et al. [31], who reported recently that amino acid sequence 300–310 of glucokinase seems to function as an NES in a fusion protein of glucokinase with green fluorescent protein. In agreement with results in the present paper, Shiota et al. also showed that the glucokinase regulatory protein was present in cytoplasm and nucleus when expressed alone in HeLa cells and was mostly translocated to the nucleus during the co-expression of glucokinase.

A green-fluorescent-protein-tagged version of the glucokinase regulatory protein has been reported to localize mostly to the nucleus in COS-7 cells, regardless of the presence or absence of glucokinase [32]. Although the predominantly nuclear localization of the chimaeric regulatory protein expressed alone in COS-7 cells is at variance with the present findings in 293T cells, it is not inconsistent with shuttling of the protein between nucleus and cytoplasm. It is conceivable that the balance between nuclear import and export might vary between cell types

and/or according to the stage of the cell cycle, the energy status or other factors.

A final question pertains to the reason for the nuclear import of the regulatory protein and glucokinase. It is intriguing to ask whether the regulatory-protein-glucokinase complex moves to the nucleus only for the purpose of inactivating glucokinase metabolically, or whether the purpose is also the regulation of as yet unidentified nuclear processes.

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