Hepatic Expression of a Targeting Subunit of Protein Phosphatase-1 in Streptozotocin-diabetic Rats Reverses Hyperglycemia and Hyperphagia Despite Depressed Glucokinase Expression*

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Glycogen-targeting subunits of protein phosphatase-1 (PP-1) are scaffolding proteins that facilitate the regulation of key enzymes of glycogen metabolism by PP-1. In the current study, we have tested the effects of hepatic expression of G_MΔC, a truncated version of the muscle-targeting subunit isoform, in rats rendered insulin-deficient via injection of a single moderate dose of streptozotocin (STZ). Three key findings emerged. First, G_MΔC expression in liver was sufficient to fully normalize blood glucose levels (from 335 ± 31 mg/dl prior to viral injection to 109 ± 28 mg/dl 6 days after injection) and liver glycogen content in STZ-injected rats. Second, this normalization occurred despite very low levels of liver glucokinase expression in the insulin-deficient STZ-injected rats. Finally, the hyperphagia induced by STZ injection was completely reversed by G_MΔC expression in liver. In contrast to these findings with G_MΔC, overexpression of another targeting subunit, G_L, in STZ-injected rats caused a large increase in liver glycogen stores but only a transient decrease in food intake and blood glucose levels. The surprising demonstration of a glucose-lowering effect of G_MΔC in the background of depressed hepatic glucokinase expression suggests that controlled stimulation of liver glycogen storage may be an effective mechanism for improving glucose homeostasis, even when normal pathways of glucose disposal are impaired.

In recent years, we and others have expressed the various targeting subunits in mammalian cells in culture or in liver of intact animals, leading to new insights into their relative metabolic potencies and regulatory properties (5). This has included work on a novel targeting subunit construct, G_MΔC, derived by truncation of the unique 735-amino acid C-terminal domain of native G_M (6). We have found that when expressed in hepatocytes, the targeting subunits stimulate glycogen synthesis in the rank order G_L > PTG > G_MΔC > G_M (6, 7). Surprisingly, G_MΔC, but not G_L or G_M, ameliorates glucose intolerance when expressed in livers of rats fed on a high fat diet (8). This appears to be explained by our finding that liver cells with overexpressed G_MΔC maintain full responsiveness to glycogenolytic signals such as forskolin and low glucose, unlike cells with overexpressed G_L or PTG, which have impaired responsiveness to these agents, resulting in accumulation of large amounts of glycogen in the fasted state (6, 8, 9).

In light of these encouraging findings, the current study has extended our investigation of the properties of G_MΔC by its expression in liver of rats with streptozotocin (STZ)-induced diabetes. Three surprising observations have been made as a result of this work. First, we find that G_MΔC expression in liver is sufficient to lower blood glucose levels and raise liver glycogen levels to normal in STZ-injected diabetic rats. Second, these corrective effects on hepatic glycogen metabolism and glucose homeostasis occur despite very low levels of liver glucokinase expression in the insulin-deficient animals. Finally, we also find that hepatic G_MΔC expression reduces food intake to normal levels in STZ-diabetic rats, which are otherwise hyperphagic.

MATERIALS AND METHODS

Recombinant Adenoviruses—A recombinant adenovirus containing the cDNA encoding a truncated G_M glycogen-targeting subunit isoform from which 735 C-terminal amino acids were removed (G_MΔC) is termed AdCMV-G_MΔC; its preparation has been described previously (6). A recombinant virus containing the cDNA encoding the full-length hepatic targeting subunit G_L (AdCMV-G_L) has also been described (6). Both the G_MΔC and G_L inserts include a C-terminal FLAG tag for ready identification of the transgene products by immunoblotting (6). As a control, some animals received a virus containing the Escherichia coli β-galactosidase gene, termed AdCMV-βGal (10). These viruses were amplified and purified for injection into animals using previously described procedures (11).

Animal Studies—Male Wistar rats (Charles River Laboratories) weighing 250–300 g were housed on a 12-h light-dark cycle and were allowed free access to water and standard laboratory chow (65% carbohydrate, 4% fat, 24% protein; Harlan Teklad laboratory diet 9100). These animals were injected with a single moderate dose of streptozotocin (60 mg/kg; Sigma S-0130) intraperitoneally, followed by daily monitoring of blood glucose levels in the ad libitum fed state, using an automated blood glucose analyzer (Hemoce AB; Angelholm, Sweden). Only those animals in which blood glucose rose to levels greater than 250 mg/dl within 3 days of STZ injection were studied further. Five days
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after STZ injection, 0.5×10^{12} particles of AdCMV-G_{m\Delta C}, AdCMV-G_{1\Delta C}, or AdCMV-JGAL were administered via tail vein injection to rats anesthetized with intraperitoneal Nembutal (50 mg/kg of body weight; Abbott). As an additional control, some animals received no viral injection. After viral administration, animals were individually caged for daily monitoring of food intake, body weight, and blood glucose levels. Six days after viral administration, animals were sacrificed in the ad libitum fed state or following 18 h of fasting. A blood sample was taken, and liver and muscle samples were excised, rapidly clamp-frozen in liquid nitrogen, and stored at −70 °C for further analysis. In a separate set of STZ-injected and virus-treated animals, urine volume and glucose concentration was monitored by four 12-h collections of urine from individual rats and measurement of urine glucose using a glucose oxidase-based assay (Sigma).

Measurement of RNA Levels in Liver—For analysis of RNA levels, liver samples were ground into powder under liquid nitrogen, and total mRNA was extracted using the TRIzol reagent (catalog no. 15956-018; Invitrogen). First-strand cDNAs were prepared according to the manufacturer’s instructions in a 50-μl total reaction mix using 2 μg of total mRNA, 3.2 μg of oligo-dT(12), and a cDNA synthesis kit (catalog no. 1-483-188; Roche Applied Science). The PCRs were carried out using 2 μl of the cDNA synthesis mixture, 0.5 μl of 20 μM stock solutions of the primer pairs for amplification of G_{m\Delta C} (5′-primer, 5′-GAAGAACCTGTGCAGAAACGC-3′; 3′-primer, 5′-TCTCTGCTGACCGCTTCTT-3′), glucokinase (5′-primer, 5′-TGCCCAACGATCTGCTCCT-3′; 3′-primer, 5′-GGCTCACACGACATGACC-3′), GLUT2 (5′-primer, 5′-CAGCTGCTTCACTGACGTCT-3′; 3′-primer, 5′-AGGCTACGTCGCAACCAATCTG-3′), glucose synthase (5′-primer, 5′-TCTCTGAGTTGCACCGACC-3′; 3′-primer, 5′-GGTAAGAGTCTGCGTGGACAT-3′), and a PCR reaction containing 2.5 units of Taq polymerase (catalog no. 1-418-432; Roche Applied Science) and 2 μl of 10 mM DNTP (catalog no. 1-814-362; Roche Applied Science) in a 50-μl total reaction mix. The annealing temperature was 45 °C, and PCR mix was collected for analysis after a variable number of cycles (25 cycles for G_{m\Delta C} and 32 cycles for glucokinase). The synthesized PCR products were run on a 1% agarose gel, stained with ethidium bromide, and detected by UV light using the VersaDoc imaging system (model 5000; Bio-Rad). The low DNA mass ladder (catalog no. 10068-013; Invitrogen) was used as a size standard, and the PCR products were quantified using the QuantityOne program (Bio-Rad).

Real time quantitative PCR methods were utilized to measure glucokinase and hexokinase 1 mRNA levels. The PCRs were carried out using 2 μl of the cDNA synthesis mixture (1:100 dilution of the cDNA synthesis mix described above), 0.5 μl of 20 μM stock solutions of the primer pairs for amplification of glucokinase (5′-primer, 5′-AAGCAGAGAACACGCGCCG-3′; 3′-primer, 5′-GTCAGAACAGC-3′), hexokinase 1 mRNA (5′-primer, 5′-AAAACCAAGAAGCCCTGGAGACC-3′; 3′-primer, 5′-ACCCATCATCCCCAAAGGCTC-3′), or the internal control α-tubulin (5′-primer, 5′-GTCAGAACAGC-3′; 3′-primer, 5′-GTCAGAACAGC-3′), and a PCR reaction containing 2.5 units of Taq polymerase (catalog no. 1-418-432; Roche Applied Science) and 2 μl of 10 mM DNTP (catalog no. 1-814-362; Roche Applied Science) in a 50-μl total reaction mix. The annealing temperature was 54 °C, and PCR data were collected using the iCycler detector from Bio-Rad. The relative abundance of mRNA was calculated by comparative threshold cycle methods (Applied Biosystems, User Bulletin 2), using α-tubulin as the internal control.

Immunoblot Analysis and Glycogen Measurements in Liver Samples—Powdered liver samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100, and proteinase inhibitors) using a Polytron homogenizer (model PT10–35; WNR Inc.), followed by freeze-thawing. Cell lysates were centrifuged at 3,000 × g for 5 min, and total protein concentration was measured by the Bradford method (12). Glycogen was measured by extraction in 10% trichloroacetic acid, precipitation with methanol, and digestion of glycogen to free glucose by incubation with 0.4 mg/ml amyloglucosidase, as previously described (13).

Following analysis of G_{m\Delta C} protein levels, samples were centrifuged at 8,000 × g for 2 min, and 2 μg of total proteins were incubated with 10 μg of antiFLAG antibody in 1 ml of buffer A (phosphate-buffered saline, 2% bovine serum albumin, 5 mM EDTA, and 100 μM phenylmethylsulfonyl fluoride) at 4 °C for 1 h. Then 20 μl of Sheep red anti-FLAG M2 affinity gel (catalog no. P-2426; Sigma) was added and incubated at 4 °C for 1 h. Samples were centrifuged at 8,000 × g and washed three times with buffer. The pellets were mixed with 50 μl of SDS-running buffer and boiled for 5 min. Samples were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer (50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1% bovine serum albumin) for 1 h and treated overnight at 4 °C with rabbit polyclonal serum specific for G_{m\Delta C} (a generous gift of Dr. Anna A. Depaoli Roach, University of Indiana Medical Center) at a dilution of 1:1000. The membranes were washed and subsequently treated with horseradish peroxidase-labeled anti-rabbit IgG secondary antibody at 4 °C for 2 h. The protein-antibody complexes were visualized using an enhanced chemiluminescence detection kit (PerkinElmer Life Sciences). Glucokinase protein levels were analyzed as described previously (6), using a rabbit polyclonal anti-glucokinase antibody (14) at a dilution of 1:5,000.

Analysis of Plasma Variables—Plasma insulin, glucagon, and leptin levels were measured by radioimmunoassay kits (catalog no. RI-13K, GL-32K, and RL-83K, respectively; Linco Research, St. Charles, MO). Plasma aspartate-aminotransferase, triglycerides, and ketones were measured using kits (procedure no. DG158-UV, 337, 310-UV, and 735, respectively; Sigma) as previously described (8). Plasma free fatty acids were measured using the FFA-Half microtest kit (catalog no. 1-383-175; Roche Applied Science).

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical significance was determined by unpaired Student’s t test using the statistics module of Microsoft Excel (Microsoft Excel X for Mac; Microsoft Corp., Redmond, WA). Statistical significance was assumed at p < 0.05.

RESULTS

Expression of G_{m\Delta C} in Livers of STZ-treated Rats—Our prior work has shown that expression of G_{m\Delta C} in liver of rats with diet-induced obesity and insulin resistance results in reversal of glucose intolerance, in concert with stimulation of liver glycogen storage (8). However, these studies did not address the utility of the targeting subunit to correct blood glucose levels in animals with frank diabetes. To investigate this point, we injected AdCMV-G_{m\Delta C} or, as a control, AdCMV-JGAL into Wistar rats that received a single moderate dose of STZ (60 mg/kg). Only animals in which blood glucose exceeded a level of 250 mg/dl within 3 days of STZ injection (ad libitum fed state) and with blood aspartate-aminotransferase activity of less than 200 units/ml after viral injection (indicative of the absence of virus-induced liver damage) were used for further study. As a first step in our analysis, we sought to confirm expression of G_{m\Delta C} specifically in animals injected with the AdCMV-GM\Delta C virus. The CDNA insert contained in this virus includes a FLAG tag to allow specific identification of the virus-encoded protein. As shown in Fig. 1, multiplex PCR analysis of liver RNA reveals an RNA band of the expected size only in animals that received the AdCMV-GM\Delta C virus and not in control animals (receiving either AdCMV-JGAL or no virus). Similarly, a protein detectable with an anti-FLAG antibody was immunoprecipitated and immunoblotted only in liver samples from AdCMV-GM\Delta C-injected animals. It should be noted that we expect systemic administration of AdCMV-G_{m\Delta C} to result in expression of G_{m\Delta C} in a largely liver-specific fashion, based on prior analysis of a wide array of tissues in rats and mice infused with this and other recombinant adenoviruses (8, 10, 15, 16).

Normalization of Blood Glucose Levels in STZ-treated Rats by Hepatic Expression of G_{m\Delta C}—Blood glucose was measured daily for 5 days after STZ treatment and then for an additional 6 days after adnoviral injection. The glucose values for individual STZ-treated animals in the ad libitum fed state are shown immediately before and 6 days after injection of AdCMV-G_{m\Delta C} (Fig. 2A) or in a control group injected with AdCMV-JGAL or left uninjected (Fig. 2B). All animals that received the AdCMV-G_{m\Delta C} virus experienced a decline in blood glucose to the normal range, whereas this never occurred in AdCMV-JGAL-injected or uninjected controls. A summation of this data is provided in Fig. 2C and shows that control STZ-injected animals had no decline in average glucose values (381 ± 11 versus 379 ± 35 mg/dl prior to and 6 days after viral injection, respectively). In sharp contrast, AdCMV-G_{m\Delta C}-injected animals experienced a decline in blood glucose from

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the average in normal controls (122 mg/dl immediately prior to and 172 mg/dl 6 days after viral injection, the latter value being indistinguishable from the average in normal controls (122 ± 10 mg/dl). We also examined blood glucose levels in rats following an overnight fast (Fig. 2D). Fasting caused a lowering of blood glucose levels into the normal range in all three groups of rats. AdCMV-GαC-treated, STZ-injected rats had blood glucose levels that were slightly and significantly lower than STZ-injected controls, but these values were not different from those in uninjected controls (75 ± 13, 89 ± 8, and 84 ± 13 mg/dl for the AdCMV-GαC-treated/STZ-injected, STZ-injected control, and uninjected control groups, respectively). Thus, AdCMV-GαC injection completely normalized blood glucose levels in STZ-diabetic rats.

Effect of GαC Expression on Circulating Hormone and Metabolite Levels—Lowering of blood glucose levels by expression of genes that regulate carbohydrate metabolism in liver has the potential to perturb lipid homeostasis, as was evident in a prior study from our laboratory involving adenovirus-mediated expression of glucokinase (15). Table I presents a profile of several key blood hormones and metabolites in the various groups of rats from the current study. With regard to key metabolic regulatory hormones, STZ injection resulted in a 68% decrease in circulating insulin levels relative to uninjected controls, with a similar decrease occurring in STZ-treated animals that received the AdCMV-GαC virus. These decreases in circulating insulin were accompanied by a 40% increase in glucacon levels in both groups. Thus, both groups of STZ-treated rats experienced a fall in insulin/glucagon ratio of -75% (from 0.12 to 0.03) despite the fact that one group was hyperglycemic (AdCMV-βGAL/uninjected controls) and the other normoglycemic (AdCMV-GαC-injected animals). Thus, lowering of blood glucose in the AdCMV-GαC-treated group was achieved by a mechanism independent of changes in insulin/glucacon ratio.

Table I also shows that there were no significant changes in circulating triglycerides, free fatty acids, ketones, or lactate in either group of STZ-injected animals (AdCMV-βGAL/uninjected or AdCMV-GαC-injected), relative to control rats that did not receive STZ. We presume that the absence of an increase in circulating lipids or ketones in the STZ-treated groups is attributable to our use of a single moderate dose of the drug that allows some residual insulin production. In sum, lowering of blood glucose by hepatic GαC expression does not perturb other indices of lipid or carbohydrate homeostasis measured in this study.

Normalization of Food Intake by Hepatic Expression of GαC in STZ-diabetic Rats

The hormone leptin plays a major role in control of food intake and feeding behavior. We therefore investigated whether the reduction in food intake in GαC-expressing animals was secondary to changes in circulating leptin levels. As shown in Fig. 4, STZ injection caused leptin levels to decrease by 82 and 70% in the AdCMV-βGAL-treated/untreated and AdCMV-GαC-treated groups, respectively. On average, leptin levels were slightly but significantly higher in AdCMV-GαC-injected animals than in the control STZ-treated rats (p < 0.01). It is unclear whether this slight increase in leptin in the former animals is of any functional significance. In fact, within a subset of several AdCMV-GαC (n = 4) or control (n = 3) STZ-treated-animals with no significant differences in their plasma leptin levels (0.70 ± 0.15 versus 0.74 ± 0.01 ng/ml in GαC-expressing versus control animals, respectively), food intake was still dramatically reduced in the GαC-expressing animals relative to controls (86 ± 4.8 versus 126 ± 2.5 mg/g/day, respectively). Furthermore, work from another laboratory shows that STZ treatment does not influence leptin sensitivity in rats (17). In sum, whereas we consider it unlikely that leptin makes a significant contribution to the complete normalization of food intake in the STZ-injected, AdCMV-GαC-treated rats, some contribution of the hormone cannot be rigorously excluded at the present time.

Another possible explanation for the decline in food intake in AdCMV-GαC-treated rats could be that the lowering of blood glucose in these animals prevented spilling of calories in the form of glucose in the urine, thus avoiding the need for compensatory hyperphagia. To test this idea, we measured urine volume and glucose concentration in a separate group of STZ-injected rats with and without GαC expression. Animals treated with STZ and AdCMV-GαC had blood glucose levels of 316 ± 50 mg/dl immediately prior to and 172 ± 67 mg/dl 6 days after viral injection (n = 3). Control animals not injected with AdCMV-GαC had blood glucose levels of 320 ± 54 and 317 ± 44 mg/dl at the same time points (n = 8). As measured in the 24-h period between the fifth and sixth day after AdCMV-GαC injection, total urine volume and urine glucose dropped from 78 to 27 ml and from 7.6 g of glucose/24 h to 0.9 g of glucose/24 h, respectively, in response to hepatic GαC expression. For comparison, animals that did not receive an injection of STZ had blood glucose levels of 120 mg/dl and no detectable glucose in their urine and produced 18 ml of urine/day (n = 7). The difference in glucose spilling in the untreated versus AdCMV-GαC-treated STZ-injected animals of 6.7 g of glucose/24 h (7.6–0.9 g) is equivalent to a loss of 26 kcal/day. This is nearly exactly matched by the reduction in food intake of 25 kcal/day in GαC expressing STZ-injected rats versus
controls. Thus, the reversal of caloric spilling in the urine is accounted for by a compensatory reduction in food intake in STZ-treated rats with hepatic expression of G<sub>mΔC</sub>.  

Expression of G<sub>mΔC</sub> in Liver of STZ-treated Rats Restores Glycogen Levels to Normal—Hepatic glycogen metabolism is impaired in all forms of diabetes (18–20). We therefore investigated the effect of G<sub>mΔC</sub> expression on liver glycogen stores in STZ-treated rats. As shown in Fig. 5, STZ treatment caused a 61% decrease in liver glycogen content relative to untreated controls despite significantly higher glucose levels in the STZ-treated animals (379 ± 35 versus 122 ± 10 mg/dl). In contrast, the expression of G<sub>mΔC</sub> in liver of STZ-treated rats increased liver glycogen levels by 4-fold relative to STZ-treated controls and by 47% relative to untreated controls (p < 0.01).  

**Hepatic Overexpression of G<sub>i</sub> Fails to Mimic the Effects of G<sub>mΔC</sub> on Blood Glucose and Food Intake**—To address the potential role of hepatic glycogen repletion in mediating reduced food intake, we decided to express an alternate targeting subunit, G<sub>i</sub>, which in our prior studies was shown to cause strong increases in liver glycogen stores, even in fasted animals (8). As shown in Fig. 5, adenovirus-mediated expression of G<sub>i</sub> in liver of STZ-injected rats caused hepatic glycogen to increase to levels 8 times higher than in STZ-injected controls and double those in AdCMV-G<sub>mΔC</sub>-treated STZ-injected animals. Unlike G<sub>mΔC</sub>-expressing rats, in which blood glucose levels were lowered between the second and fourth day after viral injection and then remained low for the remainder of the experimental period (6 days), G<sub>i</sub>-expressing rats experienced a
The same animals as described in the legend to Fig. 2A were used for measurement of food intake. Animals received either a single bolus of 60 mg/kg streptozotocin (STZ-injected) or no streptozotocin injection (No STZ). One group of STZ-injected rats was treated with AdCMV-βGAL adenosivirus or received no viral treatment (AdCMV-βGAL/no virus). A separate group of STZ-injected rats received the AdCMV-G₃ΔC virus. Food intake was measured during three successive 24-h periods, beginning 3 days day after viral treatment. Data represent the mean ± S.E. for eight animals in each STZ-treated group and six in the “no STZ” group. *, STZ-injected rats had increased food intake relative to noninjected controls, with /p < 0.001. #, AdCMV-G₃ΔC-treated, STZ-injected rats had reduced food intake relative to STZ-injected control rats, with /p < 0.001.

Fig. 4. Circulating leptin levels in STZ-injected rats. The animal treatment groups and their labeling are as described in the legend to Fig. 3. Plasma leptin levels were determined by radioimmunoassay 6 days after viral injection. Data represent the mean ± S.E. for eight animals in each STZ-injected group and six animals in the “no STZ” group. *, both the STZ-injected, AdCMV-G₃ΔC and STZ-injected control groups had leptin levels lower than the no STZ group, with /p < 0.02. #, leptin levels were higher in the STZ-injected, AdCMV-G₃ΔC-treated rats than in the STZ-injected control group, with /p < 0.01.

These data argue that liver glycogen content per se is not a primary regulator of food intake.

G₃ΔC Expression in Liver of STZ-treated Rats Exerts Metabolic Effects without Affecting Glucokinase Expression—Insulin-deficient states are known to be associated with a sharp decline in hepatic glucokinase expression (21–23), and reduction of glucokinase activity by liver-specific gene knock-out in mice results in impaired hepatic glycogen storage and glucose intolerance (24). These findings prompted us to investigate glucokinase expression in our model system. As shown in Fig. 7, STZ injection caused a sharp reduction in glucokinase mRNA and protein levels, as measured by reverse transcriptase-PCR and immunoblot analysis, respectively. Remarkably, glucokinase mRNA and protein levels were also very low in AdCMV-G₃ΔC-injected, STZ-treated rats.

Glucokinase-catalyzed phosphorylation of glucose is normally perceived as an important regulatory step in hepatic glucose balance. One possible mechanism by which G₃ΔC could have enhanced hepatic glucose disposal despite reduced glucokinase expression might be a compensatory increase in expression of other genes that are involved in hepatic glucose uptake and storage. To investigate this point, we measured the levels of GLUT-2 and glycogen synthase mRNA by semiquantitative multiplex reverse transcriptase-PCR. No significant differences in expression of these genes was noted between the AdCMV-G₃ΔC/STZ-injected, STZ-injected control, and uninjected groups (Fig. 8, A and B). Moreover, application of quantitative real time PCR methods for measurement of glucokinase and hexokinase I levels confirmed the sharp drop in expression of glucokinase in both the AdCMV-G₃ΔC-treated and control STZ-injected groups relative to uninjected controls but showed no change in expression of hexokinase I among these groups of animals (Fig. 8C).

DISCUSSION

Glycogen-targeting subunits of protein phosphatase-1 serve as important scaffolding proteins that juxtapose key enzymes of glycogen metabolism with the regulatory enzyme protein phosphatase-1. The various targeting subunit isoforms have
Fig. 6. Differential effects of $G_M^{AC}$ and $G_l$ on blood glucose and food intake in STZ-injected rats. Animals were treated as described in the legend to Fig. 5. A, blood glucose levels were measured after STZ injection but prior to administration of the indicated recombinant adenoviruses (Start), at the point of first strong decrease in blood glucose levels following viral administration (Inflection point), and 6 days after viral injection (Final). B, food intake was measured in the same animals at the infection and final time points described for A. Data represent the mean ± S.E. for 4 $G_M^{AC}$-expressing rats; 5 $G_l$-expressing rats, 10 no STZ, no virus controls; and 12 STZ-injected controls (a mixture of AdCMV-βGAL-injected and no virus). *, significant differences between the STZ-injected controls and STZ-injected, AdCMV-$G_l$ treated groups relative to other groups, with $p < 0.006$.

Fig. 7. Low levels of glucokinase expression in liver of STZ-injected, AdCMV-$G_M^{AC}$-treated rats. The animal treatment groups and their labeling are as described in the legend to Fig. 3. Animals were sacrificed for measurement of hepatic glucokinase mRNA levels by multiplex reverse transcriptase-PCR. A representative set of samples is shown in A, and quantitative analysis of this data by densitometric scanning, normalized to the internal α-tubulin control, is shown in B. *, STZ-treated animals had lower glucokinase mRNA levels regardless of whether they received AdCMV-$G_M^{AC}$. C, immunoblot analysis of glucokinase protein levels within the same liver samples as used for the RNA analysis in A and B.

different potencies for activating glycogen deposition and also affect the way cells respond to regulatory signals for glycogen synthesis and breakdown (3, 5–9, 25). Recently, we demonstrated reversal of glucose intolerance in rats fed a high fat diet by hepatic expression of $G_M^{AC}$, a novel truncated version of the muscle isoform $G_M$ but, surprisingly, not the more glycogenic liver isoform, $G_l$ (8). Further work supported the idea that the unique efficacy of $G_M^{AC}$ in this experiment was a result of its intermediate glycogenic potency, coupled with retention of full responsiveness to glycogenolytic signals such as forskolin and low glucose in cells in which it is expressed (6). The latter property distinguished $G_M^{AC}$ from other targeting subunits (6) and proved essential in preventing glycogen stores from filling up in the fasted state in high fat-fed rats, thereby allowing glycogen synthesis to be stimulated during a glucose challenge (8).

In the current study, we have extended our investigation of $G_M^{AC}$ to include its delivery to livers of animals with STZ-induced diabetes. Three new findings have emerged from this work. First, we show that $G_M^{AC}$ expression in liver is sufficient to fully normalize blood glucose levels and glycogen storage in this model of insulin-deficient diabetes. Importantly, and in contrast to what we have observed with hepatic overexpression of glucokinase (15), other circulating metabolites, including free fatty acids and triglycerides, were not perturbed in these experiments. Second, this normalization occurred despite very
The normalization of blood glucose and liver glycogen by hepatic G_mΔC expression is remarkable in that it occurred in the face of a 68% reduction in circulating insulin levels. Some insight into this outcome is derived from our prior work showing that stimulation of glycogen synthesis caused by overexpression of glycogen-targeting subunits in hepatocytes is glucose-dependent but largely insulin-independent (7, 25). Thus, our data are consistent with a model in which expression of G_mΔC increases hepatic glucose disposal when the system is challenged by elevated glucose but allows normal emptying of glycogen stores and protection against hypoglycemia in fasting or other catabolic states. This interpretation fits both our prior study in insulin-resistant high-fat-fed rats treated with Ad-CMV-G_mΔC, which were normoglycemic in the fasting state and exhibited normal glucose tolerance (8), and the current study, in which blood glucose levels were normalized in STZ-injected animals, with no evidence of hypoglycemia. Thus, our current and prior (8) studies taken together argue that appropriately regulated augmentation of hepatic glycogen storage is sufficient to reverse perturbations in glucose homeostasis caused by either impaired insulin action or impaired insulin secretion. Consistent with this conclusion, overexpression of another targeting subunit, G_A, in liver of insulin-resistant (8) or STZ-injected diabetic rats (current study) resulted in unregulated glycogen overstorage in liver and a consequent failure to stably reverse glucose intolerance or hyperglycemia, respectively.

Glucokinase is generally thought of as a key regulatory step in hepatic glucose metabolism. Consistent with this idea, patients with maturity-onset diabetes of the young, type 2, have mutations in their glucokinase gene that affect both insulin secretion and hepatic glucose disposal (20). Furthermore, tissue-specific knock out of the glucokinase gene in liver of mice results in perturbed glucose homeostasis and impaired liver glycogen storage (24), whereas overexpression of the enzyme in the liver increases glycogen deposition and lowers blood glucose (15). It was therefore quite surprising that G_mΔC expression in the liver was able to lower blood glucose levels and normalize liver glycogen content in STZ-injected rats in the face of a dramatic reduction in glucokinase expression. The mechanism by which G_mΔC overcomes the decrease in glucokinase expression in insulin-deficient animals remains to be defined. One idea is that STZ injection and/or G_mΔC expression could have caused a compensatory increase in other enzymes involved in hepatic glucose disposal, but such changes were not observed at the RNA level for the glycogen synthase, GLUT2, or hexokinase I genes. However, G_mΔC might still have activated glycogen synthesis by a “pull mechanism,” in which stimulation of glycogen synthase activity resulted in lowering of glucose 6-phosphate levels and consequent removal of product inhibition of hexokinase I activity. Alternatively, G_mΔC expression may have diverted gluconeogenic precursors away from the glucose-6-phosphatase reaction and into the glycogen storage pathway, thereby contributing to lowering of glucose levels via a decrease in hepatic glucose production. Consistent with this model, we have recently employed 2H2O NMR to demonstrate that a larger proportion of glycogen is synthesized from the level of gluconeogenic precursors in hepatocytes with overexpressed glucokinase-targeting subunits than in cells with overexpressed glucokinase (6). Also consistent with this idea, expression of the PTG-targeting subunit in liver of normal rats resulted in reduced expression of the catalytic subunit of glucose-6-phosphatase (9). Whereas these ideas will require further investigation, our data clearly establish that normalization of blood glucose can be achieved by manipulation of steps distal to glucokinase, even when this important enzyme is present at very low levels.

Another possible mechanism contributing to lowering of blood glucose in animals with hepatic G_mΔC expression is their decrease in food intake relative to controls. This is clearly not the only mechanism at work, however, based on a study by others of the effects of leptin infusion in STZ-induced diabetic rats (17). In these studies, infusion of leptin at a rate that allowed circulating levels of the hormone to be restored to those found in non-STZ-injected controls resulted in complete normalization of food intake. Hyperglycemia was partially ameliorated in these animals but clearly not normalized (glucose levels fell from 24.3 to 17.2 mM). In contrast, expression of G_mΔC in STZ-injected animals not only normalized food intake but also lowered blood glucose levels completely to normal (from 18.6 to 6.1 mM).

There are several possible mechanisms by which G_mΔC expression in liver could lead to normalization of food intake in

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**Fig. 8. GLUT2, glycogen synthase and hexokinase I mRNA levels in liver.** The animal treatment groups and their labeling are as described in the legend to Fig. 3. Animals were sacrificed for measurement of hepatic glycogen synthase (GS) and GLUT2 mRNA levels by multiplex reverse transcriptase-PCR. A representative set of samples is shown in A, and quantitative analysis of this data by densitometric scanning, normalized to the internal α-tubulin control, is shown in B. C, the levels of glucokinase (GK) and hexokinase I (HKI) mRNA in liver samples measured by quantitative real time PCR, using α-tubulin as the internal control. Data represent the mean ± S.E. for six samples/group. * significantly lower glucokinase expression in either group of STZ-injected rats relative to uninjected controls, confirming the data of Fig. 7.
Hepatic Expression of $G_M^{AC}$ in STZ-diabetic Rats

We have now established that expression of $G_M^{AC}$ in liver not only reverses glucose intolerance in insulin-resistant rats but also normalizes blood glucose in STZ-induced diabetic animals. However, we recognize that whereas $G_M^{AC}$ is an effective reagent for treatment of diabetes in rodents, its application to human diabetes is hampered by the lack of safe and efficacious vectors for delivery of foreign genes to liver of diabetic patients. Nevertheless, the surprising demonstration of a glucose-lowering effect of $G_M^{AC}$ in the background of depressed hepatic glucokinase expression and insulin insufficiency may place new focus on drugs that activate liver glycogen storage as a means of controlling blood glucose.

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STZ-injected animals. Three that were considered and tested experimentally in this study were the following: 1) $G_M^{AC}$ expression in liver affected leptin levels; 2) $G_M^{AC}$ expression reduced calorie spilling in the urine; and 3) liver glycogen repletion had a direct effect on food intake. STZ injection caused a large decrease in circulating leptin levels, probably secondary to the increased glucagon/insulin ratio and consequent reduction in fat mass in the diabetic animals. Expression of $G_M^{AC}$ in liver of STZ-injected animals resulted in a small but statistically significant rise in circulating leptin levels relative to animals that received the drug alone. Whereas we think it is unlikely that this small increase in leptin had a major impact on the reduced food intake in $G_M^{AC}$-expressing, STZ-injected rats, for reasons set forth under “Results,” the possibility cannot be rigorously excluded at present.

$G_M^{AC}$ expression caused a clear reduction in excretion of glucose in the urine. This amelioration of caloric “spilling” was proportional to the reduction in food intake. This finding is consistent with the idea that $G_M^{AC}$ expression lowered food intake secondary to its effect on blood glucose. However, the factors that allowed $G_M^{AC}$-expressing rats to sense the reduction in caloric spilling and translate this into a reduction in food intake remain to be defined, especially since these changes occurred at a time when hormones known to regulate food intake such as insulin (26) and leptin were present at low levels. It remains possible that levels of peptides that influence feeding behaviors such as glucagon-like peptide-1, CCK, or ghrelin could be altered by $G_M^{AC}$ expression, and this will require further investigation. Finally, we considered the possibility that reduced food intake in STZ-injected, $G_M^{AC}$-expressing rats might be related to changes in glucose disposal and storage in the liver. Fifty years ago, Mayer (27, 28) developed a “glucostatic theory” of feeding behavior in which changes in glucose utilization rates were proposed to regulate hunger and satiety. A “glycogenostatic” model in which hepatic glycogen stores play a central role in regulating food intake and energy balance has also been proposed (29–32), but direct and consistent experimental support for this idea has not emerged. In the current study, expression of the targeting subunit GL in STZ-injected animals caused sharp but transient decreases in food intake and circulating glucose levels. Unlike $G_M^{AC}$-expressing animals, in which both variables declined and then remained low, $G_M^{AC}$-overexpressing animals experienced a return to high rates of food consumption in parallel with the return of hyperglycemia (Fig. 6). Because the $G_M^{AC}$-expressing animals had much higher liver glycogen content than the $G_M^{AC}$ animals at sacrifice (Fig. 5), these data argue against a primary role for liver glycogen stores as a regulator of food intake. However, it remains possible that a sudden increment in hepatic glycogen storage, such as induced upon initial overexpression of GL, could generate satiety signals. Upon balance, however, our current best understanding is that the main driver of reduced food intake in $G_M^{AC}$ expressing, STZ-injected rats is the lowering of blood glucose, which in turn results in reduced caloric spilling in the urine, leading to abrogation of compensatory hyperphagia.