

Treatment of Type 2 Diabetes by Adenoviral-Mediated Overexpression of the Glucokinase Regulatory Protein

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The enzyme glucokinase (GK) plays a central role in glucose homeostasis. Hepatic GK activity is acutely controlled by the action of the GK regulatory protein (GKRP). In vitro evidence suggests that GKRP reversibly binds to GK and inhibits its activity; however, less is known about the in vivo function of GKRP. To further explore the physiological role of GKRP in vivo, we used an E1/E2a/E3-deficient adenoviral vector containing the cDNA encoding human GKRP (Av3hGKRP). High fat diet-induced diabetic mice were administered Av3hGKRP or a control vector lacking a transgene (Av3Null). Surprisingly, the Av3hGKRP-treated mice showed a significant improvement in glucose tolerance and had lower fasting blood glucose levels than Av3Null-treated mice. A coincident decrease in insulin levels indicated that the Av3hGKRP-treated mice had sharply improved insulin sensitivity. These mice also exhibited lower leptin levels, reduced body weight, and decreased liver GK activity. In vitro experiments indicated that GKRP was able to increase both GK protein and enzymatic activity levels, suggesting that another role for GKRP is to stabilize and/or protect GK. These data are the first to indicate the ability of GKRP to treat type 2 diabetes and therefore have significant implications for future therapies of this disease. *Diabetes* 50:1813–1820, 2001

Glucokinase (GK) is a high- K_m hexokinase that plays a central role as the physiologic glucose sensor in pancreatic β -cells and catalyzes the first committed step in hepatic glucose metabolism (1,2). As such, it is critical to maintaining normal blood glucose and insulin levels in vivo. In fact, decreased GK enzymatic activity has been reported in patients with type 2 diabetes (3), and autosomal-dominant heterozygous

mutations in the GK gene are associated with maturity-onset diabetes of the young (4,5). Transgenic animal studies have shown that even slight alterations in GK protein levels have profound effects on blood glucose homeostasis. Mice with increased hepatic GK activity showed lower fasting glucose and insulin levels and had improved glucose tolerance (6–8). Conversely, mice with only one copy of the GK gene (heterozygous knockout) showed increased fasting glucose levels and decreased glucose tolerance (9,10).

In the β -cell, GK activity is largely regulated by transcriptional mechanisms (1,2), whereas in the liver, GK activity is also acutely regulated by its binding to the GK regulatory protein (GKRP), localized within the nucleus (11–13). GKRP inhibits GK in an allosteric manner that is competitive with respect to glucose, and its activity is also modulated by phosphate esters of fructose. Fructose-6-phosphate binds to GKRP and greatly enhances its binding to and inhibition of GK, whereas fructose-1-phosphate promotes the dissociation of this complex (13,14). When released from GKRP, GK exits the nucleus due to the presence of a leucine-rich nuclear export signal sequence (15,16), where it can phosphorylate its substrate glucose. Mutants of *Xenopus* GK with a reduced affinity for GKRP remain in the cytosol, even in conditions of low glucose (12). Thus, in hepatocytes, GK is sequestered by GKRP within the nucleus in an inactive state during conditions of low glucose (elevated fructose-6-phosphate) and is released by GKRP to the cytosol in an active state in the presence of high glucose (or by fructose-1-phosphate). This regulation provides a sensitive means of maintaining large pools of quickly activated GK that is needed for postprandial glycolysis without causing hypoglycemia. Due to its identified function to negatively regulate GK activity, GKRP has been postulated as a candidate type 2 diabetes gene; however, no mutations have yet been found in this gene in diabetic subjects (17).

The ability to use viral or nonviral technologies to transduce specific genes of known function into diseased or normal tissues provides for the basis of gene therapy. Gene therapy can be applied to illnesses of either genetic or environmental etiologies, and its technology also allows for in vivo experiments to explore the function of particular genes of interest. Among the most carefully studied gene therapy systems are adenoviral vectors that, when handled properly, are easy to produce, stable, and safe (18,19). These vectors have the fortuitous ability to transduce nondividing terminally differentiated cells and are highly hepatotropic. Although they are nonintegrating and

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ALT, alanine aminotransferase; AUC, area under the curve; GK, glucokinase; GKRP, glucokinase regulatory protein; HBSS, Hanks' balanced salt solution; HF, high-fat diet; LF, control diet; OGTT, oral glucose tolerance test; PTG, protein targeting to glycogen; RSV, Rous sarcoma virus.

thus likely to supply only transient expression, recently developed adenoviral vector systems are capable of persisting in tissues *in vivo* for >6 months (20,21).

The objective of this study was to explore the effect of increasing the hepatic levels of GKRP protein on the diabetic phenotype of mice maintained on a high-fat diet. To do so, we have administered an adenoviral vector expressing human GKRP to these mice and assayed the fasting glucose, insulin, and leptin levels and the response to a glucose challenge. Surprisingly, we found this vector was able to correct the diabetic phenotype of these mice, and we have discussed the implications of these novel results.

RESEARCH DESIGN AND METHODS

Preparation of the recombinant adenoviruses. The 1.9-kb full-length cDNA encoding human GKRP was polymerase chain reaction amplified from human liver QUICK-Clone cDNA (Clontech, Palo Alto, CA) with primers containing *EcoRI* and *SalI* cleavage sites (5' primer: 5'-GAATTCATGCCAG-GCACAAAACGGTTT-3' and 3' primer: 5'-GTCGACTCACTGAACGTCAGGC-TCTAG-3'). The resulting product of polymerase chain reaction was then ligated into the TA-cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA), and the correct sequence was confirmed. The human GKRP cDNA fragment was excised from pCR2.1 with *EcoRI-SalI*, blunted-ended with Klenow (BRL, Gaithersburg, MD), and ligated into the *EcoRV* site of pAvS6a (21,22) to form pAvS6a-hGKRP. Finally, the 2-kb *BamHI* fragment from pAvS6a-hGKRP was ligated into the *BamHI* site of pAvS6alx to form pAvS6alx-hGKRP. The recombinant adenoviral vector was created by cre-lox-mediated recombination of pAvS6alx-hGKRP with pSQ3, containing most of the adenoviral genome, and was purified by standard procedures (21,22). The adenoviral vectors Av3Null and Av3hGK, which lack a transgene and encode human liver GK, respectively, were essentially as previously described (21,22), except that the Av3Null vector did not contain the Rous sarcoma virus (RSV) promoter and SV40 polyA signal.

In vitro experiments. Primary hepatocytes were isolated from male Sprague-Dawley rats as previously described (23). HepG2 cells (ATCC, Manassas, VA) or hepatocytes were incubated with the indicated adenoviral vectors for 2 h on a rocker in a 37°C incubator. Either 200 (Fig. 1) or 50 (Fig. 3) of the indicated viral particles/cell were used. Assays ($^3\text{H}_2\text{O}$ release and protein extraction) were performed on primary hepatocytes 48 h after viral transduction. Determination of $^3\text{H}_2\text{O}$ release from [2- ^3H] glucose (NEN, Boston, MA) was performed essentially as previously described (14,24). Protein lysates from HepG2 cells were prepared 72 h after viral transduction. **Animal diet and adenovirus treatment.** C57BL/6J male mice (3–4 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were housed in a pathogen free-barrier facility and were maintained on a 12-h light/dark cycle. These mice were maintained on either a high-fat diet (HF = 58% fat calories; D12309R) or a control diet (LF = 10% fat calories; D12310) (Research Diets, New Brunswick, NJ) for at least 4 months, after which diabetic mice were selected on the basis of elevated fasting blood glucose levels and impaired glucose tolerance (25). Mice were randomly divided into three treatment groups (Hanks' balanced salt solution [HBSS], Av3Null, or Av3hGKRP), such that each group had the same average fasting blood glucose levels. Adenoviral vector administrations via tail vein injections and retro-orbital phlebotomy were performed as previously described (26). Mice received 12×10^{10} viral particles/animal in a 250- μl injection volume of HBSS. The diet treatment was continued after the vector administration until study termination.

In vivo metabolic analyses. An oral glucose tolerance test (OGTT) was performed 2 weeks before and 2 weeks after the vector treatment as follows. The mice were fasted for 14–16 h before receiving a glucose bolus (1 g/kg). Blood samples from conscious mice were then collected by retro-orbital phlebotomy at 0, 30, and 120 min for glucose and insulin measurements. The area-above-baseline area under the curve (AUC) was calculated such that differences in the fasting glucose values did not affect the AUC values. At 1 and 3 weeks after the vector treatment, the mice were fasted overnight, and blood samples were collected for glucose determination. At 3 weeks after the vector treatment, animals were killed by cervical dislocation, and the livers were weighed and harvested for analyses. Tissue sections were either fixed in formalin (for hematoxylin and eosin staining) or snap-frozen in liquid nitrogen. All animal procedures were conducted in accordance with principles and guidelines established by the Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act (GTI Humane Care and Use Manual, 1998).

Assays. Blood glucose concentrations were measured using a handheld glucometer (Bayer, Tarrytown, NY). Plasma insulin was measured using an enzyme-linked immunoassay kit from Crystal Chem (St. Louis, MO). Plasma samples were sent to an outside laboratory (AniLytics, Gaithersburg, MD) for analysis of glucose, triglycerides, free fatty acids, lactate, and alanine aminotransferase (ALT).

GK activity measurements and Western analyses. Protein extracts were prepared from liver as previously described after an initial tissue homogenization step (Kontes Dual tissue homogenizer; Kimble/Kontes, Vineland, N.J.) (27). The supernatant was assayed for GK activity using a method essentially as described previously (8), except that the assay buffer contained 100 mmol/l Tris-HCl, pH 7.4, 100 mmol/l KCl, 6 mmol/l MgCl_2 , 1 mmol/l dithiothreitol, 5 mmol/l ATP, 1 mmol/l thioNAD, 30 U/ml glucose-6-phosphate dehydrogenase, and 0.5 or 100 mmol/l glucose. GK activity was estimated as the differences in activity when samples were assayed at 100 mmol/l (GK plus hexokinase activity) and 0.5 mmol/l glucose (hexokinase activity) and is indicated as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. Western blot analyses were performed as previously described (27). Antibodies used were rabbit anti-GK (in-house affinity purified antibody against rat β -cell GK, or Santa Cruz H-88 [Santa Cruz Biotechnology, Santa Cruz, CA]) and goat anti-GKRP (Santa Cruz sc-6340; Santa Cruz Biotechnology). Secondary antibodies were donkey anti-rabbit (Promega, Madison, WI) or donkey anti-goat (Santa Cruz sc-2020; Santa Cruz Biotechnology).

Glycogen assay. Liver samples were homogenized in 0.03 N HCl (to a final concentration of 0.5 g/ml). The homogenate (100 μl) was mixed with 400 μl of 1.25 N HCl and heated for 1 h at 100°C. Samples were centrifuged at 14,000 rpm and 10 μl supernatant was mixed with 1 ml glucose oxidase reagent (Sigma, St Louis, MO). After a 10-min incubation at 37°C, the absorbance was read at 505 nm. A standard curve using glycogen type III obtained from rabbit liver (Sigma) was also simultaneously analyzed to determine the final liver glycogen concentrations.

Statistical analysis. Results are reported as means \pm SE. The comparison of different groups was performed using unpaired Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

To explore the physiological role of GKRP in regulating glucose homeostasis *in vivo*, we used adenoviral-mediated gene transfer to overexpress human GKRP in the livers of diabetic mice. The human GKRP cDNA was cloned into a replication-deficient adenoviral vector (Av3hGKRP) containing deletions of the viral E1, E2a, and E3 genes. This vector contains an RSV promoter and a SV40 poly(A) signal (Fig. 1A). An identical vector lacking a promoter, transgene, and poly(A) signal was used as a control (Av3Null). Infection of primary rat hepatocytes with Av3hGKRP resulted in a significant increase in both GKRP mRNA (data not shown) and protein levels (Fig. 1B). The Av3Null vector had no effect on the endogenous GKRP protein levels (Fig. 1B). The effect of GKRP overexpression on the rate of glucose phosphorylation, as measured by the release of $^3\text{H}_2\text{O}$ from [2- ^3H]glucose, was also examined (Fig. 1C). After treatment with Av3hGKRP, glucose turnover was significantly decreased at 5 mmol/l glucose but not at higher glucose concentrations. Therefore, the ectopic human GKRP produced by Av3hGKRP is functional and physiologically responsive to glucose concentrations.

Male C57BL/6J mice were maintained on a high-fat diet (HF; $n = 31$) for 5 months before and during the course of the experiment to induce a diabetic phenotype (25). An age-matched group of control mice were maintained on normal chow (LF; $n = 9$) during the same period. The HF mice had a significant gain in body weight (29.2 [LF] vs. 48.3 g [HF]; $P < 0.0001$) and had elevated levels of fasting glucose (120.3 [LF] vs. 210.6 mg/dl [HF], $P < 0.0001$) and insulin (419 [LF] vs. 2,812 pg/ml [HF]; $P < 0.0001$), confirming the diabetogenic feeding protocol. The HF mice were sorted into three groups such that their fasting blood

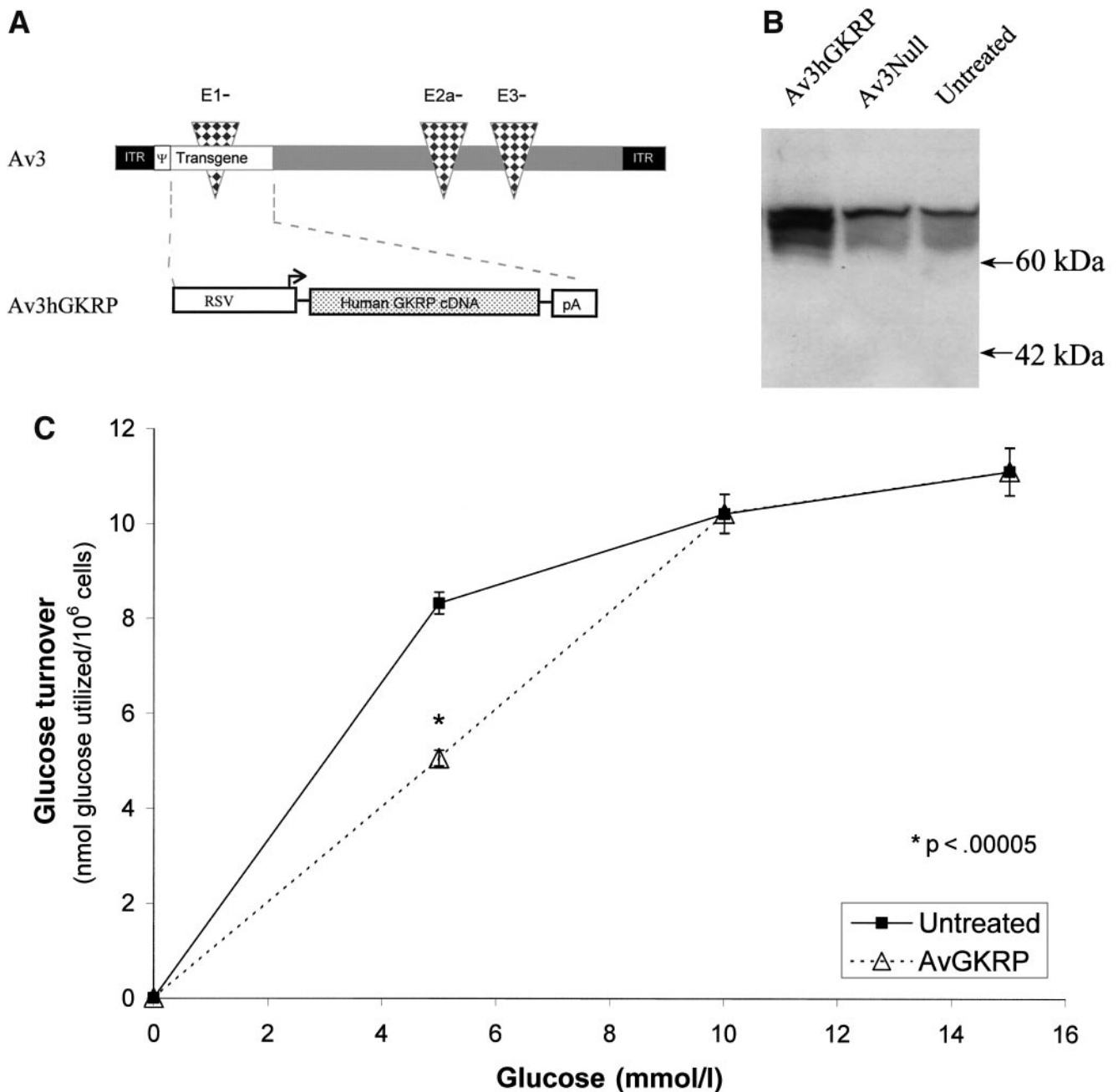


FIG. 1. Structure and function of the Av3hGKRP vector. **A:** The schematic structure of Av3hGKRP. ITR and ψ represent the inverted terminal repeats and packaging signal, respectively. E1-, E2a-, and E3- represent the deletions of these genes from the adenovirus genome. The insertion of the human GKRP expression cassette (containing an RSV promoter and a SV40 poly[A] sequence) is indicated below. **B:** Primary rat hepatocytes were transduced with 200 viral particles/cell of Av3hGKRP or Av3Null or remained untreated. Protein lysates were harvested and used for Western blot analysis using an antibody specific for GKRP 48 h after transduction. **C:** Primary rat hepatocytes were transduced with 200 viral particles/cell of Av3hGKRP or remained untreated. A $^3\text{H}_2\text{O}$ release assay for the rate of glucose phosphorylation was performed 48 h after viral transduction. Values are the means \pm SE. *A significant ($P < 0.00005$) decrease in glucose turnover at 5 mmol/l glucose was seen in the Av3hGKRP-treated cells.

glucose levels were equivalent. GKRP mice ($n = 11$) were administered 6×10^{10} particles of Av3hGKRP plus 6×10^{10} particles of Av3Null, bringing the total viral dose to 12×10^{10} particles, a dosage consistent with our previous safety (data not shown) and toxicological profiles (21). Null mice ($n = 10$) were administered 12×10^{10} particles of the Av3Null vector, and control mice ($n = 10$) were administered with an equal volume of HBSS. The vectors were administered by intravenous (tail vein) injections that, due to the hepatotropic nature of adenoviral vectors, would

allow near-complete transduction of hepatocytes preferentially to any other tissues (18,19). The age-matched LF mice ($n = 9$) were also injected with HBSS (LF:HBSS) as a nondiabetic control.

Fasting blood glucose levels were measured in the treated mice at weekly intervals after vector administration (Fig. 2). Null mice had lower fasting glucose levels than HBSS mice at all time points tested (although this may represent pre-existing differences between the groups). However, the GKRP mice showed a significant decrease in

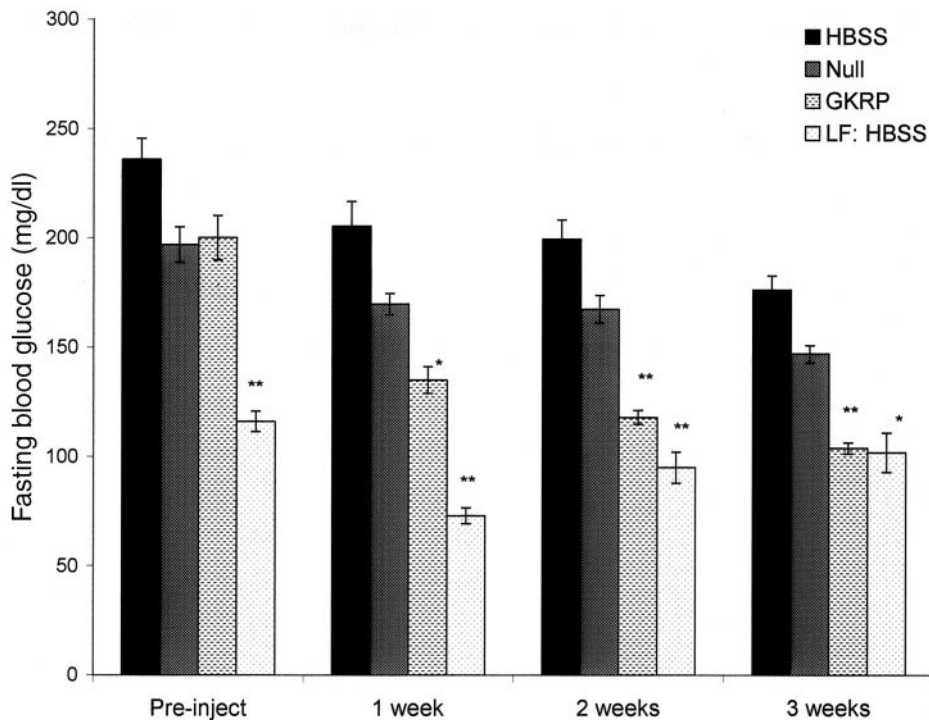


FIG. 2. Fasting blood glucose values in vector-treated mice. Blood samples were collected from overnight-fasted mice at 1 week before or 1, 2, or 3 weeks after vector administration, and whole-blood glucose values determined. HBSS, Null, and GKRP represent HF mice administered HBSS ($n = 10$), Av3Null ($n = 10$), and Av3hGKRP ($n = 11$), respectively. LF: HBSS represents chow-fed mice administered HBSS ($n = 9$). Values are the mean \pm SE. Significant differences from Null mice are indicated as follows: * $P < 0.0005$; ** $P < 0.0001$.

fasting glucose levels compared with both Null and HBSS mice at all times tested. By the end of the experiment, the blood glucose levels of the GKRP mice were statistically indistinguishable from the nondiabetic LF:HBSS group (104 vs. 102 mg/dl; $P = 0.82$).

To assay the effect of increased hepatic GKRP expression on glucose tolerance, we performed an OGTT 2 weeks after vector administration. The HF:HBSS mice had a significantly impaired glucose tolerance (decrease in AUC) compared with their LF:HBSS counterparts (Table 1), further confirming the diabetic nature of these mice. Av3Null did not significantly improve glucose tolerance by itself, compared with the pretreatment values of either the HBSS (Table 1) or Null group (data not shown). However, Av3hGKRP treatment did result in an improvement in glucose tolerance that was significant compared with both the HBSS (Table 1) and GKRP groups' pretreatment values (data not shown). In fact, the glucose tolerance curve for Av3hGKRP nearly overlapped with that of the nondiabetic LF:HBSS mice (data not shown).

Insulin resistance is a primary cause and diagnostic marker of type 2 diabetes. Therefore, we measured fasting plasma insulin levels 2 weeks after vector treatment. The HF mice displayed extreme hyperinsulinemia, consistent with an insulin-resistant phenotype (Table 1; compare HBSS and LF:HBSS). Av3Null was capable of decreasing insulin levels; however, Av3hGKRP lowered insulin levels significantly more so, to the levels seen in the LF:HBSS mice. This result was true at all points tested during the OGTT (data not shown).

To characterize the effect of Av3hGKRP treatment on hepatic function and fuel homeostasis, we assayed various parameters from fasting plasma samples taken 3 weeks after vector administration (Table 1). Elevated plasma levels of the liver enzyme ALT was observed in mice treated with either Av3Null or Av3hGKRP (Table 1). This result was expected, because adenoviral transduction often results in liver toxicity (18,19,28). An increased ability to metabolize glucose in the liver has previously been shown to result in disturbances in energy storage and

TABLE 1
The effects of Av3hGKRP on blood chemistry parameters

| | LF:HBSS | HBSS | Null | GKRP |
|--|---------------------|--------------------|-------------------|---------------------|
| Lactate (mg/dl) | 19.8 \pm 1.6* | 27.3 \pm 2.4 | 23.8 \pm 2.0 | 24.5 \pm 1.8 |
| FFAs (mmol/l) | 1.61 \pm 0.073*† | 1.15 \pm 0.044 | 1.12 \pm 0.065 | 1.37 \pm 0.074*† |
| TGs (mg/dl) | 150 \pm 10* | 125 \pm 5.2 | 124 \pm 7.3 | 150 \pm 10.0* |
| ALT (units) | 95.1 \pm 45.4† | 102.5 \pm 17.1 | 240.9 \pm 26.4* | 230.6 \pm 20.4* |
| Fasting glucose (mg/dl) | 102 \pm 9.0*† | 176.3 \pm 6.6 | 148.9 \pm 4.0* | 103.8 \pm 2.5*† |
| AUC (mg \cdot dl ⁻¹ \cdot min ⁻¹) | 8,497 \pm 531* | 14,387 \pm 1,692 | 7,473 \pm 3,785 | 5,076 \pm 767* |
| Fasting insulin (ng/ml) | 0.587 \pm 0.067*† | 2.751 \pm 0.42 | 1.51 \pm 0.11 | 0.742 \pm 0.088*† |
| Fasting leptin (ng/ml) | 3.44 \pm 1.26*† | 53.42 \pm 2.85 | 37.74 \pm 3.83* | 13.78 \pm 1.91*† |

Data are means \pm SE. *Significant differences ($P < 0.05$) from HBSS; †significant differences ($P < 0.05$) from Null mice. HBSS, Null, and GKRP represent high fat-fed mice administered HBSS ($n = 10$), Av3Null ($n = 10$), and Av3hGKRP ($n = 11$), respectively. LF:HBSS represents mice fed standard laboratory diet administered HBSS ($n = 9$). Blood samples were collected following an overnight fast at 3 weeks after Vector administration and were analyzed for the levels of circulating lactate, free fatty acids, triglycerides, ALT, and glucose. The fasting leptin and insulin values were determined at 1 and 2 weeks after vector administration, respectively.

TABLE 2

The effect of Av3hGKRP on body and liver weights, glycogen content, and GK activity

| | LF:HBSS | HBSS | Null | GKRP |
|---|---------------|-------------|-------------|---------------|
| Body weight (g) | 29.7 ± 0.7*† | 47.9 ± 0.5 | 45.9 ± 1.4 | 40.1 ± 1.0*† |
| Liver weight (g) | 1.30 ± 0.0*† | 1.99 ± 0.1 | 2.77 ± 0.2* | 2.03 ± 0.1 |
| Liver/body (%) | 4.4 ± 0.1† | 4.1 ± 0.3 | 6.0 ± 0.3* | 5.0 ± 0.2*† |
| Glycogen (mg/g) | 5.9 ± 1.6*† | 10.6 ± 0.7 | 19.9 ± 1.6* | 13.7 ± 1.3 |
| GK activity nmol · min ⁻¹ · mg ⁻¹ | 4.24 ± 0.85*† | 7.29 ± 0.70 | 6.79 ± 0.47 | 5.22 ± 0.27*† |

Data are means ± SE. *Significant differences ($P < 0.05$) from HBSS and Null mice are indicated by * and †, respectively. HBSS, Null, and GKRP represent high fat-fed mice administered HBSS ($n = 10$), Av3Null ($n = 10$), and Av3hGKRP ($n = 11$), respectively. LF:HBSS represents mice fed standard laboratory diet administered HBSS ($n = 9$).

dyslipidemia (22,31). Therefore, we measured circulating free fatty acid, triglyceride, and lactate concentrations from these samples to determine whether Av3hGKRP would do likewise. Although the GKRP mice had higher free fatty acid and triglyceride levels than the Null and HBSS mice; these levels were not increased compared with the LF:HBSS mice, confirming no abnormalities in plasma lipid deposition (Table 1). Likewise, there were no differences in the plasma lactate concentrations among the different treatment groups. Furthermore, we did not observe excessive lipid vacuolization in the liver after treatment with Av3hGKRP (data not shown).

Glycogen is a major form of stored energy within the liver. To assay whether Av3hGKRP increased energy deposition of this molecule, we quantified liver glycogen stores. Due to their high-energy diet, the HF mice showed significantly elevated glycogen stores compared with their LF counterparts (Table 2). Treatment with Av3Null unexpectedly induced a substantial increase in liver glycogen levels. The GKRP mice also showed a trend toward elevated glycogen levels compared with the HBSS mice ($P = 0.56$), but this level was considerably less than that seen for Null mice. Parallel results were also observed in the liver weights of these mice (Table 2). The livers from GKRP mice weighed about the same as those from HBSS mice, both of which did not show the hepatomegaly as seen in the Null mice.

In addition, we observed a curious weight loss shortly after administration of the Av3hGKRP vector. By the end of the 3-week experiment, these mice had lost 16% of their pretreatment body weight (Table 2). Although the Null mice also lost weight (6%; Table 2), it was to a significantly lesser extent than the GKRP mice (data not shown). The plasma levels of leptin, a hormone secreted by adipocytes that regulates feeding behavior, were modestly decreased in the Null mice and sharply decreased in the GKRP mice compared with the HBSS mice (Table 1).

To examine the effect of GKRP overexpression on GK protein levels, we performed Western blot analysis on protein lysates prepared from the livers of overnight-fasted mice. Mice treated with Av3hGKRP had no significant alterations in GK protein levels compared with Null mice (data not shown). These lysates were then used in an *in vitro* assay for GK enzymatic activity (Table 2). Interestingly, we found that HF mice had increased GK activity when compared with their LF controls. Treatment with Av3Null had little effect on GK activity. However, in contrast to what we expected based on our *in vivo* data and the Western analysis, Av3hGKRP treatment resulted in a significant loss of GK activity compared with both

Av3Null (23% decrease) and HBSS (28% decrease) treatments. RNA was then prepared from the livers of these mice and used for Northern blot analysis with a probe specific for GK. Interestingly the GKRP mice showed sharply reduced levels of GK mRNA compared with the Null or HBSS mice (data not shown), probably due to feedback resulting from the lowered plasma glucose and insulin levels in these mice.

The mechanism by which Av3hGKRP treatment was able to correct the diabetic phenotype of the HF mice remained elusive. To more carefully investigate how GKRP overexpression could lead to an increase in glucose metabolism *in vivo*, we initiated *in vitro* studies with adenoviral vectors expressing either human GK (Av3hGK) or GKRP (22,30). HepG2 cells, which lack endogenous GK and GKRP protein, expressed high levels of enzymatically active GK protein when transduced with Av3hGK (Fig. 3A and B), whereas cells transduced with Av3hGKRP expressed high levels of GKRP but showed no detectable GK protein. However, when cells were transduced with both vectors, the levels of GK protein were found to be 50% higher than that seen with Av3hGK alone (2,398 vs. 1,586 arbitrary densitometry units). Furthermore, the GK enzymatic activity from these lysates was >70% higher than that seen with Av3hGK alone (50.3 ± 0.5 vs. 29.1 ± 0.3 ; $P < 0.05$). Therefore, it seems that increasing GKRP protein levels allows increased GK protein and activity levels *in vitro*.

DISCUSSION

In this study, we have detailed the first gene therapy use of GKRP for diabetes. Type 2 diabetes in humans is a complex polygenic disease with strong environmental underpinnings, the most noteworthy of which is obesity. Therefore, we used a murine model in which diabetes was induced by high-fat feeding, because it most accurately mimicked the etiology of the human disease. We used an E1/E2a/E3-deficient adenoviral vector containing the cDNA encoding human GKRP (Av3hGKRP), which produced high levels of functional GKRP protein *in vitro* (Fig. 1) (32). Diabetic mice were administered either Av3hGKRP, Av3Null (a control vector), or the saline carrier HBSS.

Because the documented function of GKRP is to inhibit GK activity under low-glucose conditions, we expected to see a more diabetic phenotype in mice administered Av3hGKRP. However, mice overexpressing GKRP unexpectedly exhibited a decrease in fasting blood glucose levels similar to that seen in nondiabetic mice (Fig. 2). GKRP mice also had sharply reduced insulin levels (Table

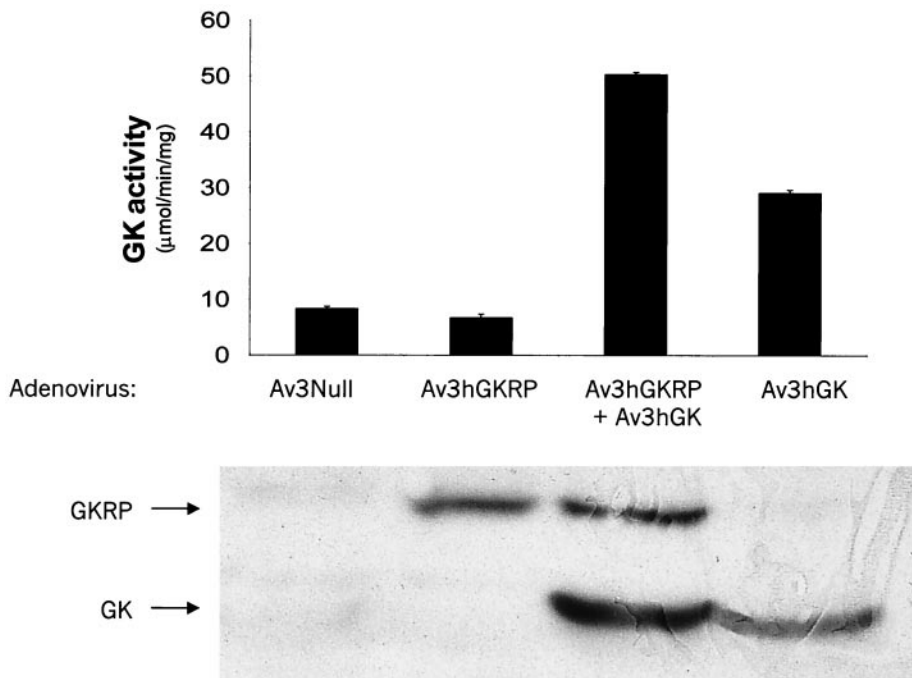


FIG. 3. The effect of Av3hGKRP treatment on GK protein and activity in vitro. HepG2 cells were transduced with the following vector combinations: Av3Null (100 particles/cell of Av3Null); Av3hGKRP (50 particles/cell each of Av3hGKRP and Av3Null); Av3hGK (50 particles/cell each of Av3hGK and Av3Null); or Av3hGKRP + Av3hGK (50 particles/cell each of Av3hGKRP and Av3hGK). At 48 h after transduction, protein lysates were harvested and used for Western blot analysis with antibodies specific for GK or GKRP and for determination of their GK enzymatic activity. Note that the Av3hGKRP + Av3hGK treatment has significantly more GK activity than any other treatment ($P < 0.05$).

1), revealing their normalized insulin sensitivity. Furthermore, they showed improved glucose tolerance during an OGTT (Table 1). In fact, by these measures, Av3hGKRP treatment completely corrected the diabetic phenotype of the animals and was capable of maintaining this effect for the duration of the 3-week study. Based on our experience with related adenoviral vectors (21,22,26), it is expected that Av3hGKRP treatment should be effective for substantially longer periods of time. The beneficial effects of GKRP overexpression were actually quite similar to those found after administration of an adenovirus expressing GK to diabetic mice (data not shown) (22,30). In the GKRP mice, there was also a trend toward higher hepatic glycogen levels compared with HBSS mice, which is consistent with the decreased or increased glycogen levels seen in GKRP knockout (33) and GK transgenic mice (6), respectively. However, the increase observed in our studies may in part be an artifact of supplementing the Av3hGKRP viral dose with Av3Null, which itself can increase glycogen content (Table 2). Further studies will be needed to clarify this result.

Numerous studies have reported a role for GKRP in sequestering GK in the hepatocyte nucleus in an enzymatically inactive state under metabolically quiescent conditions (low glucose) (11,12,15,16,32,34). However, in our in vitro studies, we found that HepG2 cells simultaneously transduced with Av3hGKRP and an adenoviral vector expressing human GK (Av3hGK) had significantly elevated GK protein and activity levels compared with cells transduced with Av3hGK alone (Fig. 3). These data suggest that GKRP may also serve to stabilize and protect a pool of GK protein (i.e., extend half-life). Consistent with this finding, recent studies report that heterozygous and homozygous mice deficient for GKRP show 30 and 75% reductions in GK protein levels and 16 and 41% reductions in GK activity levels, respectively (35). Furthermore, the GKRP knockout mice show a deregulation of the nuclear localization of GK (33,35). Therefore, it follows that increased levels of

hepatic GKRP should result in increased levels of GK protein and activity. Although GKRP would sequester GK in an inactive state under low glucose concentrations (i.e., fasted state), the increased GKRP protein levels would provide for release of an excess pool of GK into the cytosol (i.e., fed state), thereby allowing the animal to more effectively defend normal glycemia. However, the in vivo data present a more complicated situation. We found no significant changes in the GK protein levels in livers of GKRP mice compared with Null mice (data not shown). In addition, overexpression of GKRP led to a 23% decrease in GK enzymatic activity in lysates prepared from these livers (Table 2). A plausible explanation for the differences observed in GK activity in the in vivo and in vitro experiments relates to the ability of Av3hGKRP to improve the in vivo diabetic phenotype and to the different promoters for GK in these experiments. GK expression in the in vivo experiments is regulated by the endogenous GK promoter, whereas expression in the in vitro experiment was controlled by the RSV promoter. It is well known that the rate of GK transcription is increased by insulin (1,36,37), and we and others have found GK activity to be elevated in obese animals (Table 2; LF:HBSS versus HBSS; data not shown) (38). Therefore, with the Av3hGKRP-treated animals, because their insulin sensitivity improved and their insulin levels and body weights were reduced, liver GK transcription and activity may also be decreased. Our data showing that GK mRNA is reduced in GKRP mice supports this hypothesis. This feedback loop may even limit the potential of Av3hGKRP to induce hypoglycemia, which has been seen with GK overexpression (29,39).

A second possibility is that although the in vivo overexpression of GKRP leads to a decrease in overall GK activity, this activity may be applied in a more efficient manner toward metabolizing blood glucose. The subcellular compartmentalization by scaffolding proteins of enzymes or signaling proteins into clusters is often used as a means of increasing system efficiencies (40,41). In this

case, even decreased levels of overall GK activity can be applied temporally and spatially in more appropriate ways to increase the effective hepatic GK activity, similar to how increased levels of the scaffold protein targeting to glycogen (PTG) results in improved glucose tolerance (42). Finally, a possible role for the GK-associated phosphatase (GKAP) in regulating GK activity (43), as well as potentially unidentified functions of GKR, may further explain the discrepancy between our *in vivo* and *in vitro* results.

To our surprise, we observed a beneficial effect of Av3Null treatment on glucose tolerance when compared with HBSS treatment (Table 1). Although gene therapies for diabetes using adenoviral vectors have been reported, the control vectors used in these studies contained transgenes (i.e., β -gal) and were not compared with untreated or saline-treated control groups (29,42,44–46). Therefore, we know of no other reports suggesting an influence of adenoviruses or adenoviral vectors on glucose tolerance or on the expression or function of hepatic proteins involved in glucose metabolism. The adenoviral vector (Av3) used in these studies would be expected to express several adenoviral backbone genes (i.e., E4, L1) within the transduced cell. The resulting proteins, and/or the viral capsid proteins necessary for transduction, may directly affect host cell functions or may induce an immune response against the transduced cells (18,19,28). In fact, a preliminary gene expression analysis using a RNA hybridization array chip (Incyte Pharmaceuticals, Fremont, CA) has shown >20 genes differentially regulated (of ~10,000 tested) in response to treatment with Av3Null (compared with HBSS; data not shown). Furthermore, these mice had increased liver function test values (i.e., ALT), liver glycogen contents, and liver weight (Tables 1 and 2). In subsequent studies, it may be possible to minimize these effects by using ‘gutless’ adenoviral vectors, which would only express the transgene (20,47).

Several gene therapies for type 2 diabetes have been studied with mixed success, including insulin, GK, and PTG (29,42,48). Most insulin gene therapies suffer from constitutive or inappropriate timing of insulin production, which has the potential for causing hypoglycemia, and would also be ineffective for the insulin-resistant patient (48). GK, insulin, and PTG gene therapies also have the potential to cause hypoglycemic episodes, and GK overexpression has resulted in the deleterious accumulation of lipids within the liver and bloodstream (42). The fundamental problem with overexpressing these transgenes is that they deregulate metabolic pathways. In contrast, by overexpressing GKR, there is the potential to increase GK activity in a controlled manner only when it is needed. Av3hGKR treatment did not cause hypoglycemia in diabetic (Table 1) or normal mice (data not shown) nor did it result in hepatic steatosis (data not shown) or increased plasma triglyceride or free fatty acid levels (Table 1). The only unanticipated side effect we noted with Av3hGKR treatment was weight loss, which could be an added benefit to the largely obese type 2 diabetic population.

Our study and the two recent reports of mice deficient in GKR showing decreased levels of GK protein (33,35), provide support for a novel level of GK regulation: protein stability. The manipulation of a protein’s half-life is a common means of signal transduction and directly controlling

biochemical/metabolic pathways (49). Several pharmaceutical entities (small molecule compounds) designed to manipulate the half-life of specific proteins are currently under development (50,51). Our findings suggest the validity of such an approach with respect to GK. However, any approach to increase GK activity by interfering with the binding of GKR to GK risks worsening the disease it seeks to alleviate (diabetes), because prolonged disruption may result in decreased hepatic GK levels. In summary, we have developed a novel therapeutic approach for type 2 diabetes, GKR gene therapy, which we have shown to be quite effective in correcting the phenotype of diabetic mice. Our studies have also provided insight into the physiological role of GKR. In the future, it would be of interest to determine whether an adenovirus expressing both GKR and GK would show a synergistic ability to treat diabetes without the deleterious effects (hepatic steatosis and dyslipidemia) that may result from GK overexpression alone.

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