Reversal of Diet-Induced Glucose Intolerance by Hepatic Expression of a Variant Glycogen Targeting Subunit of Protein Phosphatase-1

Rosa Gasa¹, Catherine Clark¹, Ruojing Yang¹, Anna A. DePaoli-Roach², and Christopher B. Newgard¹³

¹Departments of Biochemistry and Internal Medicine & Touchstone Center for Diabetes Research, University of Texas Southwestern Medical Center Dallas, TX 75390

²Department of Biochemistry University of Indiana School of Medicine Indianapolis, IN 46202

³Address Correspondence to:
Christopher B. Newgard, Ph.D.
Touchstone Center for Diabetes Research, Room Y8.212 University of Texas Southwestern Medical Center 5323 Harry Hines Blvd.
Dallas, TX 75390; FAX: (214) 648-9191; e-mail: newgard@utsw.swmed.edu
Summary

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 GL, the muscle isoform G_m/R_gl, or a truncated version of G_m/R_gl termed G_mΔC in liver ameliorates glucose intolerance in rats fed on a high-fat diet (HF). HF animals overexpressing G_mΔC, but not GL or G_m/R_gl, 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 GL and G_mΔC overexpressing HF rats compared to control HF animals following OGTT. In a second set of studies on fed and 20 h fasted HF animals, G_mΔC overexpressing rats lowered their liver glycogen levels by 57% (from 402 ± 54 to 173 ± 27 µg glycogen/mg protein) in the fasted versus fed states, compared to only 44% in GL overexpressing animals (from 740 ± 35 to 413 ± 141 µg glycogen/mg protein). Since the OGTT studies were performed on 20 h fasted rats, this meant that G_mΔC overexpressing rats synthesized much more glycogen than GL overexpressing HF rats during the OGTT (419 versus 117 µg glycogen/mg protein, respectively), helping to explain why G_mΔC preferentially enhanced glucose clearance. We conclude that G_mΔC 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 (1-3). 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 (GK). 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 GK 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) in spatial organization and regulation of glycogen metabolism (9). Prominent members of this gene family include G_M or R Git (hereafter referred to as G_M/R Git), expressed primarily in striated skeletal muscle (10), G_L, expressed primarily in liver (11), and protein targeting to glycogen (PTG) (12, 13) and PPPR6 (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_{l} > PTG >
G_{M/R_{Gl}} (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_{M/R_{Gl}} > G_{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_{M/R_{Gl}} (16). Native G_{M/R_{Gl}} is distinct from other members of
its gene family in that it contains two consensus sequences for protein kinase A (PKA)-
mediated serine phosphorylation. One of these sites resides within the PP-1 binding site of
G_{M/R_{Gl}}, and its phosphorylation leads to dissociation of the phosphatase, contributing to
inactivation of glycogen synthesis (10, 18, 19). G_{M/R_{Gl}} 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 $G_M/R_G$ yields a 275 amino acid molecule that we have termed $G_M\Delta C$ that can be directly aligned with the similarly sized native $G_L$ and PTG proteins. Overexpression of $G_M\Delta C$ and native $G_M/R_G$ in hepatocytes reveals that the former protein is more effective at stimulating glycogen synthesis (16). Moreover, unlike PTG or $G_L$ overexpressing cells, cells with $G_M\Delta C$ 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 $G_M\Delta C$, $G_L$, and $G_M/R_G$ 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 $G_M\Delta C$ 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, Massachusetts, USA and Harlan Tekland Laboratory, Winfield, Iowa, USA) weighing 175-200 g were housed on a 12 hour light-dark cycle and were allowed free access to water and either standard laboratory chow (65% carbohydrate, 4% fat, 24% protein, Harlan Tekland Laboratory diet 7001) or high fat diet (19% lard, 1% corn oil, Harlan Tekland Laboratory diet 96001) unless otherwise specified. The rats were housed under these conditions for 7 weeks before adenovirus administration. Rats were treated with cyclosporin (15 mg/kg; Calbiochem, San Diego, California, USA) for three consecutive days starting on the day prior to adenovirus administration and Depo-Medrol (1.5 mg/kg; Pharmacia & Upjohn, Kalamazoo, MI, USA) on the day of adenoviral treatment. The preparation and testing of recombinant adenoviruses containing the cDNAs encoding Gl (AdCMV-Gl), Gm/Rgi (AdCMV-Gm/Rgi), a truncated form of Gm/Rgi with its 735 C-terminal amino acids deleted (AdCMV-GmΔC), or β-galactosidase (AdCMV-βGAL) has been described previously (15-17, 21). Aliquots of these viruses were amplified and purified for the current study as described (22). Between 0.5-1.5 x 10^{12} recombinant adenovirus particles were administered via tail vein injection to rats anesthetized with Nembutal (50 µg/g body weight intraperitoneally; Abbott Laboratories, North Chicago, Illinois, USA) or a 50:5:1 mixture of ketamine (Avoco, Fort Dodge, Iowa, USA), Rompun (Avoco), and acepromazine (Haver, Shawnee, Kansas, USA), as described elsewhere (4, 23). After viral administration, animals
were individually caged to allow monitoring of food intake and body weight before initiation of experiments.

**Animal studies.** Two experimental protocols were performed. In the first, animals were infused with AdCMV-G$_L$, AdCMV-G$_M$/R$_G$, AdCMV-G$_M$ΔC, or AdCMV-βGAL viruses. Ninety hours after virus administration, animals were fasted for 20 hours with free access to water. An oral glucose tolerance test (OGTT) was performed by anesthetizing animals with Nembutal (50 µg/g body weight intraperitoneally) and administration of a bolus of 2 g glucose/kg body weight by gavage of a 45% solution of glucose in water. Blood samples (approximately 20 µl/sample from the tail vein) were collected immediately before administration of the bolus and at 30, 60, 90, 120, 150, and 180 minutes after the bolus for measurement of circulating glucose concentrations. Animals were sacrificed immediately after the 180 minute time point for collection of blood and liver. The liver samples were rapidly frozen in liquid nitrogen and stored at -70°C until further analysis. In the second protocol, animals were infused with AdCMV-G$_L$, AdCMV-G$_M$ΔC, or AdCMV-βGAL viruses. Ninety hours after virus administration, animals were either fasted for 20 hours or allowed to continue feeding ad libitum. Thereafter, all animals were anesthetized with Nembutal (50 µg/g 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 used was based on methods described previously (15, 17). Total RNA was extracted from powdered liver or muscle.
tissue using RNeasy spin columns (QIAGEN Inc., Valencia, California, USA), following the manufacturer's instructions. First-strand cDNA was prepared using 0.5 µg total RNA, the Superscript RT kit, and random hexamer primers (GIBCO BRL, Gaithersburg, Maryland, USA), according to the manufacturer's instructions. 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) and buffer (Promega Corp., Madison, Wisconsin, USA), dNTP mix (final concentrations of 40 mM of each dNTP, except dCTP, which was present at 20 mM [GIBCO BRL]), and with or without 1.25 µCi of [α-\(^{33}\)P]-dCTP (2,000 Ci/mmol; Du Pont NEN Research Products, Boston, Massachusetts, USA) 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 GL transgene and did not amplify endogenous rat GL because the upstream primer hybridizes to 5' untranslated sequence derived from the adenovirus vector (5' primer CGAGCTCGGTACCAACTTC, and 3' primer GAAGGTGAAGCGCTCTCTG). The second set amplified a 162 bp product from either the full-length endogenous GM/RL or GM\(_{\Delta C}\) transgenes, as previously described (15). The third oligonucleotide pair specifically amplified a 900 bp fragment of GM\(_{\Delta C}\) derived by expression from the AdCMV-GM\(_{\Delta C}\) adenovirus, because the upstream primer hybridizes to sequence within the GM\(_{\Delta C}\) cDNA sequence, while the 3' primer hybridizes to the 3' untranslated region derived from the adenovirus vector (5'- primer, CTCAAGGAAGATCTTATGCAAC, and 3' primer GGTAGTTTGTCCAATTATGTCAC). The last oligonucleotide pair amplified one of the following as internal standards: a 186 bp fragment of the endogenous TATA binding protein (TBP) 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 GCGTGAGTGTATCTCCATCCA, and 3’ primer GGTAGGTGCCAGTGCGAACTT).

In experiments involving inclusion of [α-33P]-dCTP, PCR conditions were an initial incubation at 95 °C for 5 minutes followed by 22 or 24 cycles (the latter only when studying full length G\(_m\)/R\(_{Gl}\) transgene expression) cycles of 95 °C for 45 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. The final PCR reactions were mixed with 98% formamide denaturing loading buffer and separated on a 6% (wt/vol) polyacrylamide gel, containing 7 M urea. The gel was subsequently dried and exposed to a phosphoimager screen, and the resulting scan was analyzed using ImageQuant from Molecular Dynamics (Sunnyvale, California, USA). In the experiments designed to assess expression of the G\(_m\)ΔC transgene in extrahepatic tissues, PCR reactions were carried out in the absence of [α-33P]-dCTP. For these experiments, PCR conditions were an initial incubation at 95 °C for 1 minute followed by 30 cycles of 95 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes. 5 µl of the PCR reaction was resolved on an 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 RIA (Linco Research, St. Charles, Missouri, USA). Plasma triglyceride, ketone, and lactate levels were measured using kits from Sigma Chemical CO. (St. Louis, Missouri, USA). Plasma free fatty acids were measured using a kit from Roche Molecular Biochemicals (Indianapolis, IN, USA). 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 ± SEM. Statistical significance was determined by unpaired Student's t test using the statistics module of Microsoft Excel (version 5.0; Microsoft Corp., Redmond, Washington, USA). 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 Figure 1A. Animals treated with AdCMV-βGAL exhibited either no signal or background at the positions expected for the reverse transcribed and amplified fragments of GL, G_{M\Delta C}, or G_{M/R}\_Gl. Rats infused with AdCMV-GL, AdCMV-G_{M/R}\_Gl, or AdCMV-G_{M\Delta C} showed clear expression of the respective transgene mRNAs. When normalized to the internal control, TBP, GL, and G_{M\Delta C} mRNA levels were found to be indistinguishable and approximately 3-fold greater than the levels of G_{M/R}\_Gl transgene RNA (Figure 1B). The lower apparent efficiency of G_{M/R}\_Gl overexpression relative to the other two targeting subunits is consistent with our previous findings in isolated hepatocytes (15, 16). No attempt could be made to correct for the clearly lower level of G_{M/R}\_Gl expression, since infusion of higher viral titers began to have toxic effects, as assessed by an increase in the activity of a liver enzyme, aspartyl aminotransferase (PGOT) in the blood of these animals (data not shown). It should also be pointed out that our main goal was to compare the highly glycogenic targeting subunit GL with our novel construct G_{M\Delta C}.

OGTT in Wistar rats with adenovirus-mediated G_{L}, G_{M\Delta C}, or G_{M/R}\_Gl overexpression in liver. To test the capacity of the various glycogen targeting subunits to improve glucose homeostasis, we performed oral glucose tolerance tests (OGTT) in the HF animals evaluated for targeting subunit expression in Figure 1, as well as a group of rats that were fed on normal...
chow and infused with AdCMV-βGAL. As shown in Figure 2, AdCMV-βGAL-treated animals fed on normal chow had normal glucose tolerance, with a rapid decline of blood glucose from a maximum of 170 mg/dl at 30 minutes after the glucose load, and a return to basal levels by 150 minutes. In sharp contrast, AdCMV-βGAL-treated HF rats were clearly glucose intolerant, with a higher excursion of blood glucose to a peak value of 210 mg/dl at 60 minutes after the glucose bolus, and a slow decline thereafter that failed to approach baseline values by 180 minutes. Surprisingly, HF rats treated with AdCMV-G_M/R_Gl or AdCMV-G_L exhibited no significant improvement in glucose tolerance during OGTT. In contrast, HF animals treated with AdCMV-G_MΔC had glucose levels indistinguishable from those of AdCMV-βGAL-treated rats fed on normal chow, except at 150 minutes, where glucose was slightly elevated compared to the standard chow controls, but still lower than in the other three treatment groups.

**Effects of G_L, G_MΔC, or G_M/R_Gl overexpression on liver glycogen following OGTT.** To determine if the differential effects of the various glycogen targeting subunits on glucose levels in the OGTT were related to glycogen deposition, liver glycogen levels were measured in animals at the conclusion of the experiment (180 minute time point) summarized in Figure 2. Figure 3 shows that high fat feeding per se did not increase liver glycogen stores relative to feeding with normal chow (both of these control groups were treated with AdCMV-βGAL). Treatment of HF rats with AdCMV-G_M/R_Gl did not enhance glycogen accumulation compared to either control group. However, treatment of HF animals with AdCMV-G_L or AdCMV-G_MΔC resulted in 108% and 138% increases in liver glycogen, respectively, relative to the AdCMV-βGAL-treated HF controls. Thus, both G_L and G_MΔC overexpressing animals had
higher liver glycogen levels following OGTT, but only the AdCMV-GmΔC treated rats had improved glucose tolerance.

**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 Figure 2 (180 minute time point), allowing several plasma variables to be measured. As summarized in Table 1, in HF animals, overexpression of the various glycogen targeting subunit isoforms had no effect on circulating free fatty acids, ketones, lactate, or insulin relative to AdCMV-βGAL-treated controls. Treatment of animals with AdCMV-Gl 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-GmΔC-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. In order 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 employed 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 (Figure 4). RT-PCR analysis was also performed on RNA isolated from liver and muscle samples taken from 3 AdCMV-βGAL or 3 AdCMV-GmΔC-treated rats subjected to OGTT. As shown in Figure 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 Figure 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 GmΔC and G L overexpression on glucose tolerance (Figure 2), we next studied liver glycogen levels in fed and fasted HF animals treated with AdCMV-GmΔC or AdCMV-G L. Multiplex RT-PCR analysis of transgene expression levels in these animals is summarized in Figure 5. In both the AdCMV-G L 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 GmΔC to G L mRNA levels in fed versus fed or fasted versus fasted groups also revealed no significant differences.

As shown in Figure 6, AdCMV-βGAL-treated HF rats contained 317 ± 46 µg glycogen/mg protein in the fed state, and depleted this reserve by 68%, to 103 ± 15 µg.
glycogen/ mg protein, in response to a 20 h fast. Interestingly fed AdCMV-G<sub>L</sub>-treated rats accumulated 740 ± 35 µg glycogen/mg 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 glycogen/ mg protein. In sharp contrast, fed AdCMV-G<sub>MΔC</sub>-treated rats contained 402 ± 54 µg glycogen/ mg protein in liver, and reduced their glycogen stores by 57% in response to the 20 hour fast to 173 ± 27 µg glycogen/ mg protein, a value slightly higher than in fasted AdCMV-βGAL-treated controls. Importantly, the liver glycogen level in fasted AdCMV-G<sub>L</sub>-treated rats was 80% of that in AdCMV-G<sub>L</sub>-treated rats following OGTT. In contrast, liver glycogen content in fasted AdCMV-G<sub>MΔC</sub>-treated rats was only 29% of that in AdCMV-G<sub>MΔC</sub>-treated rats following OGTT. In other words, AdCMV-G<sub>MΔC</sub>-treated rats synthesized 419 µg glycogen/mg protein during the OGTT, compared to an increment of only 117 µg glycogen/mg protein in AdCMV-G<sub>L</sub>-treated animals (values obtained by subtracting the glycogen levels in fasted rats shown in Figure 6 from the glycogen levels after OGTT shown in Figure 3; note that animals were fasted for 20 h prior to OGTT). This suggests that the differential potency of G<sub>MΔC</sub> and G<sub>L</sub> for lowering of blood glucose in glucose intolerant HF rats may have been due in part to the high basal glycogen levels in G<sub>L</sub> 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 1) was measured in fed and 20 h fasted HF rats treated with the various recombinant adenoviruses (Table 2). Animals treated with AdCMV-βGAL, AdCMV-G<sub>L</sub>, or AdCMV-G<sub>MΔC</sub> 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 TG were more than twice as high in fed compared to fasted AdCMV-βGAL or AdCMV-GmΔC-treated rats. However, in AdCMV-GL treated animals, TG remained low in the fed state and was indistinguishable from fasted values. Lactate levels in fasted AdCMV-GL-infused rats were also lower than in AdCMVGmΔC or AdCMV-βGAL-treated animals (p < 0.05).
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 knock-out 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 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 $G_M/R_GI$ 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 $G_L$ (15). These findings led us to investigate the possibility that the glycogenic impact of $G_M/R_GI$ 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 $G_M/R_GI$ ($G_M\Delta C$) and demonstrated that its overexpression in hepatocytes had a more potent glycogenic effect than native $G_M/R_GI$, 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 G\textsubscript{m}\textDelta C, native G\textsubscript{m}/R\textsubscript{Gl}, and the most glycogenic of all the targeting subunits, G\textsubscript{l}. These studies were performed in Wistar rats fed on a high fat diet for a period of 7 weeks, a regimen which 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, G\textsubscript{m}\textDelta C but not G\textsubscript{l} lowers blood glucose levels towards normal during OGTT in insulin-resistant, glucose intolerant, HF rats. Native G\textsubscript{m}/R\textsubscript{Gl}, 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 G\textsubscript{m}\textDelta C and G\textsubscript{l} appears to be that animals with overexpressed G\textsubscript{m}\textDelta C experience a larger increment in hepatic glycogen storage during OGTT than animals with overexpressed G\textsubscript{l}, probably related to the much higher fasting liver glycogen levels in the latter group. Thus, at the time that the OGTT begins in fasted G\textsubscript{l} overexpressing animals, liver glycogen levels are already higher than in the fed state in AdCMV-\betaGAL controls, probably limiting the further capacity for glycogen storage. In contrast, fasted G\textsubscript{m}\textDelta C overexpressing animals have levels of liver glycogen that are only slightly higher than fasted AdCMV-\betaGAL 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 G\textsubscript{m}\textDelta C overexpressing rats was slightly, but not significantly higher than in AdCMV-\betaGAL-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 \( G_m \Delta C \) 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, FFA, and TG were normal in ad-lib fed and 20 h fasted \( G_m \Delta C \) overexpressing rats. Thus, the new \( G_m \Delta C \) 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 \( G_m \Delta C \) appears to ameliorate glucose intolerance induced by high-fat feeding, it does not reduce the high fasting insulin levels in these animals (Table 2). 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 \( G_m \Delta C \) 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 \( G_m \Delta C \) 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 $G_l$ and $G_m\Delta C$ overexpression on circulating TG levels also deserve mention. $G_m\Delta C$ overexpressing animals experienced a mild elevation in TG following OGTT, but had normal TG levels in the ad-lib fed or fasted states, while $G_l$ overexpressing rats had decreased TG levels in the fed state. Interestingly, $G_l$ but not $G_m\Delta C$ overexpression caused fat to accumulate in liver (C. Clark, J. Donkor, R. Fehn, unpublished observations). This may be related to the tendency to saturate hepatic glycogen stores in $G_l$ overexpressing animals but not $G_m\Delta C$ 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 $G_m\Delta C$ or a related activity might be introduced into liver of patients with diabetes. Current viral and non-viral 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 glycogenic potency on the one hand, and the differential responses to glycogenolytic 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 has reported on the use of a small molecule inhibitor of liver glycogen phosphorylase in lowering of blood glucose levels in diabetic rodents (33).

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.
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References


Figure Legends

Figure 1. Expression of the G_L, G_M/R_Gl, and G_MΔC transgenes in liver after OGTT. Male Wistar rats were fed a high fat (HF) diet for 7 weeks. At the end of this period, animals were treated with the AdCMV-βGAL, AdCMV-G_L, AdCMV-G_M/R_Gl or AdCMV- G_MΔC adenoviruses and allowed to feed ad libitum for 90 hours after viral administration. Animals were then fasted for 20 hours before receiving an oral glucose bolus of 2g/kg body weight. Animals were sacrificed 180 min after the oral glucose challenge for collection of liver samples. A portion of these samples was used to prepare total RNA and to examine transgene expression by multiplex PCR as described in Materials and Methods. (A) Representative gel displaying RT-PCR results for 2-4 animals in each treatment group. TATA-binding protein (TBP) was used as an internal control. Note that endogenous G_L was not amplified as primers specific for the G_L transgene were used (B) Quantitative analysis of the ratio of each transgene:TBP for all animals included in the OGTT protocol. Data represent mean ± SEM for a total of 6 G_L, 7 G_M/R_Gl and 7 G_MΔC overexpressing animals. Symbols * and *** indicate significant differences between the G_MΔC and G_L overexpressing groups relative to the G_M/R_Gl overexpressing group, with levels of significance of p<0.05 and p<0.0001, respectively. Expression levels in the G_L and G_MΔC overexpressing groups were not significantly different.

Figure 2. Oral glucose tolerance test (OGTT). Animals were treated as described in the legend for Figure 1. Tail vein blood samples were taken and glucose levels were measured at the indicated times after the glucose bolus. Data are mean ± SEM for 12 βGAL, 6 G_L, 7 G_M/R_Gl, and 7 G_MΔC overexpressing animals. The symbol * indicates those time points at which blood glucose levels were significantly lower in HF AdCMV-G_MΔC rats versus HF.
AdCMV-βGAL treated controls, with $p < 0.05$. A second control group of animals fed a standard chow (SC) diet for 7 weeks and infused with AdCMV-βGAL (std. chow, $n=8$) was also included in this protocol. Note that G$_{M\Delta C}$ overexpressing animals had glucose levels indistinguishable from those of standard chow-fed control animals with the exception of one time point (#, $p<0.01$).

**Figure 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 Figure 2. Data represent mean ± SEM for 6 G$_L$, 7 G$_{M/R\alpha L}$, 7 G$_{M\Delta C}$ overexpressing HF rats, and 12 βGAL overexpressing HF (βGAL) and 8 βGAL overexpressing standard chow fed (βGAL/std. chow) rats. The symbols * and ** indicate that G$_L$ and G$_{M\Delta C}$ overexpressing animals stored more glycogen than HF βGAL controls, with levels of significance of $p < 0.001$ and $p < 0.05$, respectively.

**Figure 4. RT-PCR analysis of G$_{M\Delta C}$ transgene expression in liver and muscle.**

Oligonucleotides specific to the G$_{M\Delta C}$ gene product expressed from the AdCMV-G$_{M\Delta C}$ adenovirus, or as an internal control, 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 Figures 1-3. As an additional control, the same oligonucleotides were used to analyze an RNA sample from cultured 293 cells treated with AdCMV-G$_{M\Delta C}$. Note that a band of 900 bp (labeled G$_{M\Delta C}$ transgene), as predicted only in cells with adenovirus-mediated expression of G$_{M\Delta C}$, is found in liver of AdCMV-G$_{M\Delta C}$ 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.
Figure 5. Expression of the $G_L$ and $G_M^\Delta C$ transgenes in liver of fasted and ad libitum fed-rats. Animals were treated as described in the legend to Figure 1 with either the AdCMV-$G_L$ or AdCMV-$G_M^\Delta C$ adenoviruses. 90 h after virus infusion, animals were either allowed to continue feeding ad libitum on the high fat diet (white bars) or were fasted for 20 h (dark bars). Livers were collected and transgene expression was measured by multiplex RT-PCR. Band intensities were normalized to EF-1$\alpha$ as the internal control. Results represent mean ± SEM for the following number of animals: fed $G_L$, n=8; fasted $G_L$, n=5; fed $G_M^\Delta C$, n=10; fasted $G_M^\Delta C$, n=11. No significant differences were found when comparing expression levels in fed and fasted animals within a virus treatment group, or when comparing fed to fed or fasted to fasted animals between viral treatment groups.

Figure 6. Liver glycogen levels in fasted and ad-libitum fed rats. Animals were treated as described in the legend to Figure 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 ± SEM for the following number of animals: fed $G_L$, n=8; fasted $G_L$, n=5; fed $G_M^\Delta C$, n=10; fasted $G_M^\Delta C$, n=11. The symbols ** and *** denote differences between the indicated groups at levels of significance of $p < 0.01$ and $p < 0.0001$, respectively.
Table 1. Plasma variables in AdCMV-βGAL, AdCMV-GL, AdCMV-GM/RGl and AdCMV-GM∆C-treated rats after the OGTT protocol. Male Wistar rats were fed a high fat (HF) diet for 7 weeks. At the end of this period, animals received the AdCMV-βGAL, AdCMV-GL, AdCMV-GM/RGl or AdCMV-GM∆C adenoviruses and were allowed to feed ad libitum for 90 hours after viral administration. Animals were then fasted for 20 hours before receiving an oral glucose bolus (2g/kg). Blood samples were collected after the 180 min time point of the OGTT for analysis of the indicated plasma variables. Data are mean ± SEM 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.

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<tr>
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<th>AdCMV-βGAL</th>
<th>AdCMV-GL</th>
<th>AdCMV-GM/RGl</th>
<th>AdCMV-GM∆C</th>
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<td>n=6</td>
<td>n=7</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
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<td>89.0±18.1</td>
<td>92.0±19.8</td>
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<td>FFAs (mmol/l)</td>
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<td>Lactate (mg/dl)</td>
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<td>Insulin (ng/ml)</td>
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Table 2. Plasma variables in fed and fasted AdCMV-βGAL, AdCMV-G_L and AdCMV-G_M∆C-treated rats.

Animals were treated as described in the legend to Table 1. 90 h after virus administration, animals were either allowed to continue feeding ad libitum on the high fat diet or were fasted for 20 hours. Blood was collected for analysis of the indicated plasma variables. Results represent means ± SEM 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.

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<td>Glucose (mg/dl)</td>
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Figure 1A

Adenovirus

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<th>GL</th>
<th>GM/RL</th>
<th>GMΔC</th>
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TBP

GL

GM/RL
Transgene expression (ratio transgene:TBP)

GL

GM/RG1

GMΔC

Figure 1B
Figure 2

Glucose (mg/dl)

Time (min)
Figure 3

Glycogen (mg/mg protein)

- βGAL
- GL
- GM/RG
- GMΔC
- βGAL/ std.chow
AdCMV-G$_M$ΔC, 293 cells

Molecular Weight Markers

Muscle

AdCMV-G$_M$ΔC
AdCMV-$\beta$GAL

Liver

AdCMV-G$_M$ΔC
AdCMV-$\beta$GAL

G$_M$ΔC Transgene

$\alpha$-tubulin
Transgene expression (ratio transgene:EF-1α)

GL

GMΔC

Figure 5
Figure 6

The bar chart shows the glycogen (mg/mg protein) levels for three conditions: βGAL, GL, and GMΔC. The chart includes error bars and statistical symbols (***, **, #) indicating significant differences. The y-axis represents glycogen levels ranging from 0 to 1000 mg/mg protein, and the x-axis lists the conditions.
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

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