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

Laura Massa,1 Simone Baltrusch,1 David A. Okar,2,3 Alex J. Lange,2 Sigurd Lenzen,1 and Markus Tiedge1

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) was recently identified as a new intracellular binding partner for glucokinase (GK). Therefore, we studied the importance of this interaction for the activity status of GK and glucose metabolism in insulin-producing cells by overexpression of the rat liver and pancreatic islet isoforms of PFK-2/FBPase-2. PFK-2/FBPase-2 overexpression in RINm5F-GK cells significantly increased the GK activity by 78% in cells expressing the islet isoform, by 130% in cells expressing the liver isoform, and by 116% in cells expressing a cAMP-insensitive liver S32A/H258A double mutant isoform. Only in cells overexpressing the wild-type liver PFK-2/FBPase-2 isoform was the increase of GK activity abolished by forskolin, apparently due to the regulatory site for phosphorylation by a cAMP-dependent protein kinase. In cells overexpressing any isoform of the PFK-2/FBPase-2, the increase of the GK enzyme activity was antagonized by treatment with anti-FBPase-2 antibody. Increasing the glucose concentration from 2 to 10 mmol/l had a significant stimulatory effect on the GK activity in RINm5F-GK cells overexpressing any isoform of PFK-2/FBPase-2. The interaction of GK with PFK-2/FBPase-2 takes place at glucose concentrations that are physiologically relevant for the activation of GK and the regulation of glucose-induced insulin secretion. This new mechanism of posttranslational GK regulation may also represent a new site for pharmacotherapeutic intervention in type 2 diabetes treatment. Diabetes 53: 1020–1029, 2004

From the 1Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany; the 2Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota; and the 3VA Medical Center, Minneapolis, Minnesota.

Address correspondence and reprint requests to Dr. Markus Tiedge, Institute of Clinical Biochemistry, Hannover Medical School, D-30623 Hannover, Germany. E-mail: tiedge.markus@mh-hannover.de.

Received for publication 17 October 2003 and accepted in revised form 5 January 2004.

L.M and S.B. contributed equally to this work.

F-2,6-P2, fructose-2,6-bisphosphate; GK, glucokinase; MOI, multiplicity of infection; PKA, cAMP-dependent protein kinase; PFK-2/FBPase-2,6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

© 2004 by the American Diabetes Association.

The enzyme glucokinase (GK) plays a pivotal role in the recognition of glucose in pancreatic β-cells and the regulation of glucose metabolism in the liver (1–7). In pancreatic β-cells, GK acts as a glucose sensor and catalyzes the rate-limiting step for initiation of glucose-induced insulin secretion (6). GK is regulated in a complex manner in pancreatic β-cells by posttranslational modifications of the enzyme protein that mainly depend on the intracellular glucose concentration (8–13). These posttranslational mechanisms of GK activity regulation are comprised of conformational changes (14,15), sulfhydryl-group conversions (16–18), and interactions with β-cell matrix proteins (13,19), insulin granules (20,21), newly identified binding partners (22,23), and GK-activating compounds (24). The hepatic GK regulatory protein, which binds and inhibits GK competitively and confers short-term regulation of GK in the liver (25,26), is not expressed in pancreatic β-cells (13). Using a peptide phage display strategy, we recently identified the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) as a binding partner of the GK (23). PFK-2/FBPase-2 modulates intracellular levels of fructose-2,6-bisphosphate (F-2,6-P2), a highly potent regulator of carbohydrate metabolism (27–29). In liver, the PFK-2/FBPase-2 enzyme is modulated by phosphorylation and dephosphorylation, which significantly affects the kinase/bisphosphatase ratio of this bifunctional enzyme (28,29). Rat pancreatic islets express the brain isoform of PFK-2/FBPase-2 (23), which does not appear to be regulated by cAMP-dependent protein kinases (PKAs) or phosphatases at the NH2-terminus of the protein, as is the liver isoform (29). However, yeast two-hybrid studies clearly indicate that the GK protein interacts with the liver as well as the islet PFK-2/FBPase-2 isoform (23). This raised the question whether the PFK-2/FBPase-2–GK interaction participates in the posttranslational regulation of GK enzyme activity. Knowledge of the physiological significance of GK regulation through interaction with PFK-2/FBPase-2 may have a central impact on the understanding of the failure of β-cell function in type 2 diabetes. To elucidate the effect of PFK-2/FBPase-2 binding to GK on GK enzyme activity and glucose metabolism, the liver and islet PFK-2/FBPase-2 isoforms were overexpressed in the present study in...
RIMn5F-GK and INS1 insulin-producing cells. The results show that overexpression of PFK-2/FBPase-2 resulted in a significant increase of GK activity in insulin-producing cells accompanied by corresponding increases in glucose metabolism.

RESEARCH DESIGN AND METHODS

Materials. Restriction enzymes and modifying enzymes for the cloning procedures were from New England Biolabs (Beverly, MA) or Fermentas (St. Leon-Rot, Germany). The SP6/T7 Transcription Kit and DIG Nucleic Acid Detection Kit were obtained from Roche (Mannheim, Germany). Hybond nylon membranes were from Amersham (Braunschweig, Germany), and InnuPrep 96-well strip plates were from Qiagen (Hilden, Germany). The enhanced chemiluminescence detection system and autoradiography films were from Amersham. Forskolin was from ICN Biomedicals (Irvine, CA). All reagents of analytical grade were from Merck (Darmstadt, Germany). All tissue culture equipment was from Gibco Life Technologies (Gaithersburg, MD).

Tissue culture. RIMn5F cells (30.31) overexpressing GK (RIMn5F-GK cells) were generated by stable transfection of the human β-cell GK cDNA (32) in the pcDNA3 vector as described previously (13,33). Cells were grown in RPMI 1640 medium supplemented with 10 mmol/l glucose, 10% (vol/vol) FCS, penicillin, streptomycin, and 250 μg/ml G418 in a humidified atmosphere at 37°C and 5% CO2. INS1 cells (passage 80–90) were grown in RPMI 1640 medium supplemented with 10 mmol/l glucose, 10% (vol/vol) FCS, 1 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, 10 mmol/l HEPES, 50 mmol/l 2-mercaptoethanol, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO2 (34).

Stable overexpression of PFK-2/FBPase-2 in RIMn5F-GK cells. PFK-2/FBPase-2 coding cDNA for rat liver (35), rat liver SS2A/HeSa double mutant (36,37), and rat ileal/brain (23,38) were subcloned as a HindIII-Aval fragment into the pcDNA3-Zeo expression vector by standard molecular biology techniques (39). RIMn5F-GK cells were transfected with the vector DNA by the use of CLONfectin (Clontech, Palo Alto, CA) as described in the manufacturer’s manual. Positive clones were selected through resistance against Zeocin (250 μg/ml) and characterized further for PFK-2/FBPase-2 expression by Northern blot and Western blot analyses. In the present study, we used the RIMn5F-GK-PPK/FBPase-2 islet clones I4 and I10, the liver clones LS and L11, and the liver mutant clones LM11 and LM12.

Recombinant adenovirus preparation and transduction of RIMn5F-GK and INS1 cells. The adenoviral vector system was provided by B. Vogelstein (Baltimore, MD) (40). Rat liver PFK-2/FBPase-2 coding cDNA was subcloned as a BglII-EcoRV fragment in the psbshuttle-CMV vector, and generation of the recombinant adenoviral plasmid was performed by homologous recombinant with the pAdEasy-1 plasmid in BL21 bacteria. The cleavage of the RV fragment in the pShuttle-CMV vector, and generation of the recombinant FBPase-2 antibody. The bispahosphate domain of rat liver PFK-2/FBPase-2 (amino acid residues 250–470) was subcloned as a BamHI-SalI fragment into the pGEX-6P-1 vector (Amersham Pharmacia Biotech, Freiburg, Germany). The glutathione-S-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech) was used for the expression and purification of the GST-fused protein in Escherichia coli BL21 bacteria. The cleavage of the glutathione-S-transferase tag was achieved by incubating the glutathione-Sepharose column with PreScission protease as described in the manufacturer’s manual. The recombinant protein was characterized through SDS-PAGE and activity measurements (42). The protein concentration was analyzed by a Bio-Rad protein assay. Thereafter, dithiothreitol and bromphenol blue were added from concentrated stocks to yield a final concentration of 100 mmol/l and 0.1%, respectively. Cellular protein (20 μg) was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by nonfat dry milk overnight at 4°C. GK immunodetection was performed as described previously (13,18). For PFK-2/FBPase-2, the blots were incubated with the described FBPase-2 antibody at a dilution of 1:10,000 followed by a 2-h incubation period with an anti-β-galactosidase-labeled secondary antibody at a dilution of 1:40,000 at room temperature. The specific protein bands were visualized by chemiluminescence using the enhanced chemiluminescence detection system and quantified by densitometry using the Gel-Pro Analyzer software. Linearity of the band intensities of the autoradiograms was verified by serial dilutions of recombinant β-cell GK or rat liver FBPase-2 protein, respectively (data not shown).

Assay of GK enzyme activity. GK activity measurements and GK Western blot analysis were performed from identical samples to achieve a direct comparison between GK protein expression and activity. The cells were homogenized in PBS (pH 7.4), and insoluble material was pelleted by centrifugation. The protein concentration was measured by a Bio-Rad protein assay. Thereafter, dithiothreitol and bromphenol blue were added from concentrated stocks to yield a final concentration of 100 mmol/l and 0.1%, respectively. Cellular protein (20 μg) was fractionated by reducing 10% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The membranes were stained by Ponceau to verify the transfer of comparable amounts of cellular protein. Nonspecific binding sites of the membranes were blocked by nonfat dry milk overnight at 4°C. GK immunodetection was performed as described previously (13,18). For PFK-2/FBPase-2, the blots were incubated with the described FBPase-2 antibody at a dilution of 1:10,000 followed by a 2-h incubation period with an anti-β-galactosidase-labeled secondary antibody at a dilution of 1:40,000 at room temperature. The specific protein bands were visualized by chemiluminescence using the enhanced chemiluminescence detection system and quantified by densitometry using the Gel-Pro Analyzer software. Linearity of the band intensities of the autoradiograms was verified by serial dilutions of recombinant β-cell GK or rat liver FBPase-2 protein, respectively (data not shown).

RESULTS

Adenoviral overexpression of wild-type liver PFK-2/FBPase-2 in RIMn5F-GK and INS1 cells and its effect on GK enzyme activity. Adenoviral infection resulted in a significant overexpression of the liver PFK-2/FBPase-2 enzyme in RIMn5F-GK insulin-producing tissue culture cells (Fig. 1A) as well as in INS1 insulin-producing tissue.
culture cells (Fig. 1B). Using three different MOIs, gradually increasing levels of PFK-2/FBPase-2 expression were detectable in both Northern and Western blot analyses (Fig. 1). RINm5F-GK and INS1 cells showed endogenous expression of PFK-2/FBPase-2 mRNA and protein, which was detectable after longer exposure times of the blots (data not shown). The increase of the PFK-2/FBPase-2 mRNA and protein expression was accompanied by a significant increase of GK enzyme activity by 40–150% in both cell lines (Fig. 1). In control experiments, adenoviral infection of the cells with the AdWT virus did not affect the abundance of PFK-2/FBPase-2 mRNA and protein. Densitometric analysis confirmed that the level of GK protein expression remained unchanged in the PFK-2/FBPase-2 overexpressing cells at all MOIs, irrespective of the significant increase of the GK enzyme activity (Fig. 1). In RINm5F-GK cells, GK protein levels were 100/113% at an MOI of 5, 100/89% at an MOI of 10, and 100/105% at an MOI of 20 (average values from four experiments, AdWT-infected control cells/AdPFK2L-infected cells) (Fig. 1). In INS1 cells, GK protein levels were 100/108% at an MOI of 20, 100/89% at an MOI of 40, and 100/105% at an MOI of 80 (average values from four experiments, AdWT-infected control cells/AdPFK2L-infected cells) (Fig. 1). GK gene expression was also not affected (data not shown). Thus, the overexpression increased the GK activity on the post-translational level.

Effects of PFK-2/FBPase-2 overexpression on glucose metabolism in RINm5F-GK and INS1 cells. RINm5F-GK and INS1 cells were infected with AdPFK2L at MOIs, which resulted in an optimal activation of GK activity (Figs. 1 and 2). At 48 h after viral infection, glucose metabolism was assessed by the glucose oxidation rate (Fig. 2). As expected, from the glucose sensor concept of GK, the production of 14CO2 from D-[U-14C]glucose increased in a concentration-dependent manner in both RINm5F-GK and INS1 cells. Cells with an activated GK after overexpression of the liver PFK-2/FBPase-2 showed significantly higher glucose oxidation rates of 50% (RINm5F-GK) and 100% (INS1) at all glucose concentra-

FIG. 1. Adenoviral overexpression of rat liver PFK-2/FBPase-2 and its effect on GK enzyme activity in RINm5F-GK (A) and INS1 (B) cells. Cells were transduced for 2 h with wild-type AdWT (lanes 1, 3, and 5 (C)) or liver PFK-2/FBPase-2 AdPFK2L (lanes 2, 4, and 6) adenoviral constructs using MOI 5, 10, and 20 (A) and MOI 20, 40, and 80 (B). The experiments were performed 48 h after the viral incubation. For Northern blot analyses, 10 µg of total RNA was loaded per lane. The blots were probed with antisense cRNA coding for rat liver PFK-2/FBPase-2 by nonradioactive hybridization. Shown are representative blots of four independent experiments. For Western blot analyses, 20 µg cellular protein was analyzed per lane by immunoblotting using a specific antibody against FBPase-2 or GK. Representative blots of four independent experiments are shown. GK activities were measured spectrophotometrically in cell extracts after sonication. Data are expressed as the percentage of enzyme activity measured in AdWT cells. Means ± SE from six to eight individual experiments are shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with enzyme activity of control AdWT cells (ANOVA/Bonferroni’s test).

FIG. 2. Effect of rat liver PFK-2/FBPase-2 overexpression on glucose metabolism in RINm5F-GK and INS1 cells. RINm5F-GK and INS1 cells were infected with AdPFK2L at MOIs, which resulted in an optimal activation of GK activity (Figs. 1 and 2). At 48 h after viral infection, glucose metabolism was assessed by the glucose oxidation rate (Fig. 2). As expected, from the glucose sensor concept of GK, the production of 14CO2 from D-[U-14C]glucose increased in a concentration-dependent manner in both RINm5F-GK and INS1 cells. Cells with an activated GK after overexpression of the liver PFK-2/FBPase-2 showed significantly higher glucose oxidation rates of 50% (RINm5F-GK) and 100% (INS1) at all glucose concentra-

Effects of PFK-2/FBPase-2 overexpression on glucose metabolism in RINm5F-GK and INS1 cells. RINm5F-GK and INS1 cells were infected with AdPFK2L at MOIs, which resulted in an optimal activation of GK activity (Figs. 1 and 2). At 48 h after viral infection, glucose metabolism was assessed by the glucose oxidation rate (Fig. 2). As expected, from the glucose sensor concept of GK, the production of 14CO2 from D-[U-14C]glucose increased in a concentration-dependent manner in both RINm5F-GK and INS1 cells. Cells with an activated GK after overexpression of the liver PFK-2/FBPase-2 showed significantly higher glucose oxidation rates of 50% (RINm5F-GK) and 100% (INS1) at all glucose concentra-
clones L5 and L11 (PFK-2/FBPase-2 is insensitive to regulation of the kinase/ islet/brain isoform of PFK-2/FBPase-2 reaction sites for PKA (Fig. 3; clones LM11 and LM12). The liver isoform (Fig. 3; clones I4 and I10), the liver isoform (Fig. 3; clones L5 and L11 (lanes 3 and 4), and RINm5F-GK cells stably overexpressing PFK-2/FBPase-2 (Fig. 3). In RINm5F-GK cells stably overexpressing PFK-2/FBPase-2, GK protein levels were 100% in control cells, 106% in the L11 clone, 95% in the L14 clone, 101% in the L5 clone, 91% in the L11 clone, 99% in the LM11 clone, and 92% in the LM12 clone (average values from four experiments) (Fig. 3). Therefore, the activation of GK enzyme activity was apparently the result of a posttranslational regulation. The data indicate that the activation of GK by PFK-2/FBPase-2 also occurs with the islet-specific isoform. The most intriguing explanation for the activation of the GK was an allosteric activation by F-2,6-P2.

PFK-2/FBPase-2 overexpression promotes glucose oxidation in RINm5F-GK cells. Rates of glucose oxidation, measured as production of $^{14}$CO$_2$ from $[$U-$^{14}$C]$\text{glucose}$, increased in a concentration-dependent manner in RINm5F-GK cells overexpressing the islet as well as the liver and the liver mutant PFK-2/FBPase-2 isoforms. In each case, they were significantly higher than in RINm5F-GK control cells not overexpressing PFK-2/FBPase-2 (Fig. 4A–C). Theoretically, the stimulatory effect on glucose metabolism in PFK-2/FBPase-2–overexpressing RINm5F-GK cells could be explained by allosteric activation of the PFK-1 by F-2,6-P$_2$. However, in control experiments, it was demonstrated that RINm5F-GK cells, in which high-affinity hexokinase activities dominate the glucose phosphorylation, do not show any changes in the glucose oxidation rate in the micromolar and millimolar concentration range after stable overexpression of liver PFK-2/FBPase-2 (data not shown). Thus, the increase of glucose metabolism in PFK-2/FBPase-2 overexpressing cells was conferred by the activity status of the GK enzyme and may be independent of F-2,6-P$_2$.

Stable overexpression of PFK-2/FBPase-2 in RINm5F-GK cells. After transfection of different isoforms of PFK-2/FBPase-2 into RINm5F-GK cells, we selected overexpressing clones for the rat islet/brain isoform (Fig. 3; clones I4 and I10), the liver isoform (Fig. 3; clones L5 and L11), and a liver mutant lacking the phosphorylation sites for PKA (Fig. 3; clones LM11 and LM12). The islet/brain isoform of PFK-2/FBPase-2 reflects the situation in insulin-producing cells, whereas the liver mutant of PFK-2/FBPase-2 is insensitive to regulation of the kinase/ bisphosphatase activity ratio (36,37). All clones showed a significant increase of PFK-2/FBPase-2 expression on both the mRNA and protein level (Fig. 3). In parallel with the level of PFK-2/FBPase-2 overexpression, there was a significant increase in the GK enzyme activity to 148 and 178% in clones I10 and I4, to 155 and 230% in clones L5 and L11, and to 130 and 216% in clones LM11 and LM12, respectively (Fig. 3). Northern blot analyses and densitometric quantification revealed that overexpression of PFK-2/FBPase-2 did not affect the GK mRNA levels in RINm5F-GK cells (Fig. 3). Comparable to the adenoviral overexpression of PFK-2/FBPase-2 (Fig. 1), the level of GK protein expression remained unchanged in all six RINm5F clones overexpressing PFK-2/FBPase-2 (Fig. 3). The mRNA and protein levels of PFK-2/FBPase-2 were 100% in control cells, 106% in the L11 clone, 95% in the L4 clone, 101% in the L5 clone, 91% in the L11 clone, 99% in the LM11 clone, and 92% in the LM12 clone (average values from four experiments) (Fig. 3).

Therefore, the activation of GK enzyme activity was apparently the result of a posttranslational regulation. The data indicate that the activation of GK by PFK-2/FBPase-2 also occurs with the islet-specific isoform. The most intriguing explanation for the activation of the GK was an allosteric activation by F-2,6-P$_2$. However, the cellular levels of F-2,6-P$_2$ were 20 ± 4 pmol/mg DNA in RINm5F-GK control cells, 20 ± 3 pmol/mg DNA in the islet PFK-2/FBPase-2 clone, 6 ± 1 pmol/mg DNA in the L11 liver PFK-2/FBPase-2 clone, and 31 ± 4 pmol/mg DNA in the LM12 liver mutant PFK-2/FBPase-2 clone (means ± SE from seven independent measurements at 10 mmoI/l glucose). Thus, the increase of GK activity brought about by overexpression of PFK-2/FBPase-2 was not due to an allosteric activation of the enzyme by F-2,6-P$_2$.
activity ratio of the liver PFK-2/FBPase-2. Because F-2,6-P2 levels in cell clones L5 and L11 significantly decreased after forskolin treatment, it is likely that the activities of the bifunctional enzyme were shifted toward the FBPase-2 because of phosphorylation by a PKA. This inhibitory effect of forskolin (10 μmol/l) was also confirmed in experiments on RINm5F-GK cells transfected with the liver PFK-2/FBPase-2 isofrom adenoviral vectors (data not shown).

In contrast, forskolin (10 μmol/l) did not abolish the significant increase of GK enzyme activity in the cell clones I10 and I4 overexpressing the islet PFK-2/FBPase-2 isoform. Forskolin (10 μmol/l) did also not abolish the significant increase of GK enzyme activity in the cell clone LM12 overexpressing the liver mutant PFK-2/FBPase-2 isoform, which lacks a regulatory phosphorylation site and thus a key component of the bisphosphatase active site of this bifunctional enzyme (Fig. 5B and C) (29,36,37).

**Fig. 4.** Effect of PFK-2/FBPase-2 overexpression on the glucose oxidation rate in RINm5F-GK cells. Measurements were performed in RINm5F-GK–PFK-2/FBPase-2 islet clone I4 (A), RINm5F-GK–PFK-2/FBPase-2 liver clone L11 (B), and RINm5F-GK–PFK-2/FBPase-2 liver mutant clone LM12 (C) (D), compared with RINm5F-GK control cells (○). Cells were grown overnight at 10 mmol/l glucose. After 48 h, cells were incubated for 1 h at 37°C in Krebs-Ringer buffer without glucose. Thereafter, glucose metabolism was measured in the presence of various glucose concentrations (0.5, 1, 2, 5, and 10 mmol/l). Glucose oxidation was calculated from the production of 14CO2. Means ± SE from five individual experiments are shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the glucose oxidation rate in control cells (Student’s t test).

**Fig. 5.** Effect of forskolin on GK enzyme activity in RINm5F-GK cells overexpressing PFK-2/FBPase-2. Measurements were performed in RINm5F-GK–PFK-2/FBPase-2 liver clones L5 and L11 (A), RINm5F-GK–PFK-2/FBPase-2 islet clones I10 and I4 (B), and RINm5F-GK–PFK-2/FBPase-2 liver mutant clone LM11 and LM12 (C). Cells were grown overnight at 10 mmol/l glucose. Thereafter, cells were incubated for 2 h in the presence of 10 μmol/l forskolin. Finally, cells were homogenized by sonication and enzyme activities were measured spectrophotometrically. Data are expressed as the percentage of enzyme activity measured in untreated RINm5F-GK control cells. Means ± SE from six to ten individual experiments are shown. *P < 0.05, **P < 0.01 compared with enzyme activity in untreated control cells; ***P < 0.001 compared with enzyme activity in the absence of forskolin (ANOVA/Bonferroni’s test).
medium from 2 to 10 mmol/l increased the GK enzyme activity significantly in control RINm5F-GK insulin-producing cells (Fig. 6). Overexpression of the islet (clone I4) as well as the liver (clone L11) and the liver mutant (clone LM12) PFK-2/FBPase-2 forms resulted in a threecold greater stimulatory effect of glucose on GK enzyme activity when the glucose concentration was increased from 2 to 10 mmol/l (Fig. 6). The stimulatory effect of PFK-2/FBPase-2 overexpression on GK enzyme activity was due to a significant increase of the V_max values to 222% (islet isoform) and 236% (liver isoform), whereas the S_0.5 values were not significantly different to those of RINm5F-GK control cells (Fig. 7, Table 1). The S_0.5 values for glucose were somewhat lower than those in pancreatic islets and liver presumably because of the high activity levels of low-K_m hexokinases in RINm5F-GK cells, which apparently interfere with the kinetic measurements of GK. Overexpression of liver PFK-2/FBPase-2 resulted in a significantly higher cooperativity for glucose with a Hill coefficient of 2.6 (Table 1). Notably, the level of GK protein expression was not affected by the overexpression of PFK-2/FBPase-2 (Fig. 6). The GK protein levels were 100% at 2 mmol/l and 107% at 10 mmol/l glucose in control cells, 101% at 2 mmol/l and 95% at 10 mmol/l glucose in the I4 clone, 101% at 2 mmol/l and 96% at 10 mmol/l glucose in the L11 clone, and 100% at 2 mmol/l and 101% at 10 mmol/l glucose in the LM12 clone (average values from three experiments).

**Effect of the anti–PFK-2/FBPase-2 antibody on GK enzyme activity in PFK-2/FBPase-2 overexpressing RINm5F-GK cells.** The significant increase of GK enzyme activity in RINm5F-GK cells after overexpression of the islet (clone I4) as well as the liver (clone L11) and the liver mutant (clone LM12) PFK-2/FBPase-2 forms was also significantly antagonized by incubation with an anti-

**DISCUSSION**

The GK enzyme is regulated in a complex manner on both the transcriptional and posttranslational level. Posttranslational mechanisms of GK regulation play a pivotal role in the adaptation of the enzyme activity to the physiological needs of the nutritional status in both liver and pancreatic β-cells. In pancreatic β-cells, glucose is a key regulator of GK enzyme activity by a currently unknown mechanism. There is evidence that the GK interacts with intracellular structures in insulin-producing cells providing an indication for an enzyme activity regulation through interaction with specific proteins (13,21,22). In a systematic random peptide phage display library screening, we recently identified the bifunctional enzyme PFK-2/FBPase-2 as a GK binding partner (23). PFK-2/FBPase-2 was transiently and stably overexpressed in RINm5F-GK and INS1 insulin-producing cells to address the functional relevance of this interaction as it relates to the regulation of the GK enzyme activity.

Overexpression of PFK-2/FBPase-2 significantly increased GK enzyme activities in both RINm5F-GK and INS1 cells. The overexpression of PFK-2/FBPase-2 stimulated GK enzyme activity at the posttranslational level, whereas GK protein levels remained unchanged in comparison to control cells. The GK activation in PFK-2/FBPase-2 overexpressing cells resulted in a higher glucose oxidation rate, demonstrating that increased GK activity is coupled with an increased metabolism of glucose in insulin-producing cells (Fig. 2). It should be mentioned in this context that in insulin-producing cell lines, glucose metabolism is partly determined by high-affinity hexokinase isoenzymes, which are expressed at considerable levels. Thus, in these model cells the kinetics of the glucose oxidation rate showed an increase in the lower millimolar concentration range, which does not necessar-

**FIG. 6. Effect of glucose on GK enzyme activity in RINm5F-GK cells.** Measurements were performed in RINm5F-GK–PFK-2/FBPase-2 islet clone 14, RINm5F-GK–PFK-2/FBPase-2 liver clone L11, and RINm5F-GK–PFK-2/FBPase-2 liver mutant clone LM12, compared with RINm5F-GK control cells. Cells were grown overnight at 10 mmol/l glucose. Thereafter, cells were incubated for 4 h at 2 or 10 mmol/l glucose. Finally, cells were homogenized by sonication, and enzyme activities were measured spectrophotometrically. For Western blot analyses, 20 μg cellular protein was analyzed per lane by immunoblotting using a specific antibody against GK. Representative blots of three independent experiments are shown. Data are expressed as per-

**FIG. 7. Kinetic characteristics of GK in RINm5F-GK cells overexpressing PFK-2/FBPase-2.** Measurements were performed in cellular extracts from RINm5F-GK–PFK-2/FBPase-2 islet clone 14 (●), RINm5F-GK–PFK-2/FBPase-2 liver clone L11 (■), and RINm5F-GK control cells (○). Cells were grown overnight at 10 mmol/l glucose. Thereafter, cells were homogenized by sonication, and GK enzyme activity was determined spectrophotometrically in the presence of the indicated concentrations of glucose. Data are expressed in units per milligram cellular protein. Means ± SE from four individual experiments are shown. *P < 0.05, **P < 0.001 compared with enzyme activity in the presence of 2 mmol/l glucose (ANOVA/Bonferroni’s test).
ily reflect the situation in pancreatic islets and liver with a predominant coupling of glucose metabolism by the GK enzyme.

To further evaluate the mechanisms by which PFK-2/FBPase-2 activates GK the cAMP-sensitive liver isoform, a liver mutant devoid of PKA phosphorylation sites and of FBPase-2 activity as well as the islet/brain PFK-2/FBPase-2 isoform were stably expressed in RINm5F-GK cells. Notably, overexpression of the wild-type and mutant liver isoform as well as the islet/brain isoenzyme of PFK-2/FBPase-2 resulted in an increased GK enzyme activity (Fig. 3). Thus, PFK-2/FBPase-2 isoforms with different kinetic properties could activate the GK enzyme in insulin-producing cells.

The bifunctional enzyme PFK-2/FBPase-2 controls the intracellular levels of F-2,6-P$_2$. The physiological relevance of F-2,6-P$_2$ in glucose metabolism has been discussed previously and is controversial (49–51). However, there is a general consensus that glucose at millimolar concentrations can increase F-2,6-P$_2$ in pancreatic islets. This means that the kinase/bisphosphatase ratio of the bifunctional enzyme is dominated by the kinase activity under physiological conditions. This raises the question of whether the activation of GK in PFK-2/FBPase-2 overexpressing cells is related to the intracellular level of F-2,6-P$_2$. In the present study, overexpression of the different PFK-2/FBPase-2 isoforms in insulin-producing cells resulted in variant kinase/bisphosphatase ratios as deduced from the intracellular concentrations of F-2,6-P$_2$. In RINm5F-GK cells, we observed low F-2,6-P$_2$ levels by overexpressing the liver isoform, high levels by overexpressing the phosphatase-deficient liver isoform, and intermediate levels by overexpressing the islet isoform. Thus, it is unlikely that the activation of GK by PFK-2/FBPase-2 is due to generation of F-2,6-P$_2$. Additionally, this contention is supported by the fact that F-2,6-P$_2$ had no significant effect on the kinetic characteristics of recombinant GK protein and is therefore not an allosteric effector of GK.

Notably, the lower intracellular levels of F-2,6-P$_2$ in RINm5F-GK cells overexpressing the liver isoform of PFK-2/FBPase-2 apparently did not impair glucose metabolism. This was evident from the glucose oxidation rates in the forskolin-treated liver PFK-2/FBPase-2 overexpressing RINm5F-GK cells, which were not significantly different from those of RINm5F-GK control cells. Thus, the lower F-2,6-P$_2$ levels in the L11 cell clone apparently did not negatively affect glycolysis in insulin-producing cells. It should be emphasized that F-2,6-P$_2$ is not an exclusive activator of PFK-1 in insulin-producing cells because long-chain acyl-CoA has been shown to be a potent stimulator of this key glycolytic enzyme (52). Thus, biofactors other than F-2,6-P$_2$ may participate in the regulation of the glycolytic pathway.

Yeast two-hybrid studies clearly show that the GK protein interacts with the phosphatase domain of the PFK-2/FBPase-2 protein (23). The interaction with the FBPase-2 domain is a prerequisite for the activation of GK because this process could be efficiently blocked by an antibody raised against the FBPase-2 domain of the enzyme. Forskolin, which shifted the activity ratio of the liver PFK-2/FBPase-2 isoform toward the FBPase-2 activity and modulated the conformation of the PFK-2/FBPase-2 homodimer, counteracted the activation of GK. Forskolin, which shifted the activity ratio of the liver PFK-2/FBPase-2 isoform toward the FBPase-2 activity and modulated the conformation of the PFK-2/FBPase-2 homodimer, counteracted the activation of GK (Fig. 5). As expected, the effect of forskolin was evident only in RINm5F-GK cells overexpressing the cAMP-sensitive liver isoform of PFK-2/FBPase-2 but not in cells overexpressing the islet/brain isoform or the mutant liver isoform, which lack the phosphorylation site for cAMP-dependent protein kinase A.

The prevention of GK activation by forskolin provides evidence that a high FBPase-2 activity of the phosphorylated liver PFK-2/FBPase-2 enzyme may significantly affect binding to the GK protein through an unfavorable conformation of the PFK-2/FBPase-2 protein. Thus, the cellular stoichiometry between the phosphorylated and dephospho-

---

**Table 1**

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>$V_{\text{max}}$ (units/mg protein)</th>
<th>$S_{0.5}$ (mmol/l)</th>
<th>$n_{\text{Hill}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.6 ± 0.6</td>
<td>3.2 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>RINm5F-GK cell clone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Islet PFK-2/FBPase I4</td>
<td>19.1 ± 0.9*</td>
<td>3.1 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Liver PFK-2/FBPase L11</td>
<td>20.3 ± 1.1*</td>
<td>3.7 ± 0.3</td>
<td>2.6 ± 0.3*</td>
</tr>
</tbody>
</table>

Data are means ± SE from four independent experiments. Glucokinase enzyme activity was measured in cell homogenates by a glucose-6-phosphate dehydrogenase–coupled spectrophotometric assay. Statistical analyses were performed with ANOVA followed by Bonferroni’s test for multiple comparisons. *$P < 0.001$ compared with RINm5F-GK control cells.

---

**Figure 8**

Effect of anti-PFK-2/FBPase-2 antibody on GK enzyme activity in RINm5F-GK-PFK2 cells. Measurements were performed in RINm5F-GK-PFK2/FBPase-2 islet clone I4, RINm5F-GK-PFK-2/FBPase-2 liver clone L11, and RINm5F-GK-PFK-2/FBPase-2 liver mutant clone LM12, compared with RINm5F-GK control cells. Cells were grown overnight at 10 mmol/l glucose. Thereafter, cells were homogenized by sonication and incubated for 1 h in the presence of FBPase-2 antibody (1:1,000). Enzyme activities were measured spectrophotometrically. Data are expressed as the percentage of enzyme activity measured in RINm5F-GK control cells in the absence of FBPase-2 antibody. Means ± SE from four to seven individual experiments are shown. ***$P < 0.001$ compared with enzyme activity in untreated control cells; ****$P < 0.001$ compared with enzyme activity in the absence of FBPase-2 antibody (ANOVA/Bonferroni’s test).
The ability of elevated F-2,6-P2 concentrations to upregulate transcriptional regulation due to tissue-specific mechanisms is a unique characteristic of the hepatic GK regulating gene expression in the absence of insulin (56,57).

This contention is supported by the fact that forskolin overexpressing high levels of liver PFK-2/FBPase-2, the remaining portion of dephosphorylated enzyme may therefore be sufficient to activate the GK enzyme despite a predominant phosphorylated form of PFK-2/FBPase-2.

It is not clear at the moment if the PFK-2 domain of the PFK-2/FBPase-2 is required for the activation of the GK enzyme. Mutation studies on recombinant liver PFK-2/FBPase-2 protein indicate that the phosphorylation of the enzyme by a PKA may induce conformational changes both at the NH2- and COOH-termini of the protein, which are critical for the change of the kinase/bisphosphatase activity ratio (29,53,54).

The binding to PFK-2/FBPase-2 may stabilize a specific conformation of the GK enzyme, resulting in a higher catalytic activity. Another feasible explanation for the activation of GK could be a protection against intramolecular sulphydryl group oxidation within the enzyme protein. In contrast to the high-affinity hexokinase isoenzymes (types I–III), the GK protein is highly susceptible to sulphydryl group oxidation, although mammalian hexokinases share the same spatial cysteine pattern (17,18). This GK sulphydryl group oxidation occurs constitutively in an environment that does not provide protection, e.g., by thiol reagents (16,17).

Importantly, PFK-2/FBPase-2 overexpression amplified the stimulatory effect of glucose on the GK enzyme activity in RINm5F-GK cells (Fig. 6) and significantly increased the Vmax of GK activity (Fig. 7, Table 1). Because the interaction did not affect the S0.5 of GK, the glucose sensor function of the enzyme remains in the physiological concentration range. Thus, PFK-2/FBPase-2 acts as an amplifier of glucose as the key regulator of GK activity on the posttranslational level (9–13,55). Glucose may act in a dual way, inducing a release of the GK protein from matrix proteins or secretory granules and, through a slow transition, increasing the intrinsic activity by conformational changes induced by the substrate glucose (13–15,21).

The posttranslational regulation of GK enzyme activity by interaction with PFK-2/FBPase-2 in insulin-producing cells must be distinguished from the activation of GK mRNA and protein expression by F-2,6-P2 in liver (56,57). The ability of elevated F-2,6-P2 concentrations to upregulate GK gene expression in the absence of insulin (56–58) is a unique characteristic of the hepatic GK regulating system due to tissue-specific promoters (4,59).

In the present study, the activation of GK enzyme activity by interaction with PFK-2/FBPase-2 has been demonstrated in insulin-producing cells. The activation of GK by PFK-2/FBPase-2 could also be demonstrated with recombinant proteins in a reconstituted in vitro system indicating that an interaction of the two proteins is sufficient to increase GK activity (S.B., unpublished data). The regulatory principle of the GK interaction with PFK-2/FBPase-2 may also be of relevance for GK activity regulation in liver because both proteins are abundantly expressed in hepatocytes. In liver, the binding of GK to PFK-2/FBPase-2 has to be integrated into the concept of GK regulation through transcriptional activation of the GK gene (4) and posttranslational modulation by the liver GK regulatory protein (26,60). An increase of the blood glucose concentration stimulates GK gene and protein expression via insulin and/or high F-2,6-P2 concentrations and the translocation of GK from the nucleus to the cytoplasm. At this point, the interaction of GK with PFK-2/FBPase-2 may contribute to the increase of the GK activity. Furthermore, the deactivation of GK by PKA in the liver is consistent with the promotion of gluconeogenesis by glucagon, which proceeds via cAMP.

In pancreatic β-cells, the GK gene is physiologically not regulated by hormones on the level of enzyme protein, which remains more or less unaffected by the nutritional status (13,61). A rationale for the stable levels of GK in pancreatic β-cells may be the indispensable glucose sensor function that does not allow drastic changes of protein expression and enzyme activity as they occur in the liver in dependence upon the induction by insulin and the repression by glucagon (7). Thus, posttranslational enzyme regulation is crucial to maintain the glucose sensor function of GK in β-cells. Moreover, the tight coupling of the GK activity to insulin release via ATP/ADP suggests that the metabolic fluxes from glucose-6-phosphate through glycolysis, the tricarboxylic acid cycle, and electron transport are all coordinated.

The interaction of GK with the bifunctional enzyme PFK-2/FBPase-2 is an entirely novel element in the posttranslational regulation of GK. There is evidence that the GK enzyme exists in two different activity states: 1) a freely diffusible state corresponding to a high intrinsic activity through interaction with activating or stabilizing proteins such as the PFK-2/FBPase-2 and 2) a matrix-bound state to inhibitory proteins (13) and insulin granules (20,21,62) corresponding to a low intrinsic activity. Glucose is the key molecule, which shifts the GK enzyme from a low activity state to a high activity state by a mechanism that is as yet not completely understood on the molecular level. However, there is evidence from kinetic data (14,63) and recent studies with fluorescently labeled GK protein (21) that glucose induces conformational changes, which are apparently responsible for the shift toward a high-activity state. Because chemical activators of GK are under development as a new promising class of antidiabetic drugs (24), the knowledge of the GK-PFK-2/FBPase-2 interaction may help to find the rationale for the physiological upregulation of GK and new perspective therapeutic strategies to activate or preserve the glucose sensor function of GK in type 2 diabetes.

ACKNOWLEDGMENTS
This work was supported by a grant from the Ministry of Science and Culture of Lower Saxony (to L.M.) and the
REFERENCES


