# Dual Role of Phosphofructokinase-2/Fructose Bisphosphatase-2 in Regulating the Compartmentation and Expression of Glucokinase in Hepatocytes

Victoria A. Payne,[1] Catherine Arden,[1] Chaodong Wu,[2] Alex J. Lange,[2] and Loranne Agius[1]

**Hepatic glucokinase is regulated by a 68-kDa regulatory protein (GKRP) that is both an inhibitor and nuclear receptor for glucokinase. We tested the role of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) in regulating glucokinase compartmentation in hepatocytes. PFK2 catalyzes formation or degradation of the regulator of glycolysis fructose 2,6-bisphosphate (fructose 2,6-$P_2$), depending on its phosphorylation state (ser-32), and is also a glucokinase-binding protein. Incubation of hepatocytes at 25 mmol/l glucose causes translocation of glucokinase from the nucleus to the cytoplasm and an increase in fructose 2,6-$P_2$. Glucagon caused phosphorylation of PFK2-ser-32, lowered the fructose 2,6-$P_2$ concentration, and inhibited glucose-induced translocation of glucokinase. These effects of glucagon were reversed by expression of a kinase-active PFK2 mutant (S32A/H258A) that overrides the suppression of fructose 2,6-$P_2$ but not by overexpression of wild-type PFK2. Overexpression of PFK2 potentiated glucokinase expression in hepatocytes transduced with an adenoviral vector–encoding glucokinase by a mechanism that does not involve stabilization of glucokinase protein from degradation. It is concluded that PFK2 has a dual role in regulating glucokinase in hepatocytes: it potentiates glucokinase protein expression by posttranscriptional mechanisms and favors its cytoplasmic compartmention. Thus, it acts in a complementary mechanism to GKRP, which also regulates glucokinase protein expression and compartmentation. *Diabetes* 54:1949–1957, 2005**

From the [1]School of Clinical Medical Sciences, Division of Diabetes, University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.; and the [2]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota.

Address correspondence and reprint requests to Loranne Agius, School of Clinical Medical Sciences-Diabetes, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH, U.K. E-mail: loranne.agius@ncl.ac.uk.

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Ad-LGK, adenovirus-encoding rat liver glucokinase; Ad-PFK2-DM, adenovirus encoding PFK2-DM; Ad-PFK2-WT, adenovirus encoding PFK2-WT; GKRP, glucokinase regulatory protein; fructose 2,6-$P_2$, fructose-2,6-bisphosphate; MEM, minimum essential medium; PFK2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; PFK2-DM, PFK2-S32A/H258A; PFK-WT, wild-type PFK2.

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Glucokinase (hexokinase IV) has a major role in the control of hepatic glucose metabolism, and small fractional changes in glucokinase expression cause large changes in glucose metabolism (1–3). Interaction of glucokinase with a 68-kDa glucokinase regulatory protein (GKRP), which affects both the catalytic activity of glucokinase (4) and its subcellular compartmentation (5), is a key component of the mechanism that accounts for the high control strength of glucokinase on hepatic glucose metabolism (3,6).

Glucokinase translocates between the cytoplasm and the nucleus of hepatocytes, depending on the substrate and hormonal conditions. It is sequestered in the nucleus bound to GKRP at low glucose concentrations and translocates to the cytoplasm in response to elevated glucose concentration or precursors of fructose 1-phosphate, which decreases the affinity of GKRP for glucokinase (5,7–10). This substrate-induced translocation is counteracted by glucagon (7) and various substrates (9) by mechanisms that are, at least in part, independent of changes in fructose 1-phosphate (10). GKRP sequesters glucokinase in the nucleus of hepatocytes (11,12). This is supported by the exclusive cytoplasmic location of glucokinase in livers of $GKRP^{-/-}$ mice (13,14) and the lack of sequestration of glucokinase in the nucleus in pancreatic β-cells unless GKRP is ectopically expressed (15). However, the effects of various agonists (16) or antagonists (9,10) of glucokinase translocation in hepatocytes are difficult to explain by the known ligands of GKRP (10,17), suggesting that other proteins may be involved.

Various candidate glucokinase-binding partners have been identified (18–21), including the bifunctional enzyme phosphofructokinase-2/fructose bisphosphatase-2 (PFK2), which regulates the synthesis and the degradation of fructose 2,6-bisphosphate (fructose 2,6-$P_2$), a major allosteric regulator of phosphofructokinase-1 (22–25). The liver isoform of PFK2 is regulated by protein kinase A–mediated phosphorylation of ser-32, which causes inactivation of the kinase and activation of the bisphosphatase (26). The inhibition of glycolysis by glucagon is explained by protein kinase A–mediated phosphorylation of PFK2 (ser-32) and depletion of fructose 2,6-$P_2$. In this study, we tested the hypothesis that PFK2 or its metabolic product and substrate, fructose 2,6-$P_2$, is a component of the mechanism by which glucagon counteracts substrate-induced translocation of glucokinase in hepatocytes.

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## RESEARCH DESIGN AND METHODS

**Hepatocyte culture and treatment with adenoviral vectors.** Hepatocytes were isolated by collagenase perfusion of the liver (7) of male Wistar rats (180–290 g body wt) obtained from B & K (Hull, U.K). The hepatocytes were suspended in minimum essential medium (MEM) supplemented with 5% (vol/vol) newborn calf serum (Life Technologies) and seeded in multiwell plates or on gelatin-coated coverslips for immunostaining (16). After 2 h, the medium was replaced by serum-free MEM containing the adenoviral vectors (2,3). Adenoviral vectors for expression of the wild-type liver isoform of PFK2 (Ad-PFK2-WT) or a double mutant (S32A/H258A) that functions as a constitutively active kinase (Ad-PFK2-DM) have been previously described (27,28). For overexpression of glucokinase, an adenovirus-encoding rat liver glucokinase (Ad-LGK) cDNA (29) was used. The adenoviral vectors were replicated in 293 cells, and stock-cell lysates were diluted as indicated and used throughout this study. Hepatocyte monolayers were incubated with MEM containing the adenoviral vectors for 2 h. For experiments using a combination of two adenoviruses (Ad-LGK and Ad-PFK2-WT or Ad-LGK and Ad-PFK2-DM), these were added simultaneously. Additional controls were performed where the two adenoviruses were added sequentially to confirm that the effects observed were not due to the difference in transfection efficiency. After removal of the medium containing the adenoviral vectors, the hepatocytes were cultured for 18 h in serum-free MEM containing 10 nmol/l dexamethasone and 5 mmol/l glucose.

**Incubations.** After an 18-h preculture, hepatocytes were incubated for 3 h in fresh MEM containing 25 mmol/l glucose without or with glucagon (as indicated). For determination of glycolysis or glucose phosphorylation, the medium contained [3-$^3$H]glucose or [2-$^3$H]glucose, respectively (1 μCi/ml). On termination of the incubations, the medium was collected for determination of $^3H_2O$ (3), and rates of glycolysis are expressed as nanomoles of glucose metabolized per 3 h per milligram protein. Enzyme activity, immunoreactivity, or fructose 2,6-$P_2$ were determined from parallel incubations without radiolabel. For determination of fructose 2,6-$P_2$, the hepatocytes were extracted in 0.1 mol/l NaOH and heated for 5 min at 80°C. Fructose 2,6-$P_2$ was determined as in Van Schaftingen (30) using pyrophosphate-dependent fructose 6-phosphate kinase purified as in Van Schaftingen et al. (31). Phosphofructokinase-2 activity was determined as in Bartrons et al. (32). Free and bound glucokinase were determined by the digitonin permeabilization assay, and free glucokinase is expressed as percent total activity (3).

**Immunoblotting.** Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. Membranes were then blotted for glucokinase immunoreactivity (rabbit antibody against human glucokinase residues 318–405; Santa Cruz) or raised against human recombinant glucokinase (a kind gift from K. Brocklehurst, AstraZeneca) or PFK2-P-ser-32, using a rabbit antibody raised against residues 31–37 [GS(phospho)SIPQF-C] conjugated to keyhole limpet hemocyanin. The phosphopeptide was synthesized by Dr. G. Bloomberg (University of Bristol), and rabbit immunization was performed by Sigma Genosys (Cambridge, U.K.).

**Immunofluorescence staining.** The hepatocyte monolayers on coverslips were rinsed in PBS and fixed in 4% paraformaldehyde/PBS (33). Treatment with $NaBH_4$ and preblocking and staining with a rabbit IgG against human glucokinase residues 318–405 (Santa Cruz) and fluorescein isothiocyanate–labeled anti-rabbit IgG was as previously described (33). Imaging was performed using a Nikon Eclipse E400 epifluorescence microscope and a Nikon DXM1200 digital camera. Three representative fields were selected for each condition, comprising between 50 and 90 nuclei. For image capture, the maximum nuclear intensity of the incubations with glucagon was set at ~250 mean pixel intensity by adjustment of the exposure time and the gain. The mean pixel intensity of the nuclei and cytoplasmic areas was analyzed from the gray images using Lucia G/F Analysis Software. For each incubation condition, the mean value for the nuclei and the cytoplasm was determined, and the results were expressed as a nuclear-to-cytoplasmic ratio. Expression of results as a ratio corrects for drifts in fluorescence intensity.

## RESULTS

**Adenoviral-mediated expression of PFK2-WT and PFK2-DM.** To test the role of PFK2 and fructose 2,6-$P_2$ in mediating the effects of glucose and glucagon on glucokinase compartmentation, we used graded titers (over an eightfold concentration range) of adenoviral vectors for wild-type PFK2 (PFK2-WT) or a kinase-active double mutant (PFK2-DM: S32/H258A) that have been previously described (27). Treatment of hepatocytes with graded titers of the two vectors resulted in a progressive increase in PFK2 activity (Fig. 1). We confirmed, using an antibody to P-ser-32, that endogenous PFK2 and overexpressed PFK2-WT were phosphorylated after incubation with glucagon (Fig. 1*B*). Cells expressing PFK2-DM showed similar phosphorylation in response to glucagon as nontransduced cells (results not shown).

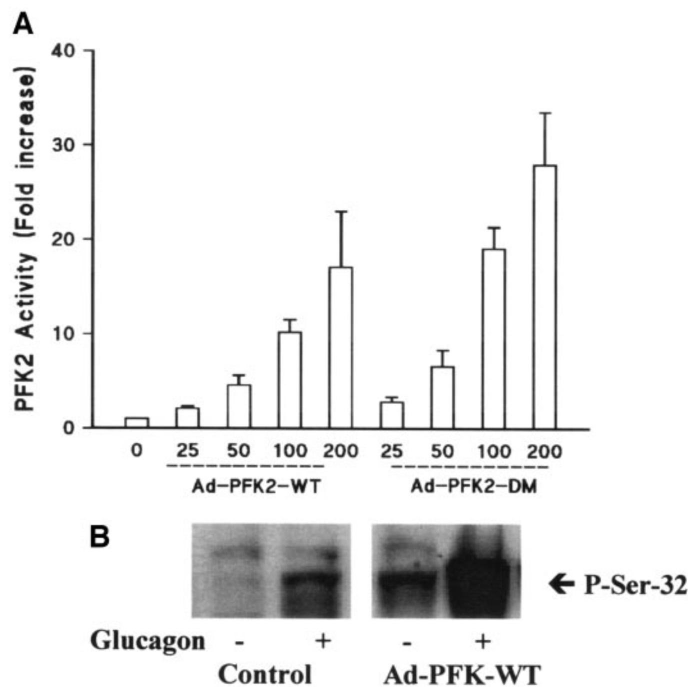


FIG. 1. PFK2 catalytic activity and immunoreactivity in cells transduced with Ad-PFK2-WT and Ad-PFK2-DM. *A*: Hepatocytes were treated with varying titers (25–200 μl/ml) of Ad-PFK2-WT or Ad-PFK2-DM and cultured for 18 h. PFK2 activity was expressed as fold increase over endogenous activity in nontransduced cells (means ± SE, $n = 5$). *B*: Hepatocytes were either nontransduced (control) or treated with 100 μl/ml of Ad-PFK2-WT and cultured for 18 h. They were then incubated for 1 h with 25 mmol/l glucose without or with 100 nmol/l glucagon, and immunoactivity to P-Ser-32 was determined.

**Effects of PFK2-WT and PFK2-DM expression on fructose 2,6-$P_2$ and glycolysis.** Since allosteric activation of phosphofructokinase-1 is one of the best characterized effects in fructose 2,6-$P_2$ (22–25), we initially determined the effects of graded expression of PFK2-WT or PFK2-DM on the fructose 2,6-$P_2$ content and rate of glycolysis in hepatocytes incubated with 25 mmol/l glucose without or with glucagon. These conditions favor dephosphorylation (34) and phosphorylation (26), respectively, of PFK2 (Fig. 1*B*). In the absence of glucagon, graded expression of either PFK2-WT or PFK2-DM increased fructose 2,6-$P_2$ by threefold but had a negligible effect on glycolysis (Fig. 2*A* and *B*). In nontransduced cells, glucagon lowered the fructose 2,6-$P_2$ content by 85% and inhibited glycolysis by 40%. In cells with maximal PFK2-WT overexpression, glucagon caused a greater suppression of fructose 2,6-$P_2$ (95%) and inhibition of glycolysis (50%) than in nontransduced cells (Fig. 2*A* and *C*), consistent with the greater immunoreactivity to P-Ser-32 (Fig. 1*B*). In contrast, expression of PFK2-DM counteracted both the suppression of fructose 2,6-$P_2$ and the inhibition of glycolysis caused by glucagon (Fig. 2*B* and *D*). Qualitatively, similar results were obtained during incubation with 10 mmol/l glucose (results not shown) compared with 25 mmol/l glucose (Fig. 2), insofar that

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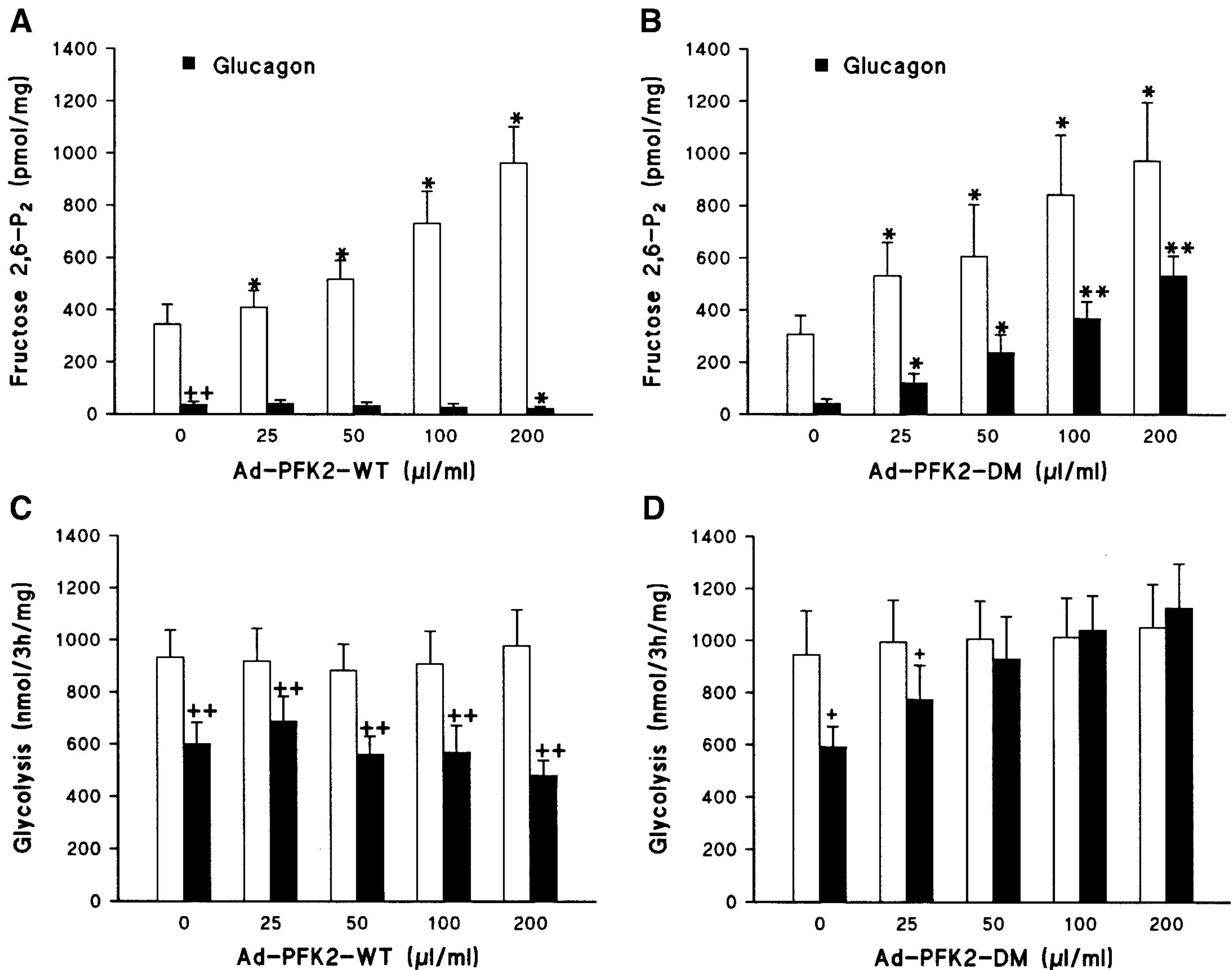


**FIG. 2. Effects of graded PFK2-WT and PFK2-DM expression on fructose 2,6-$P_2$ and glycolysis. Hepatocytes were treated with the adenoviral titers shown in Fig. 1 and cultured for 18 h. They were then incubated for 3 h in MEM containing 25 mmol/l glucose and [3-$^3$H]glucose either without (□) or with (■) 100 nmol/l glucagon for determination of fructose 2,6-$P_2$ and glycolysis. Means ± SE, $n$ = 5. *$P$ < 0.05, **$P$ < 0.005 relative to nontransduced cells, +$P$ < 0.05, ++$P$ < 0.005 effect of glucagon.**

overexpression of PFK2-DM counteracted the inhibition of glycolysis by glucagon but did not increase glycolysis in the absence of glucagon. At 10 mmol/l glucose, a higher level of PFK2-DM expression was required to increase the cell content of fructose 2,6-$P_2$, presumably because of the higher activity of the endogenous bisphosphatase at low glucose (34). A plot of glycolysis against fructose 2,6-$P_2$ for the data from Fig. 2 showed that in nontransduced cells incubated in the absence of glucagon (Fig. 3, arrow), the fructose 2,6-$P_2$ content is near saturation for glycolysis and that rates of glycolysis are also slightly higher in cells expressing PFK2-DM compared with PFK2-WT for a corresponding cell content of fructose 2,6-$P_2$ (Fig. 3).

**Ad-PFK2-DM counteracts the effect of glucagon on glucokinase translocation.** The effects of Ad-PFK2-WT and Ad-PFK2-DM on glucokinase compartmentation (Figs. 4 and 5) were determined in parallel with measurement of fructose 2,6-$P_2$ and glycolysis (Fig. 2). The total activity of glucokinase was unchanged by expression of either PFK2-WT or PFK2-DM (Fig. 4*A* and *B*). Overexpression of PFK2-WT at the highest viral titers caused a small but significant increase in free glucokinase activity in the absence of glucagon and did not counteract the suppression of free glucokinase caused by glucagon (Fig. 4*C*). Conversely, PFK2-DM counteracted the suppression of free glucokinase caused by glucagon (Fig. 4*D*), similar to counteraction of inhibition of glycolysis (Fig. 2*D*).

Glucagon caused an increase in the nuclear/cytoplasmic distribution of glucokinase, and this was counteracted by expression of PFK2-DM but not by PFK2-WT (Fig. 5). This suggests that glucagon-induced translocation of glucokinase from the cytoplasm to the nucleus may share a similar mechanism as glucagon-induced inhibition of glycolysis. Overexpression of PFK2-WT and Ad-PFK2-DM also lowered the glucokinase nuclear-to-cytoplasmic ratio in the absence of glucagon (Fig. 5). This effect was also observed in incubations with 10 mmol/l glucose (glucokinase nuclear-to-cytoplasmic ratio: control, 4.80 ± 0.55; PFK2-DM overexpression, 3.96 ± 0.48, $n$ = 6, $P$ < 0.01). This is consistent with the hypothesis that elevated fruc-

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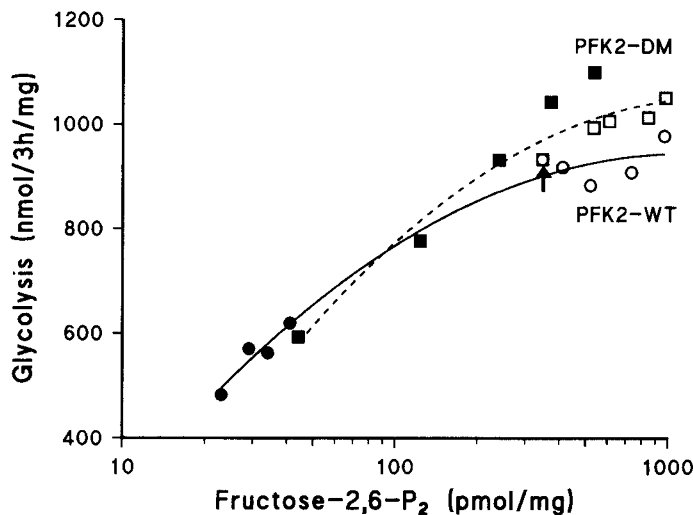


**FIG. 3. Relation between glycolysis and fructose 2,6-$P_2$ in hepatocytes overexpressing PFK2-WT or PFK2-DM. Data are from Fig. 2 for incubations without (■, ○) or with (■, ●) glucagon in cells overexpressing PFK2-WT (○, ●) or PFK2-DM (□, ■). The arrow denotes nontransduced cells incubated without glucagon.**

tose 2,6-$P_2$ favors sequestration of glucokinase in the cytoplasm.

Detritiation of [2-$^3$H]glucose was determined in parallel experiments as a further measure of glucokinase activity (35). This was modestly decreased by glucagon (1,124 ± 137 to 983 ± 125 nmol · 3 $h^{-1}$ · $mg^{-1}$, means ± SE, $n = 6$, $P < 0.05$), and the effect of glucagon was counteracted by expression of PFK2-DM (1,129 ± 155 to 1,068 ± 154 nmol · 3 $h^{-1}$ · $mg^{-1}$, $P > 0.05$) but not by overexpression of PFK2-WT (1,085 ± 156 to 980 ± 133 nmol · 3 $h^{-1}$ · $mg^{-1}$, $P < 0.05$). These changes are qualitatively similar to but much smaller than the changes in glycolysis or glucokinase translocation. Detritiation of [2-$^3$H]glucose is an approximate measure of the rate of glucose phosphorylation (35). However, it is an underestimate because loss of label is incomplete (35). Furthermore, loss of 2-tritium also occurs through an exchange reaction catalyzed by glucose 6-phosphatase (36), which may further underestimate the effects of glucagon on phosphorylation.

**Relative roles of glucokinase and fructose 2,6-$P_2$ in regulating glycolysis.** Since fructose 2,6-$P_2$ affects the rate of glycolysis by allosteric activation of phosphofructokinase-1 (22–25) and the compartmentation of glucokinase as determined by the reversal by PFK2-DM of the glucagon-induced effects on free glucokinase activity (Fig. 4) and nuclear-to-cytoplasmic ratios (Fig. 5), we further investigated the relative roles of fructose 2,6-$P_2$ and glucokinase activity in regulating glycolysis. Hepatocytes were treated with Ad-PFK2-WT or Ad-PFK2-DM (100 μl/ml) as above or with Ad-LGK to overexpress glucokinase by fourfold (14 ± 1 to 57 ± 5 mU/mg), and after an 18-h culture they were incubated with varying concentrations of glucagon (Fig. 6). Overexpression of glucokinase was associated with an increase ($P < 0.001$) in free glucokinase activity in both absolute terms (sixfold) and as percentage of total activity (Fig. 6*A*). Glucagon decreased free glucokinase in nontransduced cells and in cells overexpressing PFK2-WT but not in cells overexpressing glucokinase or PFK2-DM. Glucokinase overexpression increased ($P < 0.003$) fructose 2,6-$P_2$ by twofold, and this was suppressed by glucagon to a similar extent as in nontransduced cells (Fig. 6*B*). Rates of glycolysis were twofold higher ($P < 0.001$) in cells overexpressing glucokinase (Fig. 6*C*). When glycolysis was plotted against the respective fructose 2,6-$P_2$ content, rates of glycolysis were higher in cells overexpressing glucokinase than in untreated cells or cells overexpressing PFK2-DM or PFK2-WT for a corresponding cell content of fructose 2,6-$P_2$ (Fig. 6*D*). Rates of glycolysis were also higher ($P < 0.05$) in cells expressing PFK2-DM and incubated with 10 nmol/l glucagon than in cells overexpressing PFK2-WT and incubated with 1 nmol/l glucagon, despite a similar cell content of fructose 2,6-$P_2$. This can be in part explained by the higher free glucokinase activity ($P < 0.05$) in cells expressing PFK2-DM compared with PFK2-WT in the presence of 1–10 nmol/l glucagon (Fig. 6*C*). These results together suggest that the higher rate of glycolysis in cells expressing PFK2-DM compared with PFK2-WT (Figs. 3 and 6*D*) is determined by both the fructose 2,6-$P_2$ content and the free glucokinase.

**PFK2-WT and Ad-PFK2-DM regulate glucokinase expression.** Although PFK2-DM counteracted the effects of glucagon on both glucokinase translocation and glycolysis, neither PFK2-WT nor Ad-PFK2-DM significantly increased glycolysis (Fig. 2*C* and *D*) or total glucokinase activity (Fig. 4*A* and *B*) in the absence of glucagon. This apparent lack of metabolic response to PFK2 overexpression in the absence of glucagon may be due to a "saturating effect" of the endogenous enzyme, as suggested from the correlation with fructose 2,6-$P_2$ in Fig. 3. To further investigate this possibility, we determined the effects of combined overexpression of glucokinase and either PFK2-WT or PFK2-DM. Hepatocytes were treated with four adenoviral titers of Ad-LGK in the absence or presence of a single titer of Ad-PFK2-WT or Ad-PFK2-DM (Fig. 7). Glucokinase activity was increased by 2- to 11-fold during treatment with the glucokinase adenoviral vector alone (□) and by 6- to 17-fold during combined expression of PFK2-WT or PFK2-DM (▨ or ■). This potentiation of glucokinase activity was also observed when the two adenoviral vectors were added during sequential 2-h incubations rather than simultaneously, indicating that the effects of the combination of adenoviruses are unlikely to be due to differences in transfection efficiency.

To test whether the potentiating effect of PFK2-WT or PFK2-DM on glucokinase activity was due to increased glucokinase protein expression, glucokinase immunoreactivity was determined after treatment of hepatocytes with graded titers of Ad-PFK2-WT or Ad-PFK2-DM in the presence of a fixed titer of glucokinase adenovirus (Fig. 8). There was an increase in glucokinase activity (Fig. 8*A*) and immunoreactivity (Fig. 8*B*) with increasing titers of Ad-PFK2-WT or Ad-PFK2-DM. The correlation (Fig. 8*C*) suggests that the effect of PFK2-WT and PFK2-DM involves potentiation of glucokinase protein expression.

**Glucagon does not counteract the effect of PFK2-WT or PFK2-DM on glucokinase expression.** To test whether the increased expression of glucokinase in cells transduced with Ad-PFK2-WT or Ad-PFK2-DM is due to the increase in fructose 2,6-$P_2$, additional experiments were performed similar to the ones in Fig. 8, except that

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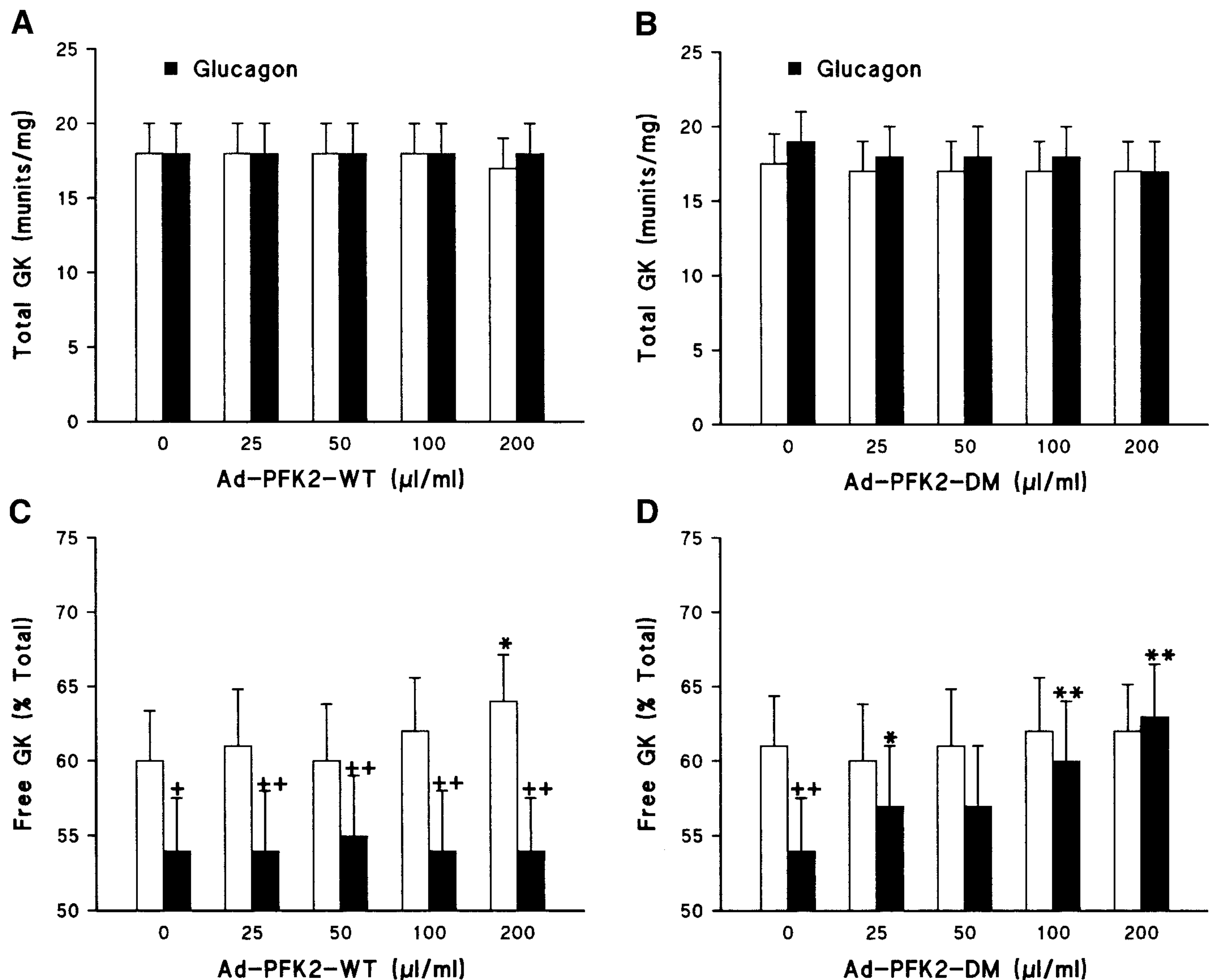


**FIG. 4. Effects of PFK2-WT and PFK2-DM expression on total glucokinase activity and binding. Experimental conditions were as in Fig. 2. Glucokinase (free and bound) was determined after a 3-h incubation with 25 mmol/l glucose without (□) or with 100 nmol/l glucagon (■) and is expressed as total (free + bound) activity (*A* and *B*) or as free glucokinase percent total activity (*C* and *D*). Means ± SE, $n = 5$. *$P < 0.05$, **$P < 0.005$ relative to nontransduced cells; +$P < 0.05$, ++$P < 0.005$ effect of glucagon.**

the hepatocytes were cultured without or with glucagon (1 µmol/l), which was added after the 2-h incubation with the adenoviral vectors. Although glucagon totally counteracted the increase in fructose 2,6-$P_2$ caused by overexpression of PFK2-WT, it did not counteract the potentiation of glucokinase expression by PFK2-WT ($n = 5$, results not shown).

**Glucokinase degradation is inhibited by GKRP but not by PFK2-WT.** To determine whether the potentiation of glucokinase expression in cells with combined glucokinase and PFK2-WT overexpression (Figs. 7 and 8) is due to stabilization of glucokinase protein from degradation by a posttranslational mechanism, hepatocytes were transduced with either Ad-LGK alone or with combined Ad-LGK and Ad-PFK2-WT at appropriate titers selected from Fig. 7 (60 µl/ml for Ad-LGK vs. 20 µl/ml for Ad-LGK + 100 µl/ml Ad-PFK2-WT) to attain the same level of glucokinase expression or with GKRP (6). After an 18-h culture, the degradation of glucokinase was determined during a 4-h incubation in the presence of 2 µmol/l cycloheximide to inhibit protein synthesis (3). Fractional degradation of glucokinase during 4 h was not significantly different in hepatocytes with combined glucokinase and PFK2-WT overexpression compared with glucokinase overexpression alone (control, 27 ± 5%; Ad-PFK2-WT, 35 ± 5%, $n = 4$). However, combined overexpression of glucokinase and GKRP, which increased glucokinase enzyme expression by twofold, relative to glucokinase expression alone, decreased glucokinase degradation (control, 27 ± 5%; GKRP, 17 ± 3%, $P < 0.01$). Combined, these results suggest that the increased expression of glucokinase in cells cotransduced with Ad-LGK and Ad-PFK2-WT (Figs. 7 and 8) is due to either stabilization of glucokinase mRNA or increased translation. This contrasts with the effects of GKRP, which stabilizes glucokinase protein from degradation (13).

## DISCUSSION

Previous work (5,7) has shown that glucagon counteracts the effects of glucose and fructose on translocation of



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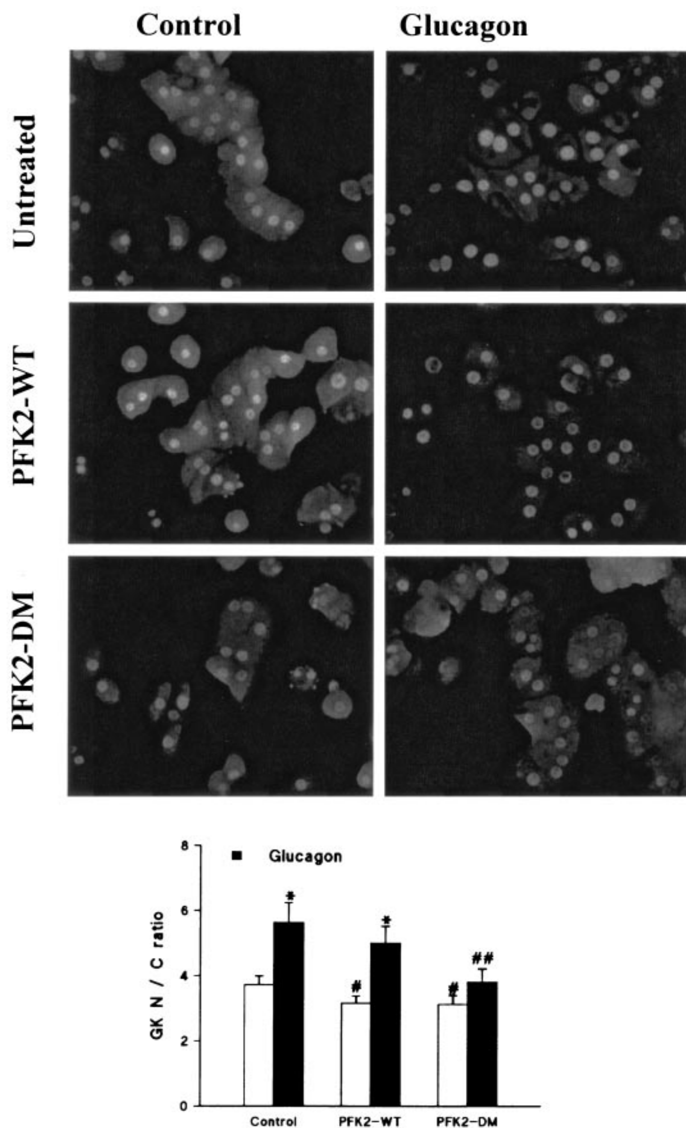


**FIG. 5. Effects of PFK2-WT and PFK2-DM expression on the nuclear/cytoplasmic distribution of glucokinase. Hepatocytes were untreated or treated with Ad-PFK2-WT or Ad-PFK2-DM (100 μl/ml). After an 18-h culture, they were incubated for 3 h with 25 mmol/l glucose without or with 100 nmol/l glucagon. *Top panels:* Representative images. Histogram shows the nuclear-to-cytoplasmic mean immunofluorescence ratios, means ± SE, $n = 6$. *$P < 0.05$ effect of glucagon, #$P < 0.05$ relative to untreated control, ##$P < 0.005$ relative to glucagon-treated control.**

glucokinase from the nucleus to the cytoplasm in hepatocytes. The translocation caused by fructose is explained by fructose 1-phosphate–mediated dissociation of glucokinase from GKRP (10). It remains debatable whether the translocation by glucose is due to an increase in fructose 1-phosphate (10) or to a conformational change in glucokinase (37). The stimulation of translocation by nonmetabolizable glucose analogs (38) supports the latter hypothesis. A decrease in fructose 1-phosphate is a possible explanation for the mechanism of action of glucagon (39). However, the inhibition of translocation by substrates that lower fructose 2,6-$P_2$, such as ethanol and glycerol, cannot be fully explained by a lowering of fructose 1-phosphate (10). Since high glucose concentration increases the fructose 2,6-$P_2$ content of hepatocytes by activation of a type-2A phosphatase, which dephosphorylates PFK2 (34,40), whereas glucagon inhibits glycolysis by fructose 2,6-$P_2$ depletion through phosphorylation of PFK2 (26), we tested the hypothesis that the effects of glucagon on compartmentation of glucokinase in hepatocytes are mediated by PFK2 phosphorylation and depletion of fructose 2,6-$P_2$. PFK2 was recently identified as a glucokinase-binding protein (20), and a physiological role for this interaction was shown in pancreatic β-cells, where overexpression of PFK2 caused an increase in glucokinase activity and glucose oxidation (21). The role of PFK2 in regulating glucokinase compartmentation or function in the hepatocyte has not been reported.

To test the role of PFK2 and of changes in the concentration of fructose 2,6-$P_2$ in mediating the effects of glucagon on glucokinase compartmentation, we used an adenoviral vector for a kinase-active PFK2 variant (S32A/H258A) to counteract the depletion of fructose 2,6-$P_2$ caused by glucagon. Graded overexpression of the kinase-active variant, but not PFK2-WT, counteracted the effects of glucagon on both glycolysis and glucokinase translocation. This supports the hypothesis that the effect of glucagon on the subcellular compartmentation of glucokinase is due to phosphorylation of PFK2 and/or depletion of fructose 2,6-$P_2$. Two mechanisms can be proposed to account for these observations. First, fructose 2,6-$P_2$ promotes the binding of glucokinase to a cytoplasmic receptor, and accordingly, depletion of fructose 2,6-$P_2$ by either glucagon or by a decline in glucose concentration allows the return of glucokinase to the nucleus. Second, the dephosphorylated form of PFK2 but not the phosphorylated form acts as a cytoplasmic receptor for glucokinase, and the kinase-active variant of PFK2 mimics the action of dephosphorylated PFK2 in retaining glucokinase in the cytoplasm. These two mechanisms are not mutually exclusive, and both fructose 2,6-$P_2$ and dephospho-PFK2 may be involved in retaining glucokinase in the cytoplasm. PFK2 binds to glucokinase through its bisphosphatase domain (20), but whether fructose 2,6-$P_2$ enhances the binding affinity of PFK2 for glucokinase is not known. The kinase-active variant does not enable us to distinguish between the effects of fructose 2,6-$P_2$ as opposed to a direct effect of PFK2 (as the phosphorylated or dephosphorylated form) through a protein-protein interaction. Nonetheless, the correlations between glycolysis and fructose 2,6-$P_2$ are suggestive of mechanisms that cannot be fully explained by fructose 2,6-$P_2$ alone. Accordingly, the higher rate of glycolysis in cells expressing the PFK2 variant compared with the PFK2-WT for a corresponding cellular content of fructose 2,6-$P_2$ (Fig. 3 and Fig. 6*D*) are suggestive of a role for either the dephosphorylated or the phosphorylated PFK2, additional to the effects of fructose 2,6-$P_2$. This could be consistent with a requirement for both fructose 2,6-$P_2$ and the dephospho-PFK2 in retaining glucokinase in the cytoplasm.

Previous work (3,6) has shown that glucokinase has a high flux control coefficient on both glycogen synthesis and glycolysis. The present study shows that in the absence of glucagon challenge, overexpression of either PFK2-WT or the kinase-active variant increases the cell content of fructose 2,6-$P_2$, but unlike glucokinase, overexpression does not increase glycolysis. This indicates that in the absence of glucagon, the cell content of fructose

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**FIG. 6. Effects of glucagon concentration on cells overexpressing PFK2-WT, PFK2-DM, or glucokinase. Hepatocytes were either nontransduced (○) or treated with Ad-LGK 20 μl/ml (□), Ad-PFK2-WT 100 μl/ml (△), or Ad-PFK2-DM 100 μl/ml (■). They were then incubated for 3 h in MEM containing 25 mmol/l glucose, [3-$^3$H]glucose (glycolysis), and the glucagon concentrations indicated. *A*: Free glucokinase (percent total). *B*: Fructose 2,6-$P_2$. *C*: Glycolysis. *D*: Glycolysis versus fructose 2,6-$P_2$. Means ± SE, $n = 5$. *$P < 0.05$, **$P < 0.005$ effect of glucagon.**

2,6-$P_2$ is saturating for glycolysis and, accordingly, that control of glycolytic flux resides entirely at the level of glucose phosphorylation. Although PFK2-DM expression caused a decrease in the nuclear/cytoplasmic distribution of glucokinase also in the absence of glucagon challenge, this was not associated with a significant increase in glycolysis. Two explanations are possible. First, the glucokinase sequestered in the cytoplasm is bound to GKRP and is therefore in a less active state. Second, the lack of stimulation of glycolysis reflects a saturating effect of endogenous PFK2.

To test for the latter possibility, we determined the combined effects of overexpression of glucokinase and PFK2. These experiments showed, unexpectedly, that PFK2 overexpression markedly potentiated glucokinase expression by the adenoviral vector. This mechanism appears to be independent of the phosphorylation state of PFK2 because it was not blocked by glucagon. Unlike the stabilizing effect of GKRP on glucokinase (13), it was not observed when protein synthesis was inhibited with cycloheximide, suggesting that PFK2 potentiates glucokinase protein expression before or during protein synthesis. Two possible

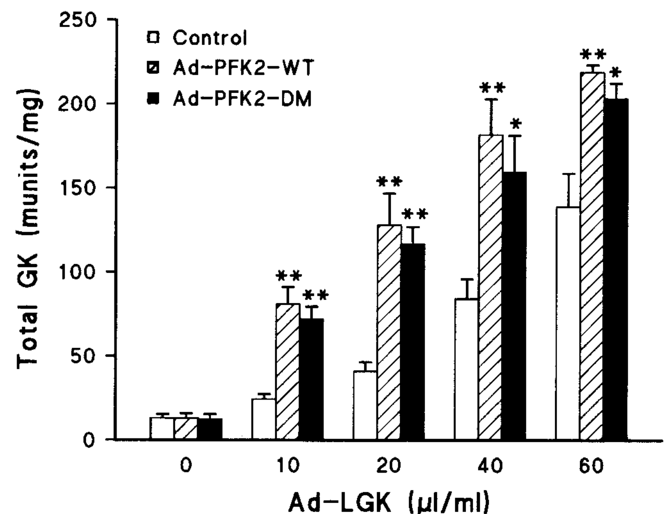


**FIG. 7. Potentiation of glucokinase expression during overexpression of PFK2-WT or PFK2-DM. Hepatocytes were treated with varying titers of Ad-LGK (10–60 μl/ml) in the absence (control) or presence of Ad-PFK2-WT (100 μl/ml) or Ad-PFK2-DM (100 μl/ml). Total glucokinase activity was determined after 18 h. Means ± SE, $n = 4$. *$P < 0.05$, **$P < 0.005$ relative to corresponding Ad-LGK alone.**

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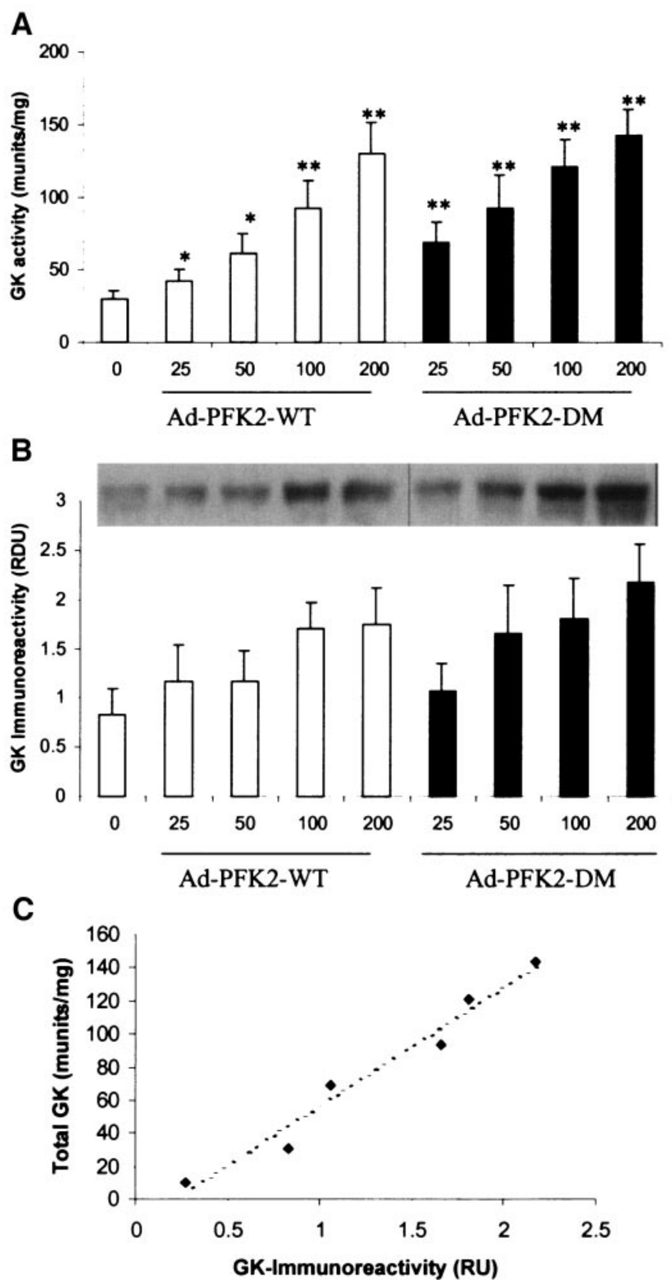


**FIG. 8. Effects of varying titers of Ad-PFK2-WT and Ad-PFK2-DM on glucokinase expression. Hepatocytes were treated with a single titer of Ad-LGK (20 μl/ml) in the absence or presence of varying titers (25–200 μl/ml) of Ad-PFK2-WT or Ad-PFK2-DM and cultured for 18 h. *A*: Total glucokinase activity. *B*: Glucokinase immunoreactivity showing representative immunoblot. *C*: Glucokinase activity versus immunoreactivity. Means ± SE, $n = 4$. *$P < 0.05$, **$P < 0.005$ relative to Ad-LGK alone.**

mechanisms are stabilization of glucokinase mRNA or stabilization of glucokinase protein during synthesis. An increase in hepatic glucokinase mRNA and protein after expression of PFK2-DM and PFK2-WT in streptozotocin-induced diabetic rats has been previously reported (41). The precise mechanism by which PFK2 protein potentiates glucokinase expression is currently being investigated.

The dual role of GKRP in regulating both glucokinase expression by a posttranslational mechanism (13) and its subcellular compartmentation has been unequivocally demonstrated in GKRP$^{-/-}$ mice, which have normal glucokinase mRNA expression but markedly decreased glucokinase protein expression and an exclusive cytoplasmic location of the protein (13,14). We show in this study that PFK2 also has a dual role in the control of hepatocyte glucokinase, it potentiates glucokinase protein expression by a different mechanism from GKRP, and in its dephosphorylated form it favors the cytoplasmic retention of glucokinase. This suggests that GKRP and PFK2 have complementary roles in favoring the sequestration of the glucokinase in the nucleus and the cytoplasm, respectively, and that they also have complementary roles in potentiating glucokinase expression. Studies involving targeted disruption of liver PFK2 should help confirm the role of PFK2 in regulating hepatic glucokinase expression. The effects of PFK2 on glucokinase activity but not protein expression in pancreatic β-cells (21) are in marked contrast to the present findings in hepatocytes and indicate that PFK2 has a different regulatory role in these tissues consistent with the expression in islet β-cells of the brain isoform, rather than the liver isoform (20).

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## REFERENCES

1. Iynedjian PB: Mammalian glucokinase and its gene. *Biochem J* 293:1–13, 1993
2. O'Doherty RM, Lehman DL, Seoane J, Gomez-Foix AM, Guinovart JJ, Newgard CB: Differential metabolic effects of adenovirus-mediated glucokinase and hexokinase I overexpression in rat primary hepatocytes. *J Biol Chem* 271:20524–20530, 1996
3. Agius L, Peak M, Newgard CB, Gomez-Foix AM, Guinovart JJ: Evidence for a role of glucose-induced translocation of glucokinase in the control of hepatic glycogen synthesis. *J Biol Chem* 27:30479–30486, 1996
4. Van Schaftingen E, Detheux M, Veiga da Cunha M: Short-term control of glucokinase activity: role of a regulatory protein. *FASEB J* 8:414–419, 1994
5. Brown KS, Kalinowski SS, Megill JR, Durham SK, Mookhtiar KA: Glucokinase regulatory protein may interact with glucokinase in the hepatocyte nucleus. *Diabetes* 46:179–186, 1997
6. De la Iglesia N, Mukhtar M, Seoane J, Guinovart JJ, Agius L: The role of the regulatory protein of glucokinase in the glucose sensory mechanism of the hepatocyte. *J Biol Chem* 275:10597–10603, 2000
7. Agius L, Peak M: Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin. *Biochem J* 296:785–796, 1993
8. Chu CA, Fujimoto Y, Igawa K, Grimsby J, Grippo JF, Magnuson MA, Cherrington AD, Shiota M: 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, 2004
9. Agius L: Control of glucokinase translocation in rat hepatocytes by sorbitol and the cytosolic redox state. *Biochem J* 298:237–243, 2004
10. Niculescu L, Veiga-da-Cunha M, Van Schaftingen E: Investigation on the mechanism by which fructose, hexitols and other compounds regulate the translocation of glucokinase in rat hepatocytes. *Biochem J* 321:239–246, 1997
11. Shiota C, Coffey J, Grimsby J, Grippo JF, Magnuson MA: Nuclear import of hepatic glucokinase depends upon glucokinase regulatory protein, whereas export is due to a nuclear export signal sequence in glucokinase. *J Biol Chem* 274:37125–37130, 1999
12. de la Iglesia N, Veiga-da-Cunha M, Van Schaftingen E, Guinovart JJ, Ferrer JC: Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. *FEBS Lett* 456:332–338, 1999
13. Farrelly D, Brown KS, Tieman A, Ren J, Lira SA, Hagan D, Gregg M, Mookhtiar KA, Hariharan N: Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: a sequestration mechanism in metabolic regulation. *Proc Natl Acad Sci U S A* 96:14511–14516, 1999
14. Grimsby J, Coffey JW, Dvorozniak MT, Magram J, Li G, Matschinsky FM,

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Shiota C, Kaur S, Magnuson MA, Grippo JF: Characterization of glucokinase regulatory protein-deficient mice. *J Biol Chem* 275:7826–7831, 2000

15. Bosco D, Meda P, Iynedjian PB: Glucokinase and glucokinase regulatory protein: mutual dependence for nuclear localization. *Biochem J* 348:215–222, 2000
16. Brocklehurst KJ, Payne VA, Davies RA, Carroll C, Vertigan HL, Wightman HJ, Aiston S, Waddell ID, Leighton B, Coghlan MP, Agius L: Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. *Diabetes* 53:535–541, 2004
17. Detheux M, Vandercammen A, Van Schaftingen E: Effectors of the regulatory protein acting on liver glucokinase: a kinetic investigation. *Eur J Biochem* 200:553–561, 1991
18. Munoz-Alonso MJ, Guillemain G, Kassis N, Girard J, Burnol AF, Leturque A: A novel cytosolic dual specificity phosphatase, interacting with glucokinase, increases glucose phosphorylation rate. *J Biol Chem* 275:32406–34012, 2000
19. Shiraishi A, Yamada Y, Tsuura Y, Fijimoto S, Tsukiyama K, Mukai E, ToyodaY, Miwa I, Seino Y: A novel glucokinase regulator in pancreatic beta cells: precursor of propionyl-CoA carboxylase beta subunit interacts with glucokinase and augments its activity. *J Biol Chem* 276:2325–2328, 2001
20. Baltrusch S, Lenzen S, Okar DA, Lange AJ, Tiedge M: 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, 2001
21. Massa L, Baltrusch S, Okar DA, Lange AJ, Lenzen S, Tiedge M: Interaction of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) with glucokinase activates glucose phosphorylation and glucose metabolism in insulin-producing cells. *Diabetes* 53:1020–1029, 2004
22. Hers HG, Van Schaftingen E: Fructose 2,6-bisphosphate 2 years after its discovery. *Biochem J* 206:1–12, 1982
23. Hue L, Rider MH: Role of fructose 2,6-bisphosphate in the control of glycolysis in mammalian tissues. *Biochem J* 245:313–324, 1987
24. Pilkis SJ, Claus TH, Kurland IJ, Lange AJ: 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: a metabolic signaling enzyme. *Annu Rev Biochem* 64:799–835, 1995
25. Okar DA, Lange AJ: Fructose-2,6-bisphosphate and control of carbohydrate metabolism in eukaryotes. *Biofactors* 10:1–14, 1999
26. Kurland IJ El-Maghrabi MR, Correia JJ, Pilkis SJ: Rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: properties of phospho- and dephospho-forms and of two mutants in which Ser32 has been changed by site-directed mutagenesis. *J Biol Chem* 267:4416–4423, 1992
27. Argaud D, Lange AJ, Becker TC, Okar DA, el-Maghrabi MR, Newgard CB, Pilkis SJ: Adenovirus-mediated overexpression of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in gluconeogenic rat hepatoma cells: paradoxical effect on Fru-2,6-P2 levels. *J Biol Chem* 270:24229–24326, 1995
28. Argaud D, Kirby T, Newgard CB, Lange AJ: Stimulation of glucose-6-phosphatase gene expression by glucose and fructose-2,6-bisphosphate. *J Biol Chem* 272:12854–12861, 1997
29. Becker TC, Noel RJ, Johnson JH, Lynch RM, Hirose H, Tokuyama Y, Bell GI, Newgard CB: Differential effects of overexpressed glucokinase and hexokinase I in isolated islets: evidence for functional segregation of the high and low Km enzymes. *J Biol Chem* 271:390–394, 1996
30. Van Schaftingen E: *Methods of Enzymatic Analysis*. 3rd ed. Bergmeyer HU, Ed. Weinheim, Germany, Verlag Chemi, 1984, p. 335–341
31. Van Schaftingen E, Lederer B, Bartrons R, Hers HG: A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers: application to a microassay of fructose 2,6-bisphosphate. *Eur J Biochem* 129:191–195, 1982
32. Bartrons R, Hue L, Van Schaftingen E, Hers HG: Hormonal control of fructose 2,6-bisphosphate concentration in isolated rat hepatocytes. *Biochem J* 214:829–897, 1983
33. Mukhtar M, Stubbs M, Agius L: Evidence for glucose and sorbitol-induced nuclear export of glucokinase regulatory protein in hepatocytes. *FEBS Lett* 462:453–458, 1999
34. Nishimura M, Fedorov S, Uyeda K: Glucose-stimulated synthesis of fructose 2,6-bisphosphate in rat liver: dephosphorylation of fructose 6-phosphate, 2-kinase:fructose 2,6-bisphosphatase and activation by a sugar phosphate. *J Biol Chem* 269:26100–26106, 1994
35. Hue L: The role of futile cycles in the regulation of carbohydrate metabolism in the liver. *Adv Enzymol Relat Areas Mol Biol* 53:247–331, 1981
36. Van Schaftingen E: Glucosamine-sensitive and insensitive detritiation of [2–3H]glucose in isolated hepatocytes: a study of the contributions of glucokinase and glucose 6-phosphatase. *Biochem J* 308:23–29, 1995
37. Zelent D, Najafi H, Odili S, Buettger C, Weik-Collins H, Li C, Doliba N, Grimsby J, Matschinsky FM: Glucokinase and glucose homeostasis: proven concepts and new ideas. *Biochem Soc Trans* 33:306–310, 2005
38. Agius L, Stubbs M: 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, 2000
39. Davies DR, Detheux M, Van Schaftingen E: Fructose 1-phosphate and the regulation of glucokinase activity in isolated hepatocytes. *Eur J Biochem* 192:283–289, 1990
40. Liu YQ, Uyeda K: The mechanism of regulation of hepatic Fru 2,6-P2 concentration upon refeeding: involvement of xylulose 5-P and cyclic-AMP. *Biochem Biophys Res Commun* 221:554–558, 1996
41. Wu C, Okar DA, Stoeckman AK, Peng LJ, Herrera AH, Herrera JE, Towle HC, Lange AJ: A potential role for fructose-2,6-bisphosphate in the stimulation of hepatic glucokinase gene expression. *Endocrinology* 145:650–658, 2004


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