Evidence for glucose and sorbitol-induced nuclear export of glucokinase regulatory protein in hepatocytes

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Abstract Glucokinase is rapidly exported from the nucleus of hepatocytes in response to a rise in glucose or fructose 1-P. We demonstrate using confocal microscopy and quantitative imaging that in contrast to previous findings, the regulatory protein of glucokinase (GKRP) also translocates from the nucleus during substrate-induced translocation of glucokinase. However, the fractional decrease in nuclear GKRP is smaller than for glucokinase and is determined by the metabolic state and not by the distribution of glucokinase. Translocation of glucokinase and GKRP is not inhibited by leptomycin B, an inhibitor of exportin-1 function. These findings highlight the importance of quantitative imaging for determining nuclear export of proteins and suggest that GKRP may have a role in nuclear export or import of glucokinase.

Key words: Glucokinase; Regulatory protein; Nuclear export; Hepatocyte

1. Introduction

Glucokinase is the predominant hexokinase in liver of most mammals [1]. It is co-expressed with a 68 kDa regulatory protein (glucokinase regulatory protein (GKRP)) which binds glucokinase and inhibits competitively with respect to glucose [2,3]. Studies in vivo and in vitro have shown that GKRP is present predominantly in the nucleus of hepatocytes [4–7], whereas glucokinase translocates between the nucleus and cytoplasm [5–9]. This translocation has been studied by a digitonin release assay, which measures the distribution of enzyme between free and bound states [10–13], and by immunostaining [5–9]. Studies on the perfused rat liver reported translocation from the nucleus of both glucokinase [8] and GKRP [4] at high glucose. However, studies on hepatocytes showed that glucokinase but not GKRP translocates during incubation with fructose or high glucose [5,6,9]. The current consensus is that GKRP acts as a nuclear retention factor to bind glucokinase in the nucleus in conditions that favour binding of glucokinase to GKRP and that conditions that dissociate glucokinase from GKRP (high glucose or fructose 1-P) cause translocation of glucokinase but not GKRP to the cytoplasm [5,6,9,12].

Using confocal fluorescence microscopy and quantitative imaging, we demonstrate that GKRP is exported from the nucleus during incubation with substrates that cause translocation of glucokinase. However, the fractional changes in translocation of GKRP are smaller than for glucokinase. This suggests that in addition to a role in nuclear retention of glucokinase, GKRP may also be involved in nuclear export or import of glucokinase.

2. Materials and methods

2.1. Materials

Antibodies against GKRP (rabbit) [14] and GST-glucokinase fusion protein (sheep) [15] were kind gifts from Drs E. Van Schaftingen and M. Magnuson, respectively. Leptomycin B was a generous gift from Novartis (Vienna, Austria).

2.2. Hepatocyte incubations

Hepatocytes were isolated from male Wistar rats (body weight 200–250 g) [16] and suspended in MEM containing 7% newborn calf serum. For immunostaining, they were seeded on 13 mm coverslips coated with 0.1% gelatin and for glucokinase determination on 24 well plates or on coverslips. After cell attachment ($\sim$4 h), the medium was replaced by serum-free MEM containing 10 nM dexamethasone and the cells were cultured for 16 h [16]. For overexpression of glucokinase, after cell attachment, hepatocytes were incubated for 2 h with appropriate titres of recombinant adenovirus encoding rat liver glucokinase cDNA [16] and then cultured for 16 h. Unless indicated otherwise, the medium contained 5 mM glucose. For determination of effects of substrates on translocation, hepatocytes were incubated for 30 min either in MEM (controls) or in MEM containing 25 mM glucose (G) and/or 200 μM sorbitol (S). For reversal of translocation (from cytoplasm to nucleus), they were incubated for 30 min in MEM with 25 mM glucose+200 μM sorbitol and were then washed and incubated for 1 h in control medium (GS-Rev). Free and bound glucokinase activities were determined by a digitonin release assay and free activity is expressed as a percentage of total activity [16].

2.3. Fixing and immunostaining

Coverslips were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS (30 min). They were treated with sodium borohydride (1 mg/ml in PBS, 10 min), 0.2% Triton X-100/PBS (10 min) and 0.2% Triton X-100/1% BSA/PBS (10 min) and incubated overnight at 4°C with primary antibodies (sheep anti-GST-glucokinase or rabbit anti-GKRP) in PBS/0.1% Triton X-100/1% BSA. Incubations with secondary antibodies (donkey anti-rabbit Texas red or FITC-labelled, donkey anti-sheep FITC-labelled, Jackson Immunoresearch) were for 1 h. They were washed in PBS, water and 100% ethanol and mounted with Mowiol containing 2.5% 1,4-diazabicyclo[2,2,2]octane. Appropriate controls were performed to confirm that there was no immunostaining due to the secondary antibodies or cross-reactivity of the secondary antibodies (against sheep versus rabbit IgG) during dual staining. For most experiments, GKRP translocation was determined from both dual staining for glucokinase (FITC-labelled secondary) and GKRP (Texas red-labelled secondary) and from single staining for GKRP (Texas red-labelled secondary) alone. In additional experiments, dual staining for glucokinase (and GKRP) was compared with single staining for glucokinase alone. This confirmed that GKRP did not interfere with glucokinase in dual staining.

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Abbreviations: GKRP, glucokinase regulatory protein

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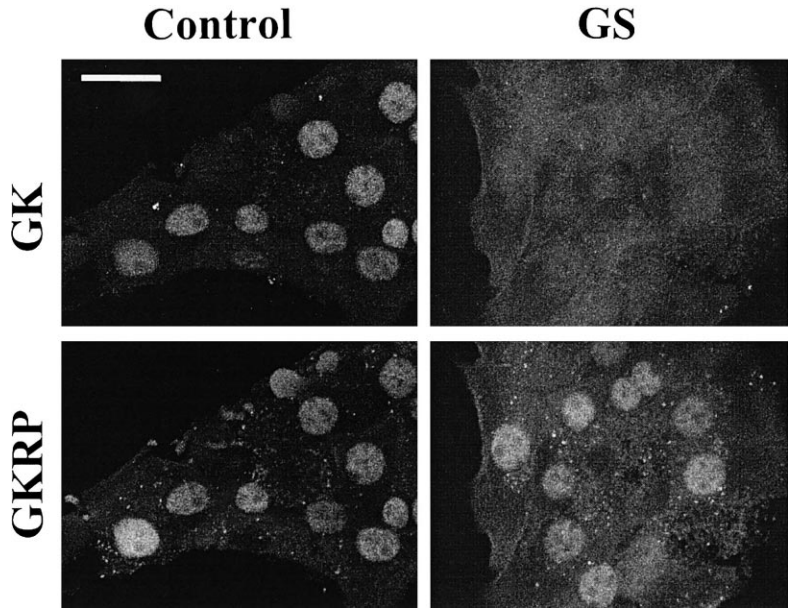

Fig. 1. Confocal images of hepatocytes dual-stained for glucokinase and GKRP. Hepatocytes were incubated for 30 min in medium containing either 5 mM glucose (control) or 25 mM glucose+200 μM sorbitol (GS) and dual-stained for glucokinase and GKRP. Scale bar, 25 μm.

2.4. Imaging

Images of stained cells were obtained using a Bio-Rad MRC-600 confocal laser scanning microscope with a 15 mW krypton–argon laser. FITC and Texas red fluorescence were imaged simultaneously using 488 and 568 nm excitation lines with appropriate filters before the photomultiplier tubes. Three or four representative fields were selected for each condition. A series of Z-sections were taken for each field with the signals from each fluorophore being recorded simultaneously. This gave a Z-section consisting of two eight bit grey-scale images of 384×512 pixels representing identical FITC and Texas red fields. The Z-sections for each field were merged using Bio-Rad CoMOS software to produce a 384 KB composite projection file. The confocal PIC images were converted to TIFF files using Confocal Assistant software (TC Brelje, Minneapolis, MN, USA) and analysed using ScionImage software (Scion, MD, USA). For measurements of nuclear intensity, the freehand selection tool was used to delineate individual nuclei. The analysis function gave the mean pixel intensity (MPI) for each nucleus. For cytoplasmic intensity, five random areas of cytoplasm were marked on each field and the MPI was determined. The MPI was expressed either as individual data per nucleus (Fig. 2) or as means ± S.E.M. for the number of nuclei or cytoplasmic areas indicated (Fig. 3). For comparisons of replicate experiments, the mean for the total number of nuclei or cytoplasmic areas was calculated for each experiment and results of replicate experiments were expressed as means ± S.E.M. (Figs. 4 and 5). Statistical analysis was determined by the paired *t*-test, except for comparisons of data sets within an experiment (Fig. 3) which was by the unpaired *t*-test.

3. Results

3.1. Intercellular heterogeneity of glucokinase and GKRP

Hepatocytes incubated with 5 mM glucose show intense nuclear staining for glucokinase and GKRP (Fig. 1A) as shown previously [5,6]. Incubation with 200 μM sorbitol and 25 mM glucose for 30 min (GS) causes clear translocation of glucokinase from the nucleus to the cytoplasm (Fig. 1B). However, GKRP staining is still more intense in the nucleus than the cytoplasm and visual inspection of the images by fluorescence or confocal microscopy did not allow for unequivocal conclusions as to whether GKRP translocates.

Intercellular heterogeneity of glucokinase expression has been demonstrated in vivo with a mosaic pattern of immunostaining intensity in both periportal and perivenous zones of the acinus [17]. Fig. 2A shows the intercellular variation in nuclear staining for glucokinase and GKRP (MPI for each nucleus). It is a representative experiment of 11 that are summarised in Table 1. There was a significant correlation ($P < 0.005$) between glucokinase and GKRP staining for control and GS-treated cells (Fig. 2A). The slope of the relation between glucokinase/GKRP (regression coefficient β) was 50% lower in GS-treated cells and this effect was reversed after

Table 1
Regression analysis for nuclear glucokinase and GKRP intensity

(n) (A/B)	A. Glucokinase/GKRP		B. GKRP/nuclear size	
	Regression coefficient (β)	Coefficient of correlation r (P)	Regression coefficient (β)	Coefficient of correlation r (P)
Control (11/5)	0.64 ± 0.09	0.80 ± 0.03 ($P < 0.005$)	−0.051 ± 0.01	0.35 ± 0.04 ($P < 0.05$)
GS-treated (11/5)	0.34 ± 0.09**	0.68 ± 0.06* ($P < 0.005$)	−0.018 ± 0.008*	0.12 ± 0.04**, not significant
GS-Rev (4)	0.64 ± 0.09	0.75 ± 0.09 ($P < 0.005$)		

Hepatocytes were incubated either in control medium or with 25 mM glucose+200 μM sorbitol (GS-treated) or after GS treatment, they were incubated in control medium for 1 h (GS-Rev). A: The relation between nuclear glucokinase (y) versus GKRP (x) (MPI for each nucleus) was determined by regression analysis ($GK = \alpha + \beta GKRP$). The regression coefficient (β) and coefficient of correlation r (P) are shown (see Fig. 2A). B: The relation between nuclear GKRP (y) versus nuclear size (x) was similarly determined (see Fig. 2B). Results are means ± S.E.M. for the numbers of experiments shown in parentheses.
*$P < 0.05$, GS-treated versus control.
**$P < 0.005$, GS-treated versus control.

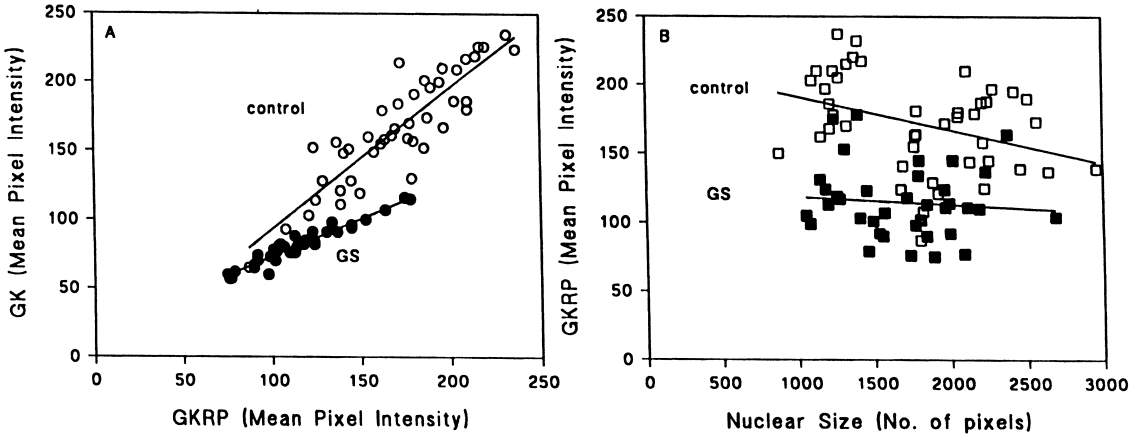

Fig. 2. Intercellular variation in nuclear glucokinase and GKRP. Experimental details were as in Fig. 1. Open symbols represent the control incubation and solid symbols the incubation with 25 mM glucose+200 μM sorbitol (GS). A: Relation between nuclear glucokinase and GKRP (MPI for each nucleus). B: Relation between nuclear GKRP (MPI for each nucleus) and nuclear size. Results are a representative experiment out of 11 (A) or five (B) that are summarised in Table 1.

removal of substrates (GS-Rev, Table 1). When the intercellular variation in GKRP staining (MPI per nucleus) was compared with the nuclear size, there was a small but significant inverse correlation ($P < 0.05$) in control but not in GS-treated cells (Fig. 2B), suggesting that smaller nuclei have a higher GKRP content per unit volume in control incubations (Table 1).

3.2. Glucose and sorbitol cause translocation of GKRP from the nucleus to the cytoplasm

The large intercellular variation in glucokinase and GKRP (Fig. 2A) makes it necessary to analyse a large number of nuclei to detect small differences. Fig. 3 shows the results of an experiment where 60 nuclei were imaged for each substrate incubation and staining condition. Hepatocytes were incubated for 30 min without or with 200 μM sorbitol (S) and/or 25 mM glucose (GS, G) or after 60 min reversal of GS-induced translocation (GS-Rev). They were dual-stained for glucokinase (FITC) and GKRP (Texas red) or single-stained for GKRP with either FITC or Texas red label. For GKRP imaging, similar results were obtained for the three staining conditions and the results of single staining with FITC (Fig. 3B) or Texas red (Fig. 3C) are shown. Incubation with 25 mM glucose and/or 200 μM sorbitol significantly decreased nuclear staining and increased cytoplasmic staining for glucokinase (Fig. 3A) and GKRP (Fig. 3B,C). The effects of GS treatment were, at least in part, reversed after 1 h in control medium (GS-Rev). The confidence intervals for the nuclear differences (control-GS-treated) are shown in Table 2.

Fig. 4 summarises data for nuclear fluorescence and nuclear/cytoplasmic fluorescence ratios for replicate experiments. In GS-treated cells, the fractional decrease in nuclear staining (Fig. 4A) was greater for glucokinase than for GKRP ($41 \pm 5\%$ decrease versus $30 \pm 7\%$, $P < 0.05$) and the changes in nuclear/cytoplasmic fluorescence ratios between control and GS treatment were also larger for glucokinase (Fig. 4B). The changes in nuclear/cytoplasmic ratios for GKRP were similar when determined by single staining for GKRP alone as compared with dual staining for GKRP and glucokinase, suggesting that there is no interference due to glucokinase during dual labelling.

3.3. Relation between fluorescence and protein content

If the relation between fluorescence and protein content was linear, it would be expected that during protein translocation from the nucleus to the cytoplasm, the ratio of the decrease in nuclear fluorescence (control-GS)/increase in cytoplasmic fluorescence (GS-control) would approximate the ratio of cytoplasmic volume/nuclear volume. This ratio for hepatocytes is about nine [18]. For the experiment in Fig. 3, the gain function of the confocal microscope (pixel value/light intensity) was adjusted to maximum sensitivity so as to improve detection of cytoplasmic changes in fluorescence. In these conditions, the decrease in nuclear fluorescence was approximately equal to the increase in cytoplasmic fluorescence, suggesting non-linearity of nuclear fluorescence relative to protein content. In experiments where the gain function was adjusted to lower sensitivity to minimise non-linearity of nuclear fluorescence, the fractional decrease in nuclear fluorescence by GS treatment was increased slightly ($47 \pm 5\%$ compared with $34 \pm 5\%$) and the ratio of changes in nuclear fluorescence/cytoplasmic fluorescence was around five. Fig. 4A summarises the fractional decrease in nuclear fluorescence for all experiments (maximum and low sensitivity). However, the nuclear/cytoplasmic ratios (Fig. 4B) could only be reliably determined for images recorded at high gain function because when this was adjusted to low sensitivity, the basal cytoplasmic fluorescence was low, resulting in large errors in nuclear/cytoplasmic

Table 2
Confidence intervals (CI) for difference between control and GS treatment for nuclear intensity data in Fig. 3

	No. of nuclei	Control mean ± S.E.M.	GS-treated mean ± S.E.M.	P	95% CI for Diff
GK-FITC (Fig. 3A)	60	208 ± 3.3	144 ± 3.7	< 0.0001	54.7–74.2
GKRP-FITC (Fig. 3B)	60	149 ± 2.9	134 ± 2.6	< 0.0001	7.4–22.6
GKRP-TR (Fig. 3C)	60	38.1 ± 1.1	31.5 ± 0.7	< 0.0001	3.9–9.2

ratios. The results together suggest that the relation between nuclear fluorescence and protein content is non-linear, such that the fractional decrease in nuclear fluorescence with GS treatment underestimates the fractional translocation of protein. We assume that a similar deviation from linearity applies for glucokinase and GKRP at comparable sensitivity.

The only subjective factor in this analysis is the 'marking' of the nuclear circumference. To exclude the possibility that differences in MPI between control and GS-treated cells may be due to differences in marking of nuclear size as a result of differences in fluorescence intensity or distribution at the nucleus periphery, we compared the nuclear size data for control and GS treatment. There was no difference in nuclear size (control 294 ± 12, GS-treated 287 ± 15, $n = 13$).

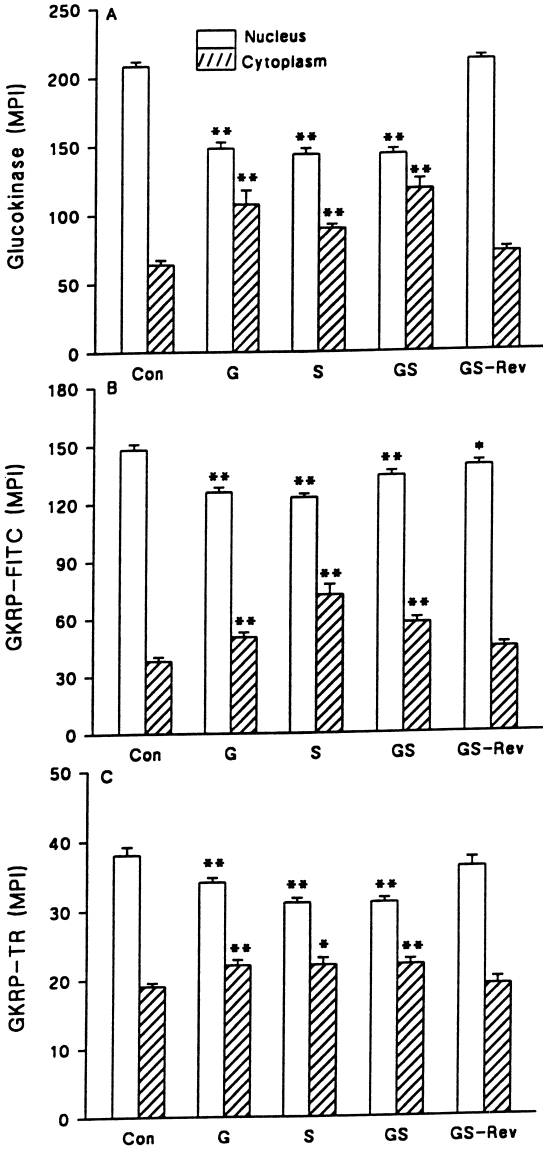

Fig. 3. Substrate-induced translocation and reversal of glucokinase and GKRP determined from nuclear and cytoplasmic fluorescence. Hepatocytes were incubated either in control medium (Con) or in medium with 25 mM glucose (G) or 200 μM sorbitol (S) or 25 mM glucose+200 μM sorbitol (GS) for 30 min or they were pre-incubated with 25 mM glucose+200 μM sorbitol for 30 min and then washed and incubated in control medium for 1 h (GS-Rev). They were either dual-stained (A) for glucokinase (FITC) and GKRP (Texas red) or stained for GKRP alone with either FITC (B) or Texas red (C) label. Results represent the MPI for either nuclear staining (open bars) or cytoplasmic staining (hatched bars) for glucokinase (A) or GKRP (B and C). Values are means ± S.E.M. for 60 nuclei or 15 cytoplasmic areas. Statistical analysis: $^{*}P < 0.05$, $^{**}P < 0.005$ relative to controls (for confidence intervals of nuclear values, see Table 2).

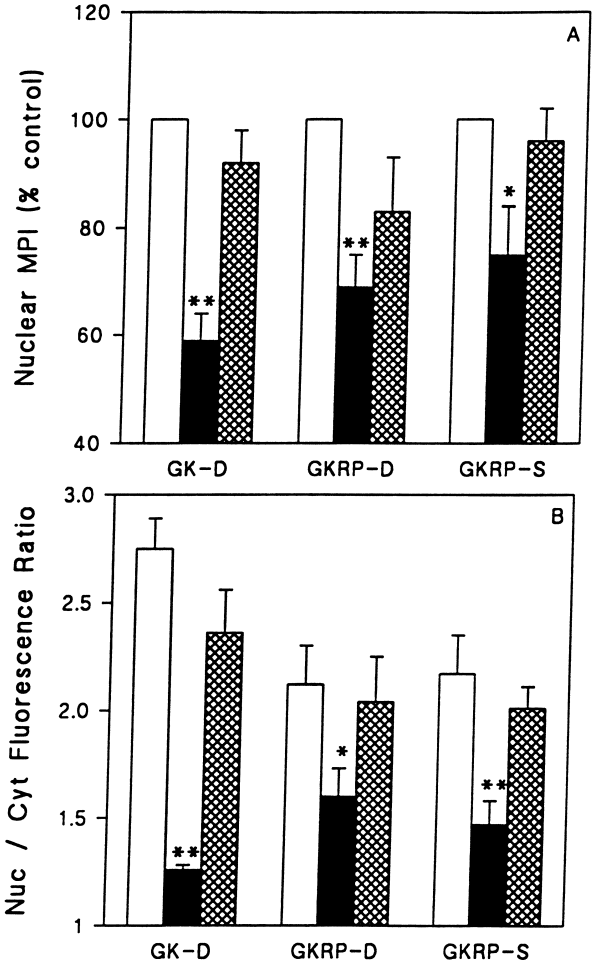

Fig. 4. Substrate-induced changes in nuclear fluorescence and nuclear/cytoplasmic fluorescence ratios. Incubation conditions: control (□), GS (■) and GS-Rev (ı) were as in Fig. 3. The cells were either dual-stained for glucokinase (FITC) and GKRP (Texas red) (GK-D or GKRP-D, respectively) or single-stained for GKRP (GKRP-S) with Texas red. Cytoplasmic and nuclear glucokinase and GKRP were determined for each experiment as in Fig. 3. A: Nuclear MPI expressed as % of control. B: Ratio of nuclear/cytoplasmic (Nuc/cyt) intensity. Means ± S.E.M. for either nine (A) or six (B) experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ relative to controls.

3.4. Effects of glucokinase overexpression

To investigate whether the distribution of GKRP between the nucleus and cytoplasm follows and/or is consequent to the distribution of glucokinase, we overexpressed glucokinase in hepatocytes by 2-fold and 3.5-fold above endogenous levels using recombinant adenovirus. This results in a marked decrease in the nuclear/cytoplasmic distribution ratio for glucokinase (Fig. 5). The gradient of GKRP between the nucleus and cytoplasm was unaffected (Fig. 5), indicating that it is not determined by the nuclear/cytoplasmic ratio of glucokinase.

3.5. Comparison with the digitonin release assay

The digitonin release assay measures the distribution of

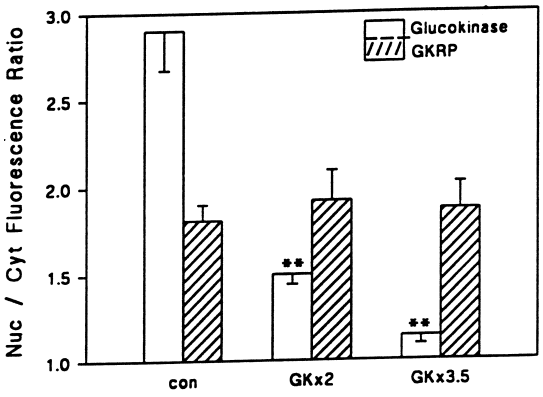

Fig. 5. Glucokinase overexpression does not decrease the nuclear/cytoplasmic ratio of GKRP. Glucokinase was overexpressed in hepatocytes by 2-fold (204 ± 21%) and 3.5-fold (353 ± 64%) relative to endogenous levels (con) and the nuclear/cytoplasmic ratios for glucokinase (open bars) and GKRP (hatched bars) were determined by dual staining after incubation of hepatocytes in medium with 5 mM glucose. Means ± S.E.M. for eight experiments. $^{**}P < 0.005$ relative to con.

glucokinase between free and bound states [10–13]. The latter most likely represents glucokinase bound to GKRP because incubation with glucose and sorbitol alters the distribution of glucokinase but not GKRP between free and bound states [12]. Fig. 6A shows that GS treatment increases the proportion of free glucokinase by 50% (31 ± 2% to 80 ± 2%) and this effect was partially reversed (80%) after 1 h in control medium, similar to the reversal of translocation determined by immunofluorescence (Fig. 4A,B).

3.6. Leptomycin B does not inhibit translocation

Previous studies showed that nuclear export of various proteins including protein kinases and G-actin involves interaction with exportin-1 [19–21] and is inhibited by leptomycin B, a fungal metabolite that blocks interaction with exportin-1 [22,23]. Exportin-1 (chromosome region maintenance-1) is localised in the nuclear pore complex and in the nucleoplasm and shuttles between the nucleus and cytoplasm. Leptomycin B did not inhibit translocation of glucokinase by sorbitol (Fig. 6B) and it did not inhibit nuclear export of glucokinase or GKRP determined by immunostaining (results not shown).

4. Discussion

This study demonstrates using confocal fluorescence microscopy and quantitative imaging that GKRP translocates from the nucleus to the cytoplasm during incubation of hepatocytes with elevated concentrations of glucose and sorbitol and this effect is, at least in part, reversible on removal of these substrates. However, the fractional changes in translocation of GKRP are smaller than for glucokinase. This may explain why previous studies using confocal fluorescence microscopy and the same or a different primary antibody [6,7] failed to observe translocation of GKRP during translocation of glucokinase. Two reasons why GKRP translocation is less apparent than for glucokinase are that the residual nuclear/cytoplasmic ratio during incubation with glucose and sorbitol is significantly higher for GKRP than for glucokinase and the changes in nuclear/cytoplasmic ratios are smaller for GKRP. The intercellular heterogeneity in GKRP expression makes it necessary to analyse a large number of nuclei in order to detect small differences in nuclear staining for GKRP. Although glucokinase shows a similar intercellular heterogeneity as GKRP as shown by the strong correlation between glucokinase and GKRP nuclear staining, its translocation from the nucleus during substrate stimulation is almost complete and thus clearly evident by qualitative imaging.

The conclusion that GKRP translocates between the nucleus and the cytoplasm in response to substrate stimulation is supported by the following evidence. First, incubation with glucose and sorbitol decreased both the nuclear fluorescence and the nuclear/cytoplasmic fluorescence ratio for GKRP and these changes were reversed following substrate removal. Similar changes in nuclear/cytoplasmic GKRP ratios were observed during single staining for GKRP alone as compared with dual staining for GKRP and glucokinase simultaneously, thereby ruling out possible artifacts resulting from dual staining. Second, there was a significant inverse correlation between nuclear staining for GKRP and nuclear size in cells incubated in control medium but not after incubation with

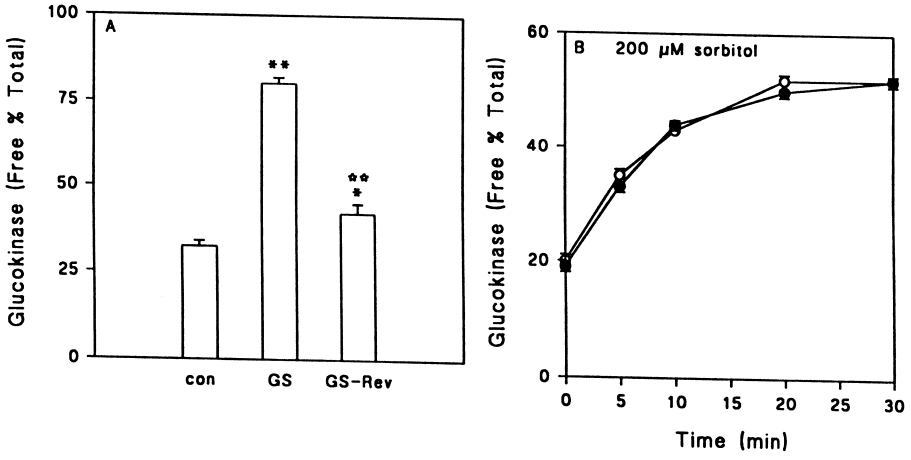

Fig. 6. Free and bound glucokinase activity. A: Incubation conditions (control, GS and GS-Rev) were as in Fig. 3. B: Time course of effects of 200 μM sorbitol in the absence (○) or presence (●) of 50 nM leptomycin B. Free and bound glucokinase activity was determined by a digitonin release assay and free activity is expressed as a percentage of total activity (16.4 ± 2.4 mU/mg). Means ± S.E.M. for seven (A) or four (B) experiments. $^{*}P < 0.05$; $^{**}P < 0.005$ relative to control; ☆☆, $P < 0.005$ relative to GS-treated.

glucose and sorbitol. Third, dissipation of the nuclear/cytoplasmic gradient of glucokinase by protein overexpression was not associated with a change in distribution of GKRP between nucleus and cytoplasm, indicating that translocation of GKRP during incubation with glucose and sorbitol cannot be explained simply by the increased distribution of glucokinase in the cytoplasm. The smaller fractional translocation of GKRP compared with glucokinase is supported by the smaller decrease in nuclear staining and nuclear/cytoplasmic fluorescence ratios for GKRP compared with glucokinase and the lower regression coefficient of glucokinase/GKRP in incubations with glucose and sorbitol.

GKRP has hitherto been considered as a nuclear retention receptor for glucokinase [5,6,9,12]. The present study suggests that it may have additional roles in nuclear export or import of glucokinase. A tentative hypothesis is that high glucose concentrations or precursors of fructose 1-P cause a conformational change in GKRP or in the glucokinase-GKRP heterodimeric complex converting GKRP from a nuclear retention factor to a nuclear export factor that triggers nuclear export of GKRP or the GKRP-glucokinase complex. The present study does not allow us to distinguish between export of GKRP and glucokinase independently or as a complex. In the latter case, the heterodimeric complex may dissociate in the cytoplasm because of differences in ionic composition between the nucleus and cytoplasm which may favour dissociation of the complex in the cytoplasm. Two possible mechanisms can be suggested for the smaller fractional translocation of GKRP compared with glucokinase. First, export of glucokinase and GKRP may occur in an equimolar ratio in tandem. In this case, the smaller fractional translocation of GKRP may be due to a molar excess of GKRP relative to glucokinase in the nucleus in the control state. Second, GKRP may be present in the nucleus in an equimolar ratio to glucokinase in the control state. However, during translocation of glucokinase and GKRP to the cytoplasm, GKRP may re-enter the nucleus because or rapid shuttling of GKRP. The strong correlation between nuclear glucokinase and GKRP fluorescence is consistent with co-ordinate control of expression of these proteins. On a tissue basis, GKRP is present in molar excess relative to glucokinase by about 2-fold (N. De la Iglesia, L. Agius, unpublished results). The decrease in regression coefficient of nuclear glucokinase/GKRP during substrate-induced nuclear export is consistent with either mechanism.

Nuclear export of several proteins that shuttle between the nucleus and the cytoplasm including protein kinases is blocked by leptomycin B [19,20] which inhibits exportin-1 function [23]. The lack of inhibition of nuclear export of glucokinase and GKRP by leptomycin B suggests that translocation of these proteins across the nuclear pore complex does not involve interaction with exportin-1.

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