The first step of glucose metabolism is the phosphorylation of glucose, catalyzed by the hexokinase family of enzymes. To address the metabolic impact of increasing glucose phosphorylation capacity in liver, rat primary hepatocytes were treated with recombinant adenoviruses containing the cDNAs encoding either rat liver glucokinase (AdCMV-GKL) or rat hexokinase I (AdCMV-HKI). Maximal glucose phosphorylation in AdCMV-GKL- and AdCMV-HKI-treated hepatocytes was increased 6.3- and 6.3-fold, respectively, compared to hepatocytes treated with an adenovirus expressing β-galactosidase. Glucose usage (measured with 3 and 20 mM 2-[3H]glucose and 5-[3H]glucose) was significantly increased in AdCMV-GKL-treated cells preincubated in 1 or 25 mM glucose. Treatment of hepatocytes with AdCMV-HKI also caused enhanced glucose utilization, but the increases were smaller and were less apparent in cells preincubated in high (25 mM) glucose. AdCMV-GKL-treated hepatocytes incubated for 48 h in the presence of variable glucose concentrations had glycogen levels that were maximally 15.0 ± 0.6-fold greater than levels in corresponding control cells. AdCMV-HKI-treated hepatocytes incubated under similar conditions had unchanged glycogen levels relative to controls. In AdCMV-GKL-treated cells, lactate output was increased to a maximum of 3.0 ± 0.4-fold (at 25 mM glucose), glucose oxidation was increased 3.5 ± 0.3-fold, and triglyceride production was unchanged relative to untreated cells. Among these three parameters, only lactate production was increased in AdCMV-HKI-treated cells, and then only at low glucose concentrations. We conclude that overexpression of glucokinase has potent effects on glucose storage and utilization in hepatocytes and that these effects are not matched by overexpression of hexokinase I.

The hexokinases (HKs), a family of four isozymes, catalyze the conversion of glucose to glucose-6-phosphate (Glc-6-P), the first committed step in glucose metabolism (1). Glucokinase (GK or HK IV) and HK I are both expressed in the pancreas and the liver but differ in their kinetic characteristics, regulation, and metabolic function (1-3). GK has an S0.5 for glucose in the physiological range (8-10 mM), and is thought to play a central role in the regulation of glucose-stimulated insulin secretion in the pancreas (2, 4) and the maintenance of glucose homeostasis by the liver (3). HK I has a low Ks for glucose (0.1 mM) and has been ascribed roles in the regulation of basal insulin secretion in the β-cell (5) and glycolysis and oxidative metabolism in other tissues (1).

Using recombinant adenoviruses, Becker et al. (5, 6) demonstrated differential metabolic effects of GK and HK I overexpression in isolated rat islets of Langerhans. GK overexpression had no effect on 2- or 5-[3H]glucose usage, lactate accumulation, glycogen content, or glucose oxidation and minimal effects on glucose-stimulated insulin secretion. Conversely, HK I overexpression significantly increased all of these metabolic parameters except glycogen content; these changes were most apparent at low glucose concentrations (3 mM). These results suggested that there is a functional segregation of the two HK isoforms in the islet, possibly by physical compartmentation (6). What is not known is if functional segregation is specific to islet cells and whether overexpression of either HK I or GK in hepatocytes can have distinct metabolic effects. Valera and Bosch (8) demonstrated that GK overexpression in two hepatoma cell lines profoundly increased glucose accumulation and lactate production, suggesting that increases in GK can alter liver glucose metabolism. Neither cell line, however, expresses endogenous GK, making it impossible to conclude that overexpression of GK or any other HK isoform in primary hepatocytes with a normal complement of GK activity would have similar metabolic effects. Additionally, it is not known if the glucokinase regulatory protein that is proposed to play a role in the acute regulation of GK activity (9) is present in the hepatoma cell lines. In the present study, therefore, we have used the recombinant adenovirus system to evaluate the metabolic impact of GK and HK I overexpression in cultured rat hepatocytes.

**MATERIALS AND METHODS**

Preparation of Recombinant Adenoviruses—Recombinant adenoviruses containing cDNAs encoding rat hexokinase I (AdCMV-HKI), rat liver glucokinase (AdCMV-GKL), rat islet glucokinase (AdCMV-GKL), or the E. coli β-galactosidase gene (AdCMV-βGal) were prepared as described previously (5-7).

**Hepatocyte Isolation, Culturing, and Treatment with Recombinant Adenovirus—**Primary hepatocytes were prepared from overnight fasted 200–300-g male Wistar rats using an adaptation of the method of Massague and Guinnovart (10). Isolated hepatocytes were incubated in a 37°C water bath for 30 min with intermittent gentle shaking to remove dead cells. After 2 centrifugations at 300 rpm for 2 min each and resuspension in DMEM containing 25 mM glucose, 10% fetal bovine
served in AdCMV-GKL-treated cells (7.1 relative to controls) that was significantly greater than in extracts from untreated or AdCMV-βGal-treated cells (p < 0.01). The symbol (#) indicates that glucose phosphorylation is significantly greater than in extracts from untreated or AdCMV-βGal-treated cells (p < 0.01).

**RESULTS**

**GK and HK I Overexpression in Rat Primary Hepatocytes**—Glucose phosphorylating capacity was measured in hepatocyte extracts 48 h after treatment with AdCMV-GKL, AdCMV-HKI, or AdCMV-βGal (Fig. 1). Preliminary experiments determined that maximal glucose phosphorylating activity was achieved 48 h post-treatment, with no further increase at 72 h (data not shown). There was no significant difference in glucose phosphorylation between untreated and AdCMV-βGal-treated cells assayed at 3 mM or 20 mM glucose. Using a radiometric assay (11), AdCMV-HKI-treated hepatocytes exhibited an increased glucose phosphorylating capacity at 3 mM glucose (18.7 ± 2.4-fold relative to controls) that was significantly greater than observed in AdCMV-GKL-treated cells (7.1 ± 1.2-fold). Analysis of the same samples at 20 mM glucose revealed a 7.1 ± 0.5-fold increase in AdCMV-GKL-treated cells relative to untreated controls, compared to a 6.3 ± 0.8-fold increase in AdCMV-HKI-treated hepatocytes (maximal values of 14.4 ± 2.4) and 12.7 ± 1.6 μmol/min/g of protein, respectively. Use of the radioisotopic assay allows addition of 10 mM Glc-6-P to the assays, which abolished AdCMV-HKI-induced increases in glucose phosphorylation (Fig. 1), but had no effect on glucose phosphorylation in AdCMV-GKL-treated cells (data not shown). These data are consistent with the known kinetic and allosteric properties of HK I and GK (1, 2). We also measured glucose phosphorylation capacity by a spectrophotometric assay. This method revealed very similar changes in fold induction for both AdCMV-GKL and AdCMV-HKI as the radiometric assay (7.5- and 5.7-fold, respectively; mean of 2–3 determinations for each condition), but the absolute values attained were higher (11.6, 87.0, and 66.6 μmol/min/g of protein for untreated, AdCMV-GKL-treated, and AdCMV-HKI-treated cells, respectively). This difference in absolute values attained with the two methods is likely due to the fact that the radiometric assay measures accumulation of U-14C-labeled glucose 6-phosphate over 90 min in whole cell extracts, whereas the spectrophotometric assay is linked to glucose 6-phosphate dehydrogenase and performed on 10,000 x g supernatants, leaving open the possibility that Glc-6-P accumulation is underestimated in the former assay because of the presence of glucose 6-phosphatase or other enzymes in the extract.

Glucose Usage and Glycolytic and Glycogenic Flux in AdCMV-GKL- and AdCMV-HKI-treated Hepatocytes—To determine the effects of GK and HK I overexpression on liver cell metabolism, a number of parameters were assessed. In AdCMV-GKL-treated hepatocytes preincubated in 25 mM glucose for 44–46 h, 2-3H glucose usage was increased 5.5 ± 0.9-fold relative to control cells when assayed at 3 mM glucose, and 2.2 ± 0.2-fold when assayed at 20 mM glucose (Fig. 2A). Similar results were obtained for 5-3H glucose usage (Fig. 2B). Glucose usage in AdCMV-GKL-treated hepatocytes incubated in 1 mM glucose for 44–46 h was similar to that observed when cells were incubated in 25 mM glucose (Fig. 2, C and D). In AdCMV-HKI-treated cells preincubated in 25 mM glucose for 44–46 h, 3mM 2-3H glucose and 5-3H glucose usage was increased 2.1 ± 0.4 and 1.7 ± 0.2-fold, respectively, but 20 mM glucose usage was unchanged (Fig. 2, A and B). However, in AdCMV-HKI-treated hepatocytes preincubated in 1 mM glucose for 44–46 h, 2-3H glucose and 5-3H glucose usage increased 1.7 ± 0.3 and 1.8 ± 0.3-fold, respectively, when assayed at 20 mM glucose.

**Fig. 1. Glucose phosphorylation in rat primary hepatocytes.** Untreated primary hepatocytes or hepatocytes treated with recombinant adenovirus containing the cDNAs of rat liver glucokinase (AdCMV-GKL), rat hexokinase I (AdCMV-HKI), or β-galactosidase (AdCMV-βGal) were incubated for 44–46 h in DMEM containing 25 mM glucose. Crude cell homogenates were prepared, and glucose phosphorylating activity was measured in the presence of 3 or 20 mM U-14C-glucose in the absence or presence of 10 mM glucose 6-phosphate over 90 min at 37°C. Values represent the mean ± S.E. of at least six independent measurements. The asterisk (*) indicates that glucose phosphorylation is significantly greater than in extracts from untreated or AdCMV-βGal-treated cells (p < 0.01). The symbol (#) indicates that glucose phosphorylation assayed at 3 mM glucose is significantly greater in AdCMV-HKI-treated hepatocytes relative to AdCMV-GKL-treated cells (p < 0.05).
Untreated, AdCMV-GKL-treated, and AdCMV-HKI-treated hepatocytes were cultured in the presence of a range of glucose concentrations (1–25 mM) for 44–46 h. As shown in Fig. 3A, increasing glucose levels caused a gradual rise in glycogen accumulation (to a maximum of 0.1 mg/mg of protein) and medium lactate (to a maximum of 10 mmol/liter) in untreated hepatocytes. In AdCMV-GKL-treated hepatocytes, however, there was a much sharper rise in lactate and glycogen accumulation, to maximum levels of 1.5 ± 0.02 mg of glycogen/mg of protein and 30.0 ± 1.2 mmol of lactate/liter at 25 mM glucose. Interestingly, significant increases in lactate were observed at the lower glucose levels tested (1–3 mM), consistent with the increases in glucose usage in AdCMV-GKL-treated cells at 3 mM glucose (Fig. 2). In contrast, the first significant increase in glycogen accumulation in AdCMV-GKL-treated cells relative to untreated cells is observed at 5 mM glucose, and the major change occurs between 5 and 10 mM glucose. In AdCMV-HKI-treated hepatocytes, glycogen accumulation was not enhanced compared to untreated cells at any glucose concentration tested (Fig. 3B). Medium lactate, however, was increased to approximately the same level as in AdCMV-GKL-treated cells at 1, 3, and 5 mM glucose. Interestingly, in cells incubated in 25 mM glucose, lactate levels were the same in AdCMV-HKI-treated and untreated cells, suggesting that overexpressed HK I is metabolically inactive at higher glucose levels. Treatment of hepatocytes with a virus expressing the islet isoform of GK (AdCMV-GKI) resulted in similar metabolic effects to those observed for the liver isoform of GK, whereas AdCMV-βGal treatment had no metabolic effects (data not shown).

FIG. 2. 2-[3H]Glucose and 5-[3H]glucose usage in GK- and HK-overexpressing primary hepatocytes. Primary hepatocytes were treated with AdCMV-GKL or AdCMV-HKI or left untreated and incubated in the presence of DMEM containing 25 mM glucose (A and B) or 1 mM glucose (C and D) for 44–46 h. The medium was removed and replaced with Hanks’ buffered saline containing 3 or 20 mM 2-[3H]glucose (A and C) or 5-[3H]glucose (B and D). Glucose usage was calculated from the production of 3H2O over a 20-min period at 37°C. Values represent the mean ± S.E. of at least four independent measurements. The asterisk (*) indicates that glucose usage in the denoted condition is statistically different from the corresponding untreated control condition (p < 0.05).
posed to isotope exchange is indicated by the fact that glycogen content increased over the 8-h measurement period by 41-fold (from 0.02 to 0.83 mg of glycogen/mg of protein) in AdCMV-GKL-treated hepatocytes studied at 15 mM glucose. We conclude that the glucose concentration required for activation of lactate accumulation is lower than that required for activation of glycogen accumulation (Fig. 3) and that this is consistent with the relative rates of synthesis of these metabolites (Fig. 4).

Glucose Oxidation and Triglyceride Accumulation in AdCMV-GKL-treated Hepatocytes—To determine if increased glycolytic flux in AdCMV-GKL-treated hepatocytes leads to elevated flux through other metabolic pathways, glucose oxidation and triglyceride levels were measured in cells incubated for 48 h in the presence of 25 mM glucose. AdCMV-GKL treatment increased glucose oxidation by 3.5 ± 0.2-fold over untreated and AdCMV-HKI-treated hepatocytes (Fig. 5A), whereas triglyceride levels were unchanged (Fig. 5B).

Mitochondrial Binding of HK I in AdCMV-HKI-treated Hepatocytes—A possible mechanism to explain the relatively small metabolic effect of HK I is a failure of the overexpressed enzyme to target to the mitochondria. Mitochondrial binding of HK I is thought to increase the activity of the enzyme by reducing its inhibition by Glc-6-P and by increasing its affinity for ATP (1). To determine the proportion of hexokinase activities that bound to mitochondria, untreated, AdCMV-GKL-treated, and AdCMV-HKI-treated hepatocytes were assayed for cytosolic and mitochondrial glucose phosphorylation activity. AdCMV-HKI-treated hepatocytes exhibited a 13.2 ± 0.9-fold increase in cytosolic glucose phosphorylation activity (asayed at 3 mM glucose) compared to untreated control cells (Fig. 6). The mitochondria-associated activity represented 24% of the total in untreated cells and 27% of the total in AdCMV-HKI-treated cells, indicating that overexpressed hexokinase I partitions normally. In contrast, AdCMV-GKL-treated cells exhibited no increase in GK activity associated with the mitochondrial fraction, despite a 9.1 ± 1.4-fold increase in cytosolic GK activity (assayed at 20 mM glucose) (Fig. 6).
DISCUSSION

Glucose phosphorylation in "glucose-sensing" tissues such as the liver and islets of Langerhans has long been perceived to be controlled by glucokinase, the high $K_m$ member of the hexokinase gene family, even though both tissues express other hexokinases, notably hexokinase I. Hexokinase and glucokinase enzymatic activities are present at approximately equal levels in islet cell extracts, but glucose usage studies suggest that the low $K_m$ activity is inhibited in intact islet cells, allowing glucokinase to regulate glucose-stimulated insulin secretion (2, 4). In liver extracts, glucokinase represents approximately 85% of the total glucose phosphorylating activity (3, 15), ensuring that metabolic pathways such as glycogen synthesis and glycolysis are only enhanced at glucose concentrations that approach the glucokinase $K_m$ and that exceed levels typically found in the circulation in the fasting state. Recent studies from our laboratory have shown that glucokinase and hexokinase I undergo functional partitioning in the islets of Langerhans such that overexpression of hexokinase I has multifaceted metabolic effects, whereas overexpressed glucokinase has no metabolic impact (5, 6). The current study was undertaken to determine whether similar regulatory mechanisms exist in primary hepatocytes and whether overexpression of either isoform of hexoki-

FIG. 5. Glucose oxidation and triglycerides in GK and HK-I overexpressing primary hepatocytes. Primary hepatocytes were treated with AdCMV-GKL or AdCMV-HKI or left untreated and incubated for 40 h in DMEM containing 25 mM glucose. Oxidation of 20 mM [U-14C]glucose (A) was measured as described under "Materials and Methods." Data are expressed as fold increase relative to untreated cells, which oxidized glucose at a rate of 14.6 pmol/min/g of protein. Triglycerides (B) were determined colorimetrically as described under "Materials and Methods." Values for both glucose oxidation and triglycerides represent the mean ± S.E. of six independent measurements. The asterisk (*) indicates that glucose oxidation in the denoted group is significantly different from untreated controls (p < 0.01).

FIG. 6. Glucose phosphorylation activity in mitochondrial and cytosolic fractions from primary hepatocytes overexpressing GK or HK I. Primary hepatocytes were treated with AdCMV-GKL or AdCMV-HKI or left untreated and incubated in the presence of DMEM containing 25 mM glucose for 44–46 h. Cells were homogenized and separated into mitochondrial (A) and cytosolic (B) fractions as described under "Materials and Methods." Glucose phosphorylating activity was measured in the presence of 3 or 20 mM [U-14C]glucose in the absence or presence of 10 mM glucose-6-phosphate over 90 min at 37°C. Values represent the mean ± S.E. of four independent measurements. The asterisk (*) indicates that glucose phosphorylation in the denoted group is significantly greater than in the corresponding untreated control group (p < 0.001).
ing triglyceride formation, suggesting that overexpression of GK in vivo may be a mechanism for overcoming the elevated hepatic glucose output that contributes to hyperglycemia in non-insulin-dependent diabetes mellitus.

Overexpression of glucokinase in rat hepatocytes has a major metabolic impact (this study), in contrast to the absence of any measurable metabolic effects when the enzyme is overexpressed in isolated rat islets of Langerhans (6). In addition to its obvious effects in isolated hepatocytes, glucokinase overexpression also activates glycogen synthesis and lactate production in hepatoma cells (8). Furthermore, when overexpressed in the monkey kidney cell line CV-1, glucokinase causes a clear increase in in situ glucose phosphorylation (6). Similar enhancement of in situ glucose phosphorylation is not observed in AdCMV-GK-treated intact islets, despite large increases in enzyme activity in islet homogenates from the same group of experiments. These results suggest that overexpressed glucokinase is somehow suppressed in intact islet cells, either by a regulatory factor or by failure to effectively couple to more distal metabolic steps. Such a stringent regulatory mechanism clearly is not operative in the hepatocyte.

Overexpression of hexokinase I in hepatocytes has limited effects on glycolytic flux (glucose usage and lactate output) relative to glucokinase overexpression, despite equivalent increases in enzyme activity measured in hepatocyte extracts. The proportion of hexokinase activity associated with mitochon-dria-enriched cellular fractions is the same in control and AdCMV-HKI-treated cells, suggesting that the relatively weak metabolic effect induced by hexokinase I overexpression is not due to lack of access to mitochondrial binding sites. Metabolic activity of overexpressed hexokinase I was more readily demonstrable in hepatocytes that were preincubated in 1 mM glucose than in cells preincubated at 25 mM glucose. Thus, AdCMV-HKI-treated hepatocytes preincubated in 25 mM glucose for 44–46 h exhibited no increase in glucose usage when assayed at 20 mM glucose but a significant increase when assayed at 3 mM glucose. The corollary experiment, preincubation in 1 mM glucose, resulted in increased glucose usage at both 3 and 20 mM glucose. These data suggest that overexpressed hexokinase I becomes inhibited at higher glucose concentrations, most likely via increases in the levels of its allosteric effector, glucose 6-phosphate (1, 2). In support of this interpretation, AdCMV-HKI-treated hepatocytes incubated at 25 mM glucose have glucose 6-phosphate levels that are approximately 6-fold greater than levels found in untreated hepatocytes, with smaller increases occurring in AdCMV-HKI-treated hepatocytes incubated in 1 mM glucose for the same time period.2

In glucokinase-overexpressing cells, activation of glycolysis, an energy-producing pathway, occurs at glucose levels that are in or below the fastest range (1–5 mM), whereas the stimulation of glycogen synthesis occurs in the range of glucose concentrations associated with the fed state (5–10 mM). These results suggest that activation of glycogen synthesis is dependent upon a threshold concentration of glucose, whereas activation of glycolysis is not. Guinovart and co-workers (17–19) have proposed that glucose activates glycolysis through an increase in glucose 6-phosphate, which causes an activation of glycogen synthase. Glucose 6-phosphate also triggers the translocation of glycogen synthase from a free to a membrane-bound state (20, 21). Glucose 6-phosphate-regulated activation of glycogen synthase is in fact enhanced s AdCMV-GKL-treated hepatocytes,2 which, when coupled with the increased efficiency of substrate flux through the phosphorylation step, can explain the large increase in glycogen synthesis and accumulation.

The ability of overexpressed glucokinase, but not hexokinase I, to stimulate glycogen synthesis may also be influenced by the cellular localization of glucokinase. In a series of studies, Agius and Peak (22, 23) have demonstrated that glucokinase is translo-cated from a “bound” to a “free” state in response to glucose, fructose, or sorbitol. These investigators have hypothesized that translocation occurs in response to metabolic signals that cause dissociation of glucokinase from its regulatory protein. Furthermore, fructose and sorbitol are suggested to cause dissociation because they are precursors of fructose-1-phosphate, which decreases the affinity of the regulatory protein for glucokinase (9), whereas elevated glucose concentrations are thought to cause translocation by competitive inhibition of binding of the regulatory protein to glucokinase. Immunocytochemical data demonstrate that the regulatory protein and glucokinase are co-localized at the nucleus of hepatocytes cultured at low glucose concentrations and that glucokinase is translocated to the cytosol in response to administration of glucose or insulin (24, 25). Translocation of glucokinase to the cytosol has been shown to activate incorporation of 2-[3H], [U-14C]glucose into glycogen (22). These studies suggest that translocation of glucokinase from the bound to free state is important in the activation of glycogen synthesis. Our data also suggest that hexokinase does not participate in this regulation, even when the enzyme is overexpressed, probably because of a distinct subcellular localization relative to glucokinase.

The foregoing observations are consistent with the following model. At glucose concentrations of less than 5 mM, glucokinase exists at a discrete subcellular site by virtue of its association with the glucokinase regulatory protein. Glucose 6-phosphate that is generated by glucokinase when it is in the bound state is efficiently funneled into glycolysis. Increased glycolytic flux to lactate at low glucose concentrations in AdCMV-GKL-treated cells occurs either because the enzyme in the bound state has a decreased $K_m$ for glucose, or more likely, because the increase in total enzyme concentration at the bound site increases catalytic efficiency at low glucose without altering affinity. At glucose concentrations in excess of 5 mM that approach the $K_m$ of glucokinase, the activity of the enzyme increases sharply, resulting in a dramatic increase in the accumulation of hexose phosphates, including glucose 6-phosphate. These conditions induce activation of glycogen synthase and translocation of this enzyme and glucokinase, possibly to similar or adjacent sites, thereby activating glycogen synthesis.

Overexpression of glucokinase clearly enhances glucose storage and utilization in isolated hepatocytes. These results suggest gene therapy strategies that may be relevant to non-insulin-dependent diabetes mellitus. In normal mammals, the liver acts as an organ of net glucose synthesis and output, switching to an organ of net glucose uptake and storage in the fed state. In non-insulin-dependent diabetes mellitus, the liver fails to respond to postprandial increases in glucose and insulin levels by appropriately suppressing glucose output. Rossetti and colleagues (26, 27) have demonstrated a reduction in the glucoki-nase:glucose 6-phosphatase ratio in the liver of partially pan-createthymized rats, a model of non-insulin-dependent diabetes mellitus. Our results suggest that supplementation of glucokinase activity in such animals may enhance glucose clearance, possibly to an extent that will result in a decrease in circulating glucose concentrations. The recombinant adenovirus system has been used to deliver genes to the liver of intact animals with high efficiency (7, 16, 28) and should allow this hypothesis to be tested.

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