Glycogen-targeting subunits of protein phosphatase-1 facilitate interaction of the phosphatase with enzymes of glycogen metabolism. Expression of one family member, PTG, in the liver of normal rats improves glucose tolerance without affecting other plasma variables but leaves animals unable to reduce hepatic glycogen stores in response to fasting. In the current study, we have tested whether expression of other targeting subunit isoforms, such as the liver isoform G\textsubscript{L} or a truncated version of G\textsubscript{M}/RGl termed G\textsubscript{M}/RGl, or a truncated version of G\textsubscript{M}/RGl termed G\textsubscript{M}/RGl, expressed primarily in liver ameliorates glucose intolerance in rats fed on a high fat diet (HF). HF animals overexpressing G\textsubscript{M}/RGl, but not G\textsubscript{L} or G\textsubscript{M}/RGl, exhibited a decline in blood glucose of 35–44 mg/dl relative to control HF animals during an oral glucose tolerance test (OGTT) such that levels were indistinguishable from those of normal rats fed on standard chow at all but one time point. Hepatic glycogen levels were 2.1–2.4-fold greater in G\textsubscript{L}- and G\textsubscript{M}/RGl-overexpressing HF rats compared with control HF animals following OGTT. In a second set of studies on fed and 20-h fasted HF animals, G\textsubscript{M}/RGl-overexpressing rats lowered their liver glycogen levels by 57% (from 402 ± 54 to 173 ± 27 µg of glycogen/mg of protein) in the fasted versus fed states compared with only 44% in G\textsubscript{L}- overexpressing animals (from 740 ± 35 to 413 ± 141 µg of glycogen/mg of protein). Since the OGTT studies were performed on 20-h fasted rats, this meant that G\textsubscript{M}/RGl-overexpressing rats synthesized much more glycogen than G\textsubscript{L}-overexpressing HF rats during the OGTT (419 versus 117 µg of glycogen/mg of protein, respectively), helping to explain why G\textsubscript{M}/RGl preferentially enhanced glucose clearance. We conclude that G\textsubscript{M}/RGl has a unique combination of glycogenic potency and responsiveness to glycogenolytic signals that allows it to be used to lower blood glucose levels in diabetes.

Hepatic glycogen storage is impaired in all major forms of diabetes, contributing to the development of hyperglycemia (1–9). This suggests that one possible means of improving glycemic control might be to enhance glucose disposal by stimulating hepatic glycogen synthesis. One method for increasing liver glycogen content is to increase the activity of the glucose phosphorylating enzyme, glucokinase. Indeed, overexpression of this enzyme in liver of normal rats (4) or mice (5, 6) lowers blood glucose levels with a commensurate increase in glycogen stores. However, these changes are accompanied by increases in circulating free fatty acids, triglycerides, and lactate (4), consistent with the large increase in glycolytic flux caused by overexpression of glucokinase in hepatocytes or hepatoma cells (7, 8).

More specific stimulation of glycogen synthesis in liver may be achievable by manipulation of the expression of proteins that function distal to the glucose phosphorylation step. In particular, recent studies have highlighted an important role for glycogen-targeting subunits of protein phosphatase-1 (PP-1)\textsuperscript{1} in spatial organization and regulation of glycogen metabolism (9). Prominent members of this gene family include G\textsubscript{M} or RGl (hereafter referred to as G\textsubscript{M}/RGl), expressed primarily in striated skeletal muscle (10), G\textsubscript{L}, expressed primarily in liver (11), and protein targeting to glycogen (PTG) (12, 13) and PPRR6 (14), expressed in a wide range of tissues. These proteins bind to glycogen and protein phosphatase-1 and have differential capacities for binding to glycogen synthase, glycogen phosphorylase, and phosphorylase kinase (9–14).

It has become apparent that a major challenge inherent in considering glycogen-targeting subunits as molecules for enhancing hepatic glucose disposal is to choose or design a protein with the optimal combination of regulatory features. Thus, overexpression of glycogen-targeting subunits reveals that all family members tested stimulate glycogen deposition in rat hepatocytes but with clear differences in potency in the order G\textsubscript{L} > PTG > G\textsubscript{M}/RGl (15). Cells with overexpressed targeting subunits also exhibit differences in response to glycogenolytic agents such as glucagon and forskolin in the order (from more to less responsive) of G\textsubscript{M}/RGl > G\textsubscript{L} > PTG (15, 16). To date, we have performed one in vivo study in which hepatic overexpression of PTG in normal rats was shown to improve glucose tolerance without perturbation of lipid homeostasis (17). However, these animals also had markedly elevated liver glycogen levels in the fed state and almost no reduction in hepatic glycogen stores in response to an overnight fast, suggesting that they might be more susceptible to perturbations in glycemic control during prolonged fasting, sustained exercise, or other stressful circumstances.

These findings have recently led us to design and test a novel form of glycogen-targeting subunit derived from G\textsubscript{M}/RGl (16). Native G\textsubscript{M}/RGl is distinct from other members of its gene family in that it contains two consensus sequences for protein kinase

\textsuperscript{1} The abbreviations used are: PP-1, protein phosphatase-1; PTG, protein targeting to glycogen; HF, high fat diet; OGTT, oral glucose tolerance test; RT, reverse transcription; TG, triglyceride.
A-mediated serine phosphorylation. One of these sites resides within the PP-1 binding site of G\textsubscript{M/RGl}, and its phosphorylation leads to dissociation of the phosphatase, contributing to inactivation of glycogen synthesis (10, 18, 19). G\textsubscript{M/RGl} is also distinguished from other targeting subunits by virtue of its large C terminus that includes a hydrophobic domain that mediates binding of the protein to sarcoplasmic reticulum in muscle (10, 20). Removal of 735 C-terminal amino acids from native G\textsubscript{M/RGl} yields a 275-amino acid molecule that we have termed G\textsubscript{M}\textsubscript{AC} that can be directly aligned with the similarly sized native G\textsubscript{L} and PTG proteins. Overexpression of G\textsubscript{M}\textsubscript{AC} and native G\textsubscript{M/RGl} in hepatocytes reveals that the former protein is more effective at stimulating glycogen synthesis (16). Moreover, unlike PTG- or G\textsubscript{L}\textsubscript{-overexpressing cells, cells with G\textsubscript{M}\textsubscript{AC} overexpression retain responsiveness to glycogenolytic signals such as forskolin or lowering of media glucose concentrations. These promising findings have led us, in the current study, to compare the metabolic impact of G\textsubscript{M}\textsubscript{AC}, G\textsubscript{L}, and G\textsubscript{M/RGl} overexpression in whole animals. These studies have been performed in rats fed on a high fat diet for 7 weeks to cause a syndrome of insulin resistance and glucose intolerance such as is seen in early stage type 2 diabetes. We find that G\textsubscript{M}\textsubscript{AC} is unique among the molecules tested in its capacity to reverse diet-induced glucose intolerance.

MATERIALS AND METHODS

Animal Maintenance and Administration of Recombinant Adenoviruses—All procedures were carried out in accordance with animal care guidelines of the University of Texas Southwestern Medical Center at Dallas and the National Research Council. Male Wistar rats (Charles River Laboratories, Wilmington, MA and Harlan Tekland Laboratory, Windfield, IA) weighing 175–200 g were housed under these conditions for 7 weeks before adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration and Depo-Medrol (1.5 mg/kg; Pharmacia & Upjohn) for 3 consecutive days starting on the day prior to adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration, Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem) for 3 consecutive days starting on the day prior to adenovirus administration.

Animal Studies—Two experimental protocols were performed. In the first, animals were infused with AdCMV-G\textsubscript{L}, AdCMV-G\textsubscript{M/RGl}, AdCMV-\textsubscript{G}M\textsubscript{AC}, or AdCMV-\textsubscript{G}M\textsubscript{GAL} viruses. Ninety hours after virus administration, animals were fasted for 20 h without access to water. An oral glucose tolerance test (OGTT) was performed by anesthetizing animals (50 mg/kg of body weight intraperitoneally), blood samples were taken, and liver was excised and rapidly frozen in liquid nitrogen and stored at −70 °C until further analysis. In the second protocol, animals were infused with AdCMV-G\textsubscript{L}, AdCMV-G\textsubscript{M/RGl}, AdCMV-\textsubscript{G}M\textsubscript{GAL} viruses. Ninety hours after virus administration, animals were either fasted for 20 h or allowed to continue feeding ad libitum. Thereafter, all animals were anesthetized with Nembutal (50 μg/kg of body weight intraperitoneally), blood samples were taken, and liver was excised and rapidly frozen in liquid nitrogen and stored at −70 °C until further analysis. Measurement of Glycogen-targeting Subunit Expression in Liver or Muscle by Semiquantitative Multiplex RT-PCR—The procedure was used on methods described previously (15, 17). Total RNA was extracted from powdered muscle that included the spin columns (Qiagen Inc., Valencia, CA) following the instructions of the manufacturer. First-strand cDNA was prepared using 0.5 μg of total RNA, the Superscript RT kit, and random hexamer primers (Invitrogen) according to the instructions of the manufacturer. The cDNA was diluted 1:6 in distilled water, and PCR was carried out using 5 μl of the diluted cDNA and a PCR mix containing Taq DNA polymerase (2.5 U/μl of reaction buffer (Promega Corp., Madison, WI), dNTP mix (final concentrations of 40 μM of each dNTP except dCTP, which was present at 20 μM; Invitrogen) and with or without 1.25 μl of [α-32P]dCTP (2,000 Ci/mmol; PerkinElmer Life Sciences) in a 25-μl reaction volume. Four primer sets (5 pmol of each primer) were used in these studies. The first set specifically amplified a 181-bp fragment from the G\textsubscript{L} transgene and did not amplify endogenous rat G\textsubscript{L} because the upstream primer hybridizes to 5′ untranslated sequence derived from the adenovirus vector (5′ primer, CGAGCTCGTACCAATCTC; 3′ primer, GAAGGT-GAACGCTCTTCGT). The second set amplified a 162-bp product from either the full-length endogenous G\textsubscript{M/RGl} or G\textsubscript{M}\textsubscript{AC} transgene as described previously (15). The third oligonucleotide pair specifically amplified a 900-bp fragment of G\textsubscript{M}\textsubscript{AC} derived by expression from the AdCMV-G\textsubscript{M}\textsubscript{AC} adenovirus because the upstream primer hybridizes to sequence within the G\textsubscript{M}\textsubscript{AC} cDNA sequence, while the 3′ primer hybridizes to the 3′ untranslated region derived from the adenovirus vector (5′ primer, CTCAAAAAAGAAAGATCTTTAGCAAC; 3′ primer, GTGATTTT-GTCCAAATTTGCAC). The last oligonucleotide pair amplified one of the following as internal standards: a 186-bp fragment of the endogenous TATA-binding protein transcript, a 201-bp fragment of the elongation factor-1α (EF-1α) mRNA (17), or a 250-bp fragment of the α-tubulin gene (5′ primer, GATACATGCATTTACATACTAC; 3′ primer, GTTAGGTT-GCCATGCTGAACTT). In experiments involving inclusion of [α-32P]dCTP, PCR conditions were as follows: an initial incubation at 95 °C for 5 min followed by 22 or 24 cycles (the latter only when studying full-length G\textsubscript{M/RGl} transgene expression) of 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 30 s. The final PCR products were mixed with 98% formamide denaturing loading buffer and separated on a 6% (w/v) polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen, and the resulting scan was analyzed using ImageQuant from Molecular Dynamics (Sunnyvale, California). In the experiments designed to assess expression of the G\textsubscript{M}\textsubscript{AC} transgene in extrathyroid tissues, PCRs were carried out in the absence of [α-32P]dCTP. For these experiments, PCR conditions were an initial incubation at 95 °C for 1 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min. DNA products were purified on a 0.8% agarose gel, and products were visualized by incubation of the gel with 0.6 μg/ml ethidium bromide.

Plasma and Tissue Analysis—Plasma insulin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO). Plasma triglyceride, ketone, and lactate levels were measured using kits from Sigma Chemical Co. Plasma free fatty acids were measured using a kit from Roche Molecular Biochemicals. Plasma glucose was measured using a HemoCue Glucose Analyzer (HemoCue AB, Angelholm, Sweden). Liver glycogen content was measured by an amyloglucosidase-based assay as described elsewhere (24).

Statistical Analysis—Data are expressed as the mean ± S.E. Statistic significance was determined by unpaired Student’s t test using the statistics module of Microsoft Excel (version 5.0; Microsoft Corp., Redmond, WA). Statistical significance was assumed at p < 0.05.

RESULTS

Expression of Glycogen-targeting Subunits in Rat Liver—Adenovirus-mediated expression of the various glycogen-targeting subunit isoforms in liver was evaluated by semiquantitative multiplex RT-PCR analysis in animals fed on the high fat diet (HF). A representative gel is shown in Fig. 1A. Animals treated with AdCMV-\textsubscript{G}M\textsubscript{GAL} exhibited either no signal or background at the positions expected for the reverse-transcribed and amplified fragments of G\textsubscript{L}, G\textsubscript{M}\textsubscript{AC}, or G\textsubscript{M/RGl}. Rats infused with AdCMV-G\textsubscript{L}, AdCMV-G\textsubscript{M/RGl}, or AdCMV-G\textsubscript{M}\textsubscript{AC} showed clear expression of the respective transgene mRNAs. When normalized to the internal control, TATA-binding protein, G\textsubscript{L}
Glycogen following OGTT To determine whether the differences between the three treatment groups were not significantly different.

Effects of GL, GMΔC, or GM/RGl Overexpression on Liver Glycogen following OGTT—To determine whether the differences in glycogen stores relative to feeding with normal chow (both of the experiment (180-min time point) summarized in Fig. 3) were not significantly different.

Effects of GL, GMΔC, or GM/RGl Overexpression on Liver Glycogen following OGTT—To determine whether the differences in glycogen stores relative to feeding with normal chow (both of the experiment (180-min time point) summarized in Fig. 3) were not significantly different.
Effects of Glycogen-targeting subunit Overexpression on Circulating Metabolites and Hormones after OGTT—A large aliquot of blood was collected from animals at the conclusion of the OGTT experiment summarized in Fig. 2 (180-min time point), allowing several plasma variables to be measured. As summarized in Table 1, in HF animals, overexpression of the various glycogen-targeting subunit isomers had no effect on circulating free fatty acids, ketones, lactate, or insulin relative to AdCMV-βGAL-treated controls. Treatment of animals with AdCMV-GM or AdCMV-GM/RGl also did not alter circulating triglyceride (TG) levels. However, AdCMV-GMΔC treatment did cause an 80% increase in TG levels relative to those of AdCMV-βGAL-treated controls that was significant at the level of p = 0.045.

Reversal of Glucose Intolerance in AdCMV-βGALΔC-infused Rats Is Not Due to “Leaky” Expression of the Transgene in Muscle—In previous studies involving systemic infusion of recombinant adenoviruses to deliver the glucokinase or glucose-6-phosphatase genes in rats, we found no evidence of transgene expression in extrahepatic tissues such as muscle, fat, brain, or kidney and only very low levels of expression in lung (4, 28). However, even modest expression of targeting subunits in a large tissue mass such as muscle could potentially affect the conclusions of the current study. To eliminate this possibility, we used RT-PCR to measure expression of the GmΔC transcript in liver and skeletal muscle of AdCMV-βGAL- and AdCMV-GMΔC-infused animals. This assay used an oligonucleotide pair that specifically amplifies the transcript derived from the adenovirus construct and not endogenous Gm/RGl. As a positive control, treatment of 293 cells with AdCMV-GMΔC and RT-PCR analysis of RNA derived from such cells resulted in amplification of a band of the predicted size of 900 nucleotides (Fig. 4). RT-PCR analysis was also performed on RNA isolated from liver and muscle samples taken from three AdCMV-βGAL- or three AdCMV-GMΔC-treated rats subjected to OGTT.

As shown in Fig. 4, a band of the same size as that in the AdCMV-GMΔC-treated 293 cells was clearly detected in liver samples of AdCMV-GMΔC-treated, but not AdCMV-βGAL-treated, rats. However, a band of this size was not amplified from muscle RNA regardless of whether the animals were treated with AdCMV-GMΔC. These findings clearly demonstrate that the improved glucose tolerance reported in Fig. 2 is due to expression of GmΔC in liver and not in muscle.

Reversal of Glucose Intolerance by Targeting Subunit of PP-1

FIG. 3. Liver glycogen levels after the OGTT. Animals were sacrificed for collection of liver samples at the 180-min point of the OGTT shown in Fig. 2. Data represent mean ± S.E. for six GmC, seven GmRGl, and seven GmΔC-overexpressing HF rats and 12 β-galactosidase-overexpressing HF (βGAL) and eight β-galactosidase-overexpressing standard chow-fed (βGAL/std. chow) rats. The symbols * and ** indicate that GmC and GmΔC-overexpressing animals stored more glycogen than HF β-galactosidase controls with levels of significance of p < 0.001 and p < 0.005, respectively.

βGAL or three AdCMV-GMΔC-treated rats subjected to OGTT. As shown in Fig. 4, a band of the same size as that in the AdCMV-GMΔC-treated 293 cells was clearly detected in liver samples of AdCMV-GMΔC-treated, but not AdCMV-βGAL-treated, rats. However, a band of this size was not amplified from muscle RNA regardless of whether the animals were treated with AdCMV-GMΔC. These findings clearly demonstrate that the improved glucose tolerance reported in Fig. 2 is due to expression of GmΔC in liver and not in muscle.

Regulation of Glycogen Metabolism in Response to Fasting and Feeding in HF Rats with Hepatic Overexpression of Glycogen-targeting Subunits—In an effort to better understand the differential effects of GmC and Gm overexpression on glucose tolerance (Fig. 2), we next studied liver glycogen levels in fed and fasted HF animals treated with AdCMV-GMΔC or AdCMV-GL. Multiplex RT-PCR analysis of transgene expression levels in these animals is summarized in Fig. 5. In both the AdCMV-GmC- and AdCMV-GMΔC-treated groups, transgene expression tended to be lower in fasted animals, but this difference was not significant in either group. Comparison of GmC to Gm mRNA levels in fed versus fed or fasted versus fasted groups also revealed no significant differences.

As shown in Fig. 6, AdCMV-βGAL-treated HF rats contained 317 ± 46 μg of glycogen/mg of protein in the fed state and depleted this reserve by 68%, to 103 ± 15 μg of glycogen/mg of protein, in response to a 20-h fast. Interestingly, fed AdCMV-GmC-treated rats accumulated 740 ± 35 μg of glycogen/mg of protein, 2.3 times more than fed AdCMV-βGAL-treated controls, and were only able to lower glycogen by 44% in response to fasting to a level of 413 ± 141 μg of glycogen/mg of protein. In sharp contrast, fed AdCMV-GMΔC-treated rats contained 402 ± 54 μg of glycogen/mg of protein in liver and reduced their glycogen stores by 57% in response to the 20-h fast to 173 ± 27 μg of glycogen/mg of protein, a value slightly higher than that in fasted AdCMV-βGAL-treated controls. Importantly, the liver glycogen level in fasted AdCMV-GmC-treated rats was 80% of that in AdCMV-GmΔC-treated rats following OGTT. In contrast, liver glycogen content in fasted AdCMV-GmΔC-treated rats was only 29% of that in AdCMV-GMΔC-treated rats following OGTT. In other words, AdCMV-GmΔC-treated rats synthesized 419 μg of glycogen/mg of protein during the OGTT compared with an increment of only 117 μg of glycogen/mg of protein in AdCMV-GmC-treated animals (values obtained by subtracting the glycogen levels in fasted rats shown in Fig. 6 from the glycogen levels after OGTT shown in Fig. 3; note that animals were fasted for 20 h prior to OGTT). This suggests that the differential potency of GmC and Gm for lowering of blood glucose in glucose-intolerant HF rats may have been due in part to the high basal glycogen levels in GmC-overexpressing rats that impaired further glycogen storage during OGTT.

Effects of Glycogen-targeting Subunit Overexpression on Circulating Metabolites and Hormones in Fasted and Fed Rats—The same group of plasma variables assayed after OGTT (Table I) was measured in fed and 20-h fasted HF rats treated with the various recombinant adenoviruses (Table II). Animals treated with AdCMV-βGAL, AdCMV-GmC, or AdCMV-GmΔC all showed expected changes in plasma glucose, free fatty acids, lactate, and ketones as a function of fasting and feeding. Insulin levels remained high in the fasted state in all three groups of animals, but this is not unexpected given the known effect of high fat feeding to cause insulin resistance and consequent fasting hyperinsulinemia (23). Circulating TGs were more than twice as high in fed compared with fasted AdCMV-βGAL- or AdCMV-GMΔC-treated rats. However, in AdCMV-GmC-treated animals, TG remained
AdCMV-βGAL-, AdCMV-GL-, AdCMV-GM/RGlu-, and AdCMV-GMΔC-treated rats after the OGTT protocol.

Male Wistar rats were fed a high fat diet for 7 weeks. At the end of this period, animals received the AdCMV-βGAL, AdCMV-GL, AdCMV-GM/RGlu, or AdCMV-GMΔC adenoviruses and were allowed to feed as ad libitum. Animals were then fasted for 20 h before receiving an oral glucose bolus (2 g/kg). Blood samples were collected after the 180-min time point of the OGTT for analysis of the indicated plasma variables. Data are mean ± S.E. for the number of animals indicated in each group. The symbol * indicates a significant difference compared to the AdCMV-βGAL-treated control group with $p = 0.045$. FFAs, free fatty acids.

Preparation of the Table:

| Variable                  | AdCMV-βGAL (n = 12) | AdCMV-GL (n = 6) | AdCMV-GM/RGlu (n = 7) | AdCMV-GMΔC (n = 7) |
|---------------------------|----------------------|------------------|-----------------------|---------------------|
| Triglycerides (mg/dl)     | 106.2 ± 14.2         | 89.0 ± 18.1      | 92.0 ± 19.8           | 191.8 ± 33.3*       |
| FFAs (mmol/l)             | 0.26 ± 0.02          | 0.20 ± 0.02      | 0.23 ± 0.03           | 0.28 ± 0.03         |
| Ketones (mg/dl)           | 2.84 ± 0.58          | 2.06 ± 0.53      | 2.33 ± 0.72           | 2.74 ± 0.73         |
| Lactate (mg/dl)           | 16.0 ± 3.1           | 9.1 ± 1.3        | 11.4 ± 1.0            | 10.9 ± 1.4          |
| Insulin (ng/ml)           | 3.78 ± 0.39          | 2.90 ± 0.41      | 3.65 ± 0.75           | 4.67 ± 0.38         |

**Discussion**

Regulation of carbohydrate metabolism in liver is perturbed in type 2 diabetes, resulting in increased hepatic glucose production. Factors contributing to this imbalance include increased gluconeogenesis and impairment of hepatic glycogen storage. One approach to improving hepatic glucose balance in diabetes might be to increase the glycolytic rate or, conversely, to decrease the rate of gluconeogenesis. Consistent with this idea, hepatic overexpression of glucokinase (4–6, 25) or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (26) has been shown to lower blood glucose levels in normal or diabetic animals. Furthermore, overexpression of phosphoenolpyruvate carboxykinase (27), the glucose-6-phosphatase catalytic subunit (28), or the transcriptional co-activator PGC-1, which stimulates expression of the genes encoding several gluconeogenic enzymes (29), all result in hyperglycemia. However, overexpression of glucokinase or 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase results in increases in the levels of circulating lipids (4, 26), raising the concern that therapies that enhance glycolytic rate may also exacerbate the hyperlipidemia associated with type 2 diabetes. It has also been surprising to learn that liver-specific knockout of phosphoenolpyruvate carboxykinase has minimal effects on glucose homeostasis but causes hepatic steatosis (30), suggesting an important role for this enzyme in integration of carbohydrate and lipid metabolism that may preclude its use as a target in diabetes therapy.

In light of the potential complications associated with drugs directed at enzymes of glycolysis or gluconeogenesis, our group has been investigating the utility of glycogen-targeting subunits of protein phosphatase-1 for lowering of blood glucose in diabetes. The advantage of this approach would be to stimulate

**Diagram 4**

RT-PCR analysis of GmΔC transgene expression in liver and muscle. Oligonucleotides specific to the GmΔC gene product expressed from the AdCMV-GmΔC adenovirus or, as an internal control, to α-tubulin were used for multiplex RT-PCR analysis of liver and muscle samples from a subset of the animals used for OGTT as described in Figs. 1–3. As an additional control, the same oligonucleotides were used to analyze an RNA sample from cultured 293 cells treated with AdCMV-GmΔC. Note that a band of 900 bp (labeled GmΔC Transgene), as predicted only in cells with adenovirus-mediated expression of GmΔC, is found in liver of AdCMV-GmΔC-treated rats or in 293 cells treated with this virus but not in liver samples from AdCMV-βGAL-treated rats or in any of the muscle samples.

**Diagram 5**

Expression of the GGl and GmΔC transgenes in liver of fasted and as ad libitum fed rats. Animals were treated as described in the legend to Fig. 1. 90 h after virus administration, animals were either allowed to continue feeding ad libitum (white bars) or were fasted for 20 h (dark bars). Liver samples were taken for measurement of glycogen content. Results are mean ± S.E. for the following number of animals: fed GGl, n = 8; fasted GGl, n = 5; fed GmΔC, n = 10; fasted GmΔC, n = 11. The symbols ** and *** denote differences between the indicated groups at levels of significance of $p < 0.01$ and $p < 0.0001$, respectively.
Reversal of Glucose Intolerance by Targeting Subunit of PP-1

Table II

Plasma variables in fed and fasted AdCMV-βGAL-, AdCMV-GL-, and AdCMV-GMΔC-treated rats

Animals were treated as described in the legend to Table I. 90 h after virus administration, animals were either allowed to continue feeding ad libitum on the high fat diet or were fasted for 20 h. Blood was collected for analysis of the indicated plasma variables. Results represent means ± S.E. for the indicated number of animals in each group. The symbol * indicates variables that are statistically different from the corresponding AdCMV-βGAL-treated group with p < 0.05. FFAs, free fatty acids.

|                      | AdCMV-βGAL | AdCMV-GL | AdCMV-GMΔC |
|----------------------|------------|----------|------------|
|                      | Fed (n = 8) | Fast (n = 9) | Fed (n = 5) | Fast (n = 11) |
| Glucose (mg/dl)      | 141 ± 3    | 112 ± 3  | 130 ± 5    | 114 ± 5      |
| Triglycerides (mg/dl)| 136.1 ± 28.9 | 56.0 ± 5.4 | 67.5 ± 7.9* | 68.1 ± 17.1 |
| Ketones (mg/dl)      | 0.33 ± 0.01 | 0.34 ± 0.01 | 0.29 ± 0.01 | 0.46 ± 0.10 |
| Ketones (μmol/l)     | 3.94 ± 0.83 | 7.75 ± 1.25 | 8.43 ± 1.76 | 7.24 ± 1.05 |
| Lactate (mg/dl)      | 11.3 ± 1.3 | 10.8 ± 1.2 | 11.8 ± 1.3 | 7.6 ± 1.11* |
| Insulin (ng/ml)      | 4.91 ± 0.4 | 2.95 ± 0.5 | 4.18 ± 0.4 | 4.22 ± 0.9 |

glucose disposal by diverting it into an inert storage polymer, glycogen, and away from the glycolytic pathway. Some support for the concept was gained in studies involving overexpression of PTG in liver of normal rats fed on standard chow, which resulted in a modest improvement in oral glucose tolerance and no discernable perturbation of lipid homeostasis (17). However, these animals had significant increases in hepatic glycogen stores in the fed state and, of greater concern, failed to lower glycogen levels in response to fasting, thus resembling patients with glycogen storage diseases.

More recently we have learned that the various glycogen-targeting subunit isoforms affect regulation of glycogen and glucose metabolism in different ways when overexpressed in isolated hepatocytes. One set of studies revealed that while overexpression of the muscle-specific isoform GM/RGl had the weakest effect on glycogen synthesis, it also allowed cells to retain appropriate regulation of glycogenolysis by forskolin, a property not equally shared by cells with overexpressed PTG or Gt (15). These findings led us to investigate the possibility that the glycogenic impact of GM/RGl could be improved by deletion of its unique C-terminal tail that includes a putative sarcoplasmic reticulum association domain. To this end, we prepared a truncated form of GM/RGl (GMΔC) and demonstrated that its overexpression in hepatocytes had a more potent glycogenic effect than native GM/RGl but with retention of glycogenolytic responsiveness to forskolin, a fall in media glucose, or the combination of both glycogenolytic signals (16).

These in vitro findings led us to compare, in the current study, the metabolic effects of hepatic overexpression of GMΔC, native Gt/RGl, and the most glycogenic of all the targeting subunits, Gt (15). These studies were performed in Wistar rats fed on a high fat diet for a period of 7 weeks, a regimen that causes a syndrome resembling early stage type 2 diabetes, including glucose intolerance, mild fasting hyperglycemia, insulin resistance, hyperinsulinemia, increased circulating and tissue lipids, and hyperleptinemia (23). This study reveals that at similar levels of overexpression in liver, GMΔC but not Gt lowers blood glucose levels toward normal during OGTT in insulin-resistant, glucose-intolerant, IFF rats. Native Gt/RGl, which consistent with our previous findings could not be overexpressed as efficiently as the other targeting isoforms (15, 16), also did not improve glucose tolerance.

The explanation for the difference in effect of GMΔC and Gt appears to be that animals with overexpressed GMΔC experience a larger increment in hepatic glycogen storage during OGTT than animals with overexpressed Gt, probably related to the much higher fasting liver glycogen levels in the latter group. Thus, at the time that the OGTT begins in fasted Gt-overexpressing animals, liver glycogen levels are already higher than in the fed state in AdCMV-βGAL controls, probably limiting the further capacity for glycogen storage. In contrast, fasted GMΔC-overexpressing animals have levels of liver glycogen that are only slightly higher than fasted AdCMV-βGAL controls and are also able to store much more during the subsequent OGTT than the controls due to the glycogenic effect of the overexpressed targeting subunit. Interestingly, liver glycogen content in fed GMΔC-overexpressing rats was slightly but not significantly higher than that in AdCMV-βGAL-treated controls. This suggests that glycogen metabolism is regulated in a near-normal fashion during typical physiologic cycles (e.g. overnight fasting and feeding) but that GMΔC contributes to enhanced efficiency of glucose disposal when the system is challenged, such as during the OGTT experiment. Consistent with this notion, plasma variables such as glucose, insulin, free fatty acids, and TG were normal in ad libitum fed and 20-h fasted GMΔC-overexpressing rats. Thus, the new GMΔC molecule appears to combine just the right level of glycogenic potency with retention of sensitivity to diverse glycogenolytic signals, allowing it to minimally perturb fuel homeostasis under normal conditions but to assist in disposing of a glucose load in otherwise glucose-intolerant animals.

What then is the real therapeutic potential of the approach outlined here? One important concern is that while hepatic overexpression of GMΔC appears to ameliorate glucose intolerance induced by high fat feeding, it does not reduce the high fasting insulin levels in these animals (Table II). We have previously shown that the elevated insulin levels in rats fed on the high fat diet is linked to insulin resistance and that insulin levels can be normalized in these animals by infusion of a recombinant adenovirus containing the leptin cDNA (23). One mechanism by which GMΔC might have reversed insulin resistance is via activation of fatty acid oxidation in liver to compensate for the diversion of glucose away from glycolysis and oxidative pathways and into the glycogen storage pathway. If liver becomes more dependent on fat for energy production as a result of GMΔC overexpression, this could potentially enhance mobilization of lipids from peripheral tissues such as muscle and fat. Given the correlation between intramyocellular lipid stores and insulin resistance (31, 32), this could ultimately lead to an increase in insulin sensitivity. Perhaps the duration of transgene expression in the current study (5 days) was simply too short to reveal such an effect, or alternatively, an improvement in insulin sensitivity occurred that was not linked to a fall in circulating insulin levels. Further work will be required to test these possibilities. Until such work is carried out, our method should be treated simply as a means of improving glucose tolerance.

The contrasting effects of Gt and GMΔC overexpression on circulating TG levels also deserve mention. GMΔC-overexpressing animals experienced a mild elevation in TG following OGTT but had normal TG levels in the ad libitum fed or fasted states, while Gt-overexpressing rats had decreased TG levels in the fed state. Interestingly, Gt but not GMΔC overexpression

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
caused fat to accumulate in liver. This may be related to the tendency to saturate hepatic glycogen stores in \( \text{G}_\text{C} \)-overexpressing animals but not \( \text{G}_\text{M} \)\text{C}-overexpressing animals, which in turn may have modulated hepatic lipid metabolism and/or mobilization of lipids from peripheral tissues. These issues will require further investigation.

It is also unclear how \( \text{G}_\text{M} \)\text{C} or a related activity might be introduced into liver of patients with diabetes. Current viral and nonviral methods for hepatic gene delivery are not sufficiently robust or safe for human therapy. Until gene delivery methods are improved, a better approach may be to develop drugs that interact with endogenous targeting subunit isoforms. This will require a better understanding of the structure/function relationships that govern isoform-specific function (progress in this area has been recently reviewed in Ref. 9). Such insights may ultimately allow the differences in glycojenic potency on the one hand and the differential responses to glycolytic signals on the other to be understood in terms of protein domains that can be specifically targeted with small molecules. It is interesting to note that the group of Treadway and associates (33) has reported on the use of a small molecule inhibitor of liver glycogen phosphorylase in lowering of blood glucose levels in diabetic rodents. Surprisingly, this agent did not cause hypoglycemia even in normal fasted animals. With our approach it appears even less likely that hypoglycemia would occur given that we are stimulating hepatic glucose disposal rather than inhibiting glucose production while leaving regulation of glycogen phosphorylase largely intact. Further testing of both approaches under more stressful conditions will be required.

Acknowledgments—We are grateful to Kimberly Jones-Ross and Paul Anderson for expert technical assistance and Dr. Per Bo Jensen for assistance with multiplex RT-PCR.

REFERENCES

1. Magnuson, I., Rothman, D. L., Katz, L. D., Shulman, R. G., and Shulman, G. I. (1992) J. Clin. Invest. 90, 1323–1327
2. Cline, G. W., Rothman, D. L., Magnusson, I., Katz, L. D., and Shulman, G. I. (1994) J. Clin. Invest. 94, 2569–2576
3. Velho, G., Petersen, K. F., Perseghin, G., Hwang, J. H., Rothman, D. L., Puesa, M. E., Cline, G. W., Frueh, P., and Shulman, G. I. (1996) J. Clin. Invest. 98, 1755–1761
4. O’Doherty, R. M., Lehman, D., Telemaque-Potts, S., and Newgard, C. B. (1999)

\(^2\) C. Clark, J. Donkor, and R. Fehn, unpublished observations.

5. Hariharan, N., Farrell, D., Hagan, D., Hillyer, D., Arbeeny, C., Sabrah, T., Treuel, A., Brown, K., Kalinowski, S., and Moorkhitar, K. (1997) Diabetes 46, 1–16
6. Niswender, K. D., Shiota, M., Postic, C., Cherrington, A. D., and Magnuson, M. A. (1997) J. Biol. Chem. 272, 22570–22575
7. O’Doherty, R. M., Lehman, D. L., Seoane, J., Gomez-Puix, A. M., Guinovart, J. J., and Newgard, C. B. (1999) J. Biol. Chem. 274, 20524–20530
8. Valera, A., and Bosch, F. (1994) Eur. J. Biochem. 222, 533–539
9. Newgard, C. B., Brady, M. J., O’Doherty, R. M., and Saltiel, A. R. (2000) Diabetes 49, 1867–1974
10. Tang, M. P., Bondor, J. A., Swiderek, K. M., and DePaoli-Roach, A. A. (1991) J. Biol. Chem. 266, 15782–15789
11. Doherty, R. M., Moehs, H. G., Morrise, N., Cohen, P., and Cohen, P. T. (1995) FEBS Lett. 375, 294–298
12. Printen, J. A., Brady, M. J., and Saltiel, A. R. (1997) Science 275, 1475–1478
13. Doherty, M. J., Young, P. R., and Cohen, P. T. (1996) FEBS Lett. 399, 339–343
14. Armstrong, G. W., Browne, J. G., Cohen, P., and Cohen, P. T. (1997) FEBS Lett. 418, 210–214
15. Gasa, R., Jensen, P. B., Berman, H. K., Brady, M. J., DePaoli-Roach, A. A., and Newgard, C. B. (2000) J. Biol. Chem. 275, 26396–26403
16. Yang, R., Cao, L., Gasa, R., Brady, M. J., Sherry, A. D., and Newgard, C. B. (2002) J. Biol. Chem. 277, 1514–1523
17. O’Doherty, R. M., Jensen, P. B., Anderson, P., Jones, J. G., Berman, H. K., Kearney, D., and Newgard, C. B. (2000) J. Clin. Invest. 105, 479–488
18. Dent, P. L., Smith, S. B., and Cohen, P. (1999) FEBS Lett. 456, 302–308
19. Wu, J., Kleiner, U., and Brautigan, D. L. (1996) Biochemistry 35, 5225–5230
20. Hubbard, M. J., Dent, P., Smythe, C., and Cohen, P. (1996) FEBS Lett. 388, 245–249
21. Herz, J., and Gerard, R. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2812–2816
22. Becker, T., Noel, R., Coats, W. S., Gomez-Puix, A., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) Methods Cell Biol. 43, 161–189
23. Buettner, R., Newgard, C. B., Rhodes, C. J., and O’Doherty, R. M. (2000) Am. J. Physiol. 278, E563–E569
24. Newgard, C. B., Hirsch, L., Foster, D. W., and McGarry, J. D. (1983) J. Biol. Chem. 258, 8046–8052
25. Ferre, T., Pujol, A., Efren, R., Bosch, F., and Valera, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 11, 9151–9154
26. Wu, C., Okar, D. A., Newgard, C. B., and Lange, A. J. (2001) J. Clin. Invest. 107, 91–98
27. Valera, A., Pujol, A., Pelegrin, M., and Bosch, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6822–6826
28. Trinh, K., O’Doherty, R., Anderson, P., Lange, A. J., and Newgard, C. B. (1999) J. Biol. Chem. 274, 31615–31620
29. Yoon, J. C., Puigserver, P., Chen, G., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C. R., Granner, D., Newgard, C. B., and Spiegelman, B. M. (2001) Nature 413, 131–138
30. She, P., Shiota, H., Shelton, K. D., Chaklyk, R., Postic, C., and Magnuson, M. A. (2000) Mol. Cell. Biol. 20, 6508–6517
31. Stein, D. T., Dobbins, R., Szczepaniak, L., Malloy, C., and McGarry, J. D. (1997) Diabetes 46, Suppl. 1, A33 (abstr.)
32. Krssak, M., Petersen, K. F., Dresser, A., DiPietro, L., Vogel, S. M., Rothman, D. L., Shulman, G. J., and Roden, M. (1999) Diabetes 48, 113–116
33. Martin, W. H., Hooper, D. J., Arments, S. J., Stock, I. A., Mepherson, R. K., Danley, D. E., Stevenson, R. W., Barrett, E. J., and Treadway, J. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1776–1781
Reversal of Diet-induced Glucose Intolerance by Hepatic Expression of a Variant Glycogen-targeting Subunit of Protein Phosphatase-1
Rosa Gasa, Catherine Clark, Ruojing Yang, Anna A. DePaoli-Roach and Christopher B. Newgard

J. Biol. Chem. 2002, 277:1524-1530.
doi: 10.1074/jbc.M107744200 originally published online November 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107744200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 16 of which can be accessed free at
http://www.jbc.org/content/277/2/1524.full.html#ref-list-1