REGULATION OF HEPATIC GLYCOGEN METABOLISM BY GLYCOGEN TARGETING SUBUNITS OF PROTEIN PHOSPHATASE 1

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DEDICATION

This Dissertation is dedicated to Dr. Richard Fehn. Without your encouragement and friendship this would not have been possible.

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REGULATION OF HEPATIC GLYCOGEN METABOLISM BY GLYCOGEN TARGETING SUBUNITS OF PROTEIN PHOSPHATASE 1

by

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DISSERTATION

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Glycogen targeting subunits of protein phosphatase 1 play a critical role in fuel homeostasis through the regulation of glycogen metabolism. Adenovirus-mediated overexpression of the liver subunit G_L, the muscle subunit G_M, or a truncated version of G_M, $G_M\Delta C$ increased hepatic glycogen content in high fat fed rats, a model of insulin resistance. Rats expressing $G_M\Delta C$ and G_L had similar amounts of hepatic glycogen following an oral glucose tolerance test, but only $G_M\Delta C$ expression improved glucose tolerance. The explanation for this difference is that animals with overexpressed $G_M\Delta C$ experience a larger increment in hepatic

glycogen storage during OGTT than animals with overexpressed G_L, probably related to the much higher fasting liver glycogen levels in the latter group. Since it is possible to improve glucose tolerance via expression of glycogen targeting subunits, the remaining research focused on designing and testing a dominant-negative glycogen targeting subunit, PTG-VF. Overexpression of PTG-VF caused an 83% reduction in glycogen content in hepatocytes indicating that the activity of glycogen targeting subunits is necessary for glycogen accumulation. Further studies found that PTG-VF was more potent in blocking glycogen synthesis in hepatocytes with overexpressed G_L than PTG. PTG-VF expression increases phosphoryalse a levels, which preferentially inhibits G_L through its C-terminal phosphorylase a binding site that is lacking in PTG. Removal of the phosphorylase a binding site from G_L renders the subunit less susceptible to inhibition by PTG-VF. PTG-VF was overexpressed in rats fed on standard chow (SC) or high fat (HF) diet to determine if suppression of glycogen targeting subunit activity could cause glucose intolerance or diabetes. Hepatic glycogen stores were decreased by PTG-VF, but this did not lead to alterations in glucose homeostasis.

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PRIOR PUBLICATIONS

Gasa, R., C. Clark, R. Yang, A. DePoali-Roach, and C. Newgard. (2002). "Reversal of Diet-induced Glucose Intolerance by Hepatic Expression of a Variant Glycogen-targeting Subunit of Protein Phosphatase-1." J. Biol. Chem. **277**(2): 1524-1530.

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Clark, C., M. Brady, R. Gasa, and C. Newgard. "Development and Testing of a Dominant-negative Glycogen Targeting Subunit of Protein Phosphatase 1." Manuscript in preparation.

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Akt – protein kinase B

βGal – beta-galactosidase

cAMP – 3',5'-cyclic adenosine monophosphate

CMV – cytomegalovirus (promoter)

EF-1α – elongation factor-1 α

FFA – free fatty acid

G6P – glucose-6-phosphate

G6Pase – glucose-6-phosphatase

GK – glucokinase

GKRP – glucokinase regulatory protein

GLUT2 – glucose transporter 2

GSK-3 – glycogen synthase kinase-3

GTS – glycogen-targeting subunit of protein phosphatase 1

HF – high fat

IDDM – Insulin dependent diabetes mellitus

mRNA – messenger ribonucleic acid

NIDDM – Non-insulin dependent diabetes mellitus

OGTT – oral glucose tolerance test

PBS – phosphate buffered saline

PCR – polymerase chain reaction

pGOT – plasma aspartate aminotransaminase

pGPT – plasma alanine aminotransaminase

PK – phosphorylase kinase

PKA – cAMP-dependent protein kinase

PMSF – phenylmethylsufonyl fluoride

PP-1 – protein phosphatase 1

PTG – protein targeted to glycogen

SC – standard chow

TG – triglyceride

Tris – tris (hydroxymethyl) animomethane

CHAPTER ONE

INTRODUCTION

Diabetes Mellitus

Diabetes mellitus is a common endocrine disorder that affects approximately 6% of the world population and is characterized by defects in insulin secretion and/or insulin action leading to hyperglycemia (American Diabetes 2005; Adeghate, Schattner et al. 2006). While there are several sub-classes of diabetes, most of the cases can be classified into two broad categories known as type 1 and type 2 diabetes (American Diabetes 2005).

Type 1, or insulin-dependent diabetes mellitus (IDDM), is caused by auto-immune destruction of the insulin-secreting beta cells of the Islets of Langerhans and accounts for 5-10% of the cases of diabetes (American Diabetes 2005). The loss of the beta cells leads to insulin deficiency. Since insulin facilitates the uptake of glucose by peripheral tissues, the insulin deficient state associated with type 1 diabetes results in impaired glucose uptake and ultimately persistent hyperglycemia. Most of the complications associated with type 1 diabetes arise from inadequate control of blood glucose, leading to protein modification by non-enzymatic glycosylation and production of toxic byproducts of glucose metabolism, ultimately leading to damage of tissues and organs.

Type 2, or non-insulin dependent diabetes mellitus (NIDDM), accounts for approximately 90-95% of diabetes cases (American Diabetes 2005). Most individuals with type 2 diabetes

are obese and suffer from insulin resistance and a relative deficiency in insulin. Early on in the disease there is a compensatory increase in beta cell mass and insulin output in response to the insulin resistance (Polonsky, Given et al. 1988; Butler, Janson et al. 2003). However, this early compensatory increase in beta cell mass and insulin production can not be sustained indefinitely, and beta cell mass and function ultimately decline, leading to hyperglycemia. Early stages of type 2 diabetes can often be effectively treated by changes in diet and exercise leading to improved insulin sensitivity and thus a decreased demand on the beta cells. However, in the absence of such lifestyle changes, the only recourse for patients is pharmacologic therapy with drugs that enhance insulin secretion or insulin action.

The liver plays a major role in maintaining metabolic fuel homeostasis. Insulin and glucagon from the pancreas tightly regulate hepatic glucose metabolism. During the fed state when levels of insulin are increased, the liver serves as a major organ of glucose disposal by storing the sugar in the form of glycogen, or by catabolism through glycolysis and the pentose monophosphate shunt, and conversion to lipids via de novo lipogenesis. Conversely, during fasting when insulin levels are low and glucagon levels are high the liver becomes a major organ of glucose production through the breakdown of glycogen (glycogenolysis) and the formation of glucose from gluconeogensis. In both major forms of diabetes mellitus this highly regulated control of hepatic glucose metabolism is disrupted, leading to inappropriately high levels of hepatic glucose production.

Hepatic glycogen metabolism

The liver is able to convert glucose and other precursors to glycogen for storage during the fasted-to-fed transition (shown schematically in Figure 1.1). Conversely, glycogen is degraded to produce free glucose during the fed-to-fasted transition. The metabolism of glycogen is controlled through a complex mix of hormonal and metabolic signals.

Glucagon or β-adrenergic agonists bind to their receptors on liver cells during the catabolic state (e.g., fasting or stress) and this initiates a "glycogenolytic cascade" (Sutherland and Robison 1969). The GαS heterotrimeric GTP-binding protein activates adenylate cyclase leading to the accumulation of cAMP and activation of cAMP-dependent protein kinase (PKA). Phosphorylation and activation of phosphorylase kinase is mediated by PKA. Phosphorylase kinase is then able to phosphorylate and activate glycogen phosphorylase leading to the phosphorolytic cleavage of the α-1,4-glycosidic bonds at the nonreducing termini of the glycogen particle. The ability of glycogen phosphorylase to catalyze phosphorolytic rather than hydrolytic cleavage of the α-1,4-glycosidic bonds produces glucose 1-phosphate rather than free glucose as the product of the reaction. Glucose-1-phosphate is then readily converted to G6P by phosphoglucomutase and used for production of glucose through the glucose-6-phosphatase reaction or for oxidation to produce ATP (Newgard, Hwang et al. 1989).

Synthesis of glycogen predominates during the fasted-to-fed transition. The formation of glycogen in the liver can occur via a direct pathway, in which G6P is produced through the phosphorylation of glucose obtained from the circulation, or an indirect pathway, in which G6P is formed from gluconeogenic precursors (McGarry, Kuwajima et al. 1987). G6P is

then converted to glucose 1-phosphate by phosphoglucomutase and to UDP-glucose by UDP-glucose pyrophosphorylase. The initiation of glycogen synthesis occurs through the self-glucosylation of glycogenin where the glucosyl residues are transferred from UDP-glucose (Tavridou and Agius 2003). Glycogen synthase uses UDP-glucose as its substrate to catalyze the addition of glucose residues to the glycogen particle via the formation of α-1,4-glycosidic bonds. The highly branched structure of mature glycogen is created when a branching enzyme links blocks of glucose residues via α -1,6-glycosidic linkages (McGarry, Kuwajima et al. 1987).

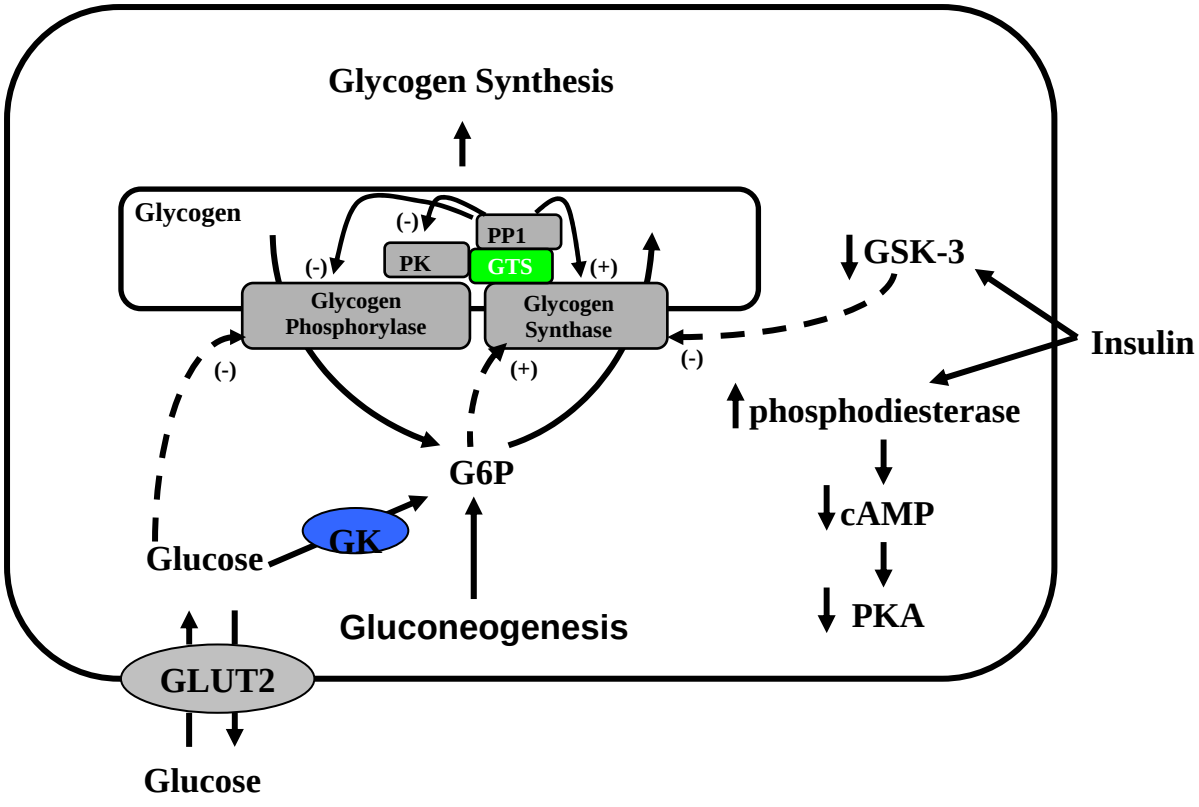


Figure 1.1. Regulation of enzymes of glycogen metabolism in liver cells. The figure shows some of the key regulatory steps controlling hepatic glycogen metabolism. GK, glucokinase; G6P, glucose-6-phosphate; GTS, glycogen-targeting subunits; PK, phosphorylase kinase; PP-1, protein phosphatase 1.

Enzymes of glycogen metabolism

Glucose Transporter

A family of facilitated glucose transporter proteins plays a role in the efficient transport of glucose into and out of mammalian cells (Bell, Kayano et al. 1990; Thorens, Charron et al. 1990). Bidirectional flow of glucose occurs through all of the isoforms of the facilitated glucose transporters depending on the relative concentration of glucose on the outside versus the inside of the cell. The major glucose transporter isoform of the liver is GLUT-2, which has a high Km for glucose (Johnson, Newgard et al. 1990). The high Km of GLUT-2 allows for alterations in its activity in response to changes in blood glucose levels to help coordinate uptake and release of glucose from the liver.

Glucokinase

Glucose gets phosphorylated to form glucose-6-phosphate (G6P) in the liver primarily through the actions of glucokinase (GK), also known as hexokinase IV (Hughes, Quaade et al. 1991; Jetton, Liang et al. 1994; Matschinsky 2002). Glucokinase, like GLUT-2, has a high Km for glucose and is not allosterically inhibited by G6P (Matschinsky 2002). The high

Kms of both GLUT-2 and glucokinase allow the liver to regulate glucose metabolism in response to changes in the blood glucose concentration.

Glucokinase is acutely controlled by the glucokinase regulatory protein (GKRP) (Malaisse, Malaisse-Lagae et al. 1990; Detheux, Vandekerckhove et al. 1994). During fasting GKRP binds to glucokinase in the cytosol thereby inhibiting its acitivity. In addition, GKRP translocates the glucokinase protein into the nucleus and away from the cytosolic pool of glucose further reducing its acitivity (Toyoda, Miwa et al. 1995; Brown, Kalinowski et al. 1997; Shiota, Coffey et al. 1999). In contrast, during the fasted-to-fed transition glucokinase dissociates from GKRP and is translocated into the cytosol, thereby facilitating phosphorylation of glucose to G6P.

Glucokinase expression is regulated at the transcriptional level by insulin. Levels of glucokinase mRNA fall during fasting and increase in response to feeding or insulin injection (Iynedjian, Ucla et al. 1987; Andreone, Printz et al. 1989; Iynedjian, Pilot et al. 1989). Thus, increased phosphorylation of glucose in liver is regulated acutely by translocation of glucokinase from the nucleus to the cytoplasm, and this is complemented by a more gradual increase in the expression of glucokinase in response to insulin.

Glycogen Synthase

The rate limiting step of glycogen synthesis is catalyzed by the enzyme glycogen synthase. The activity of glycogen synthase is regulated through covalent modification, allosteric activation, and enzymatic translocation. The phosphorylation sites and their

regulation *in vitro* have been well defined in rabbit skeletal muscle. At least ten phosphorylation sites are located in muscle glycogen synthase and these are located in the amino and carboxyl termini of the protein (Bai, Zhang et al. 1990; Roach 1990). Site 1a is phosphorylated by PKA. The phosphorylation of site 2 is mediated by phosphorylase kinase. Casein kinase II phosphorylates site 5, which is permissive for the activity of glycogen synthase kinase-3 (GSK-3). GSK-3 is able to sequentially phosphorylate sites 4, 3c, 3b, and 3a (Bai, Zhang et al. 1990). Phosphorylation of glycogen synthase generally leads to inactivation, however the degree of inactivation is dependent upon the phosphorylation site. Phosphorylation of sites 5, 1a, and 1b has little or no impact on glycogen synthase activity. Moderate levels of inactivation occur with the phosphorylation of site 2. The main sites of phosphorylation that lead to inactivation of glycogen synthase are sites 3a, 3b, and 3c (Roach 1990). Liver glycogen synthase is less well characterized then skeletal muscle glycogen synthase. However, it is known that muscle sites 1a and 1b are absent in the liver isoform (Bai, Zhang et al. 1990). As with the muscle isoform, phosphorylation by GSK-3 is potentiated by the prior phosphorylation of glycogen synthase by casein kinase II (Roach 1990).

Activation of glycogen sythase is dependent upon its dephosphorylation. The main enzyme responsible for this dephosphorylation is protein phosphatase 1 (Lawrence and Roach 1997). Insulin plays a role in the activation of glycogen synthase, but the mechanism of action has not been fully defined. One component of the mechanism involves insulin-stimulated phosphorylation of GSK-3 via a branch of the insulin signaling pathway that includes PI-3 kinase and Akt-1 (protein kinase B) (Frame and Cohen 2001). Phosphorylation

of GSK-3 leads to a decrease in activity of this kinase, which in turn leads to decreased phosphorylation of glycogen synthase and an increase in its enzymatic activity.

Glycogen synthase is also allosterically regulated by G6P. Binding of G6P to glycogen synthase promotes the dephosphorylation and thus activation through conformational changes (Bollen, Keppens et al. 1998; Tavridou and Agius 2003). G6P also plays a role in the dephosphorylation of glycogen synthase by protein phosphatase 1 by making glycogen synthase a better substrate for PP-1 (Bollen, Keppens et al. 1998).

Regulation of glycogen synthase activity also occurs through its intracellular localization (Ferrer, Favre et al. 2003). In the absence of glucose in hepatocytes glycogen synthase is localized to the cytoplasm and is translocated to the periphery of the hepatocyte as the concentration of glucose increases. Translocation of glycogen synthase is not dependent upon its activation, but rather is a result of the levels of G6P. Glycogen deposition begins at the cell cortex and progresses in towards the center of the cell. Through this mechanism, translocation of glycogen synthase to the cell periphery may bring the enzyme in proximity with the glycogen particle, thus enhancing the efficiency of the enzyme (Fernández-Novell, Ariño et al. 1992; Fernández-Novell, Bellido et al. 1997).

Glycogen Phosphorylase

Glycogen degradation is catalyzed by the enzyme glycogen phosphorylase. The activity of glycogen phosphorylase is regulated by phosphorylation of Ser-14 by phosphorylase kinase. Phosphorylation converts glycogen phosphorylase from its inactive state,

phosphorylase b, to its active form, phosphorylase a (Bollen, Keppens et al. 1998; Ferrer, Favre et al. 2003). While other isoforms of glycogen phosphorylase are also regulated allosterically, the liver isoform is primarily regulated by phosphorylation and dephosphorylation (Newgard, Hwang et al. 1989; Bollen, Keppens et al. 1998; Ferrer, Favre et al. 2003). However, liver glycogen phosphorylase is also regulated through the binding of glucose to phosphorylase a making it a better substrate for dephosphorylation and inactivation by PP-1 (Newgard, Hwang et al. 1989; Bollen, Keppens et al. 1998).

Phosphorylase Kinase

Phosphorylase kinase is the enzyme responsible for the activation of glycogen phosphorylase and the enzymatic properties of the liver isoform are poorly understood (Bollen, Keppens et al. 1998). It is known that the muscle isoform contains one catalytic gamma subunit and three regulatory subunits (alpha, beta, and delta subunits) (Brushia and Walsh 1999). The three regulatory subunits inhibit the activity of the gamma subunit. However, the alpha and beta subunits can be phosphorylated by PKA thereby activating the enzyme (Bollen, Keppens et al. 1998; Brushia and Walsh 1999).

Glycogen Targeting Subunits of Protein Phosphatase 1

Protein phosphatase 1 (PP-1) is a serine/threonine phosphatase that regulates diverse cellular processes through the interaction of its catalytic subunit with a wide family of

regulatory subunits. Four mammalian catalytic subunit gene products were initially identified and designated PP1α, PP1β, PP1γ_1 and PP1γ_2. The last two isoforms arise from alternate splicing of PP1γ. The catalytic subunit isoforms exhibit distinct tissue and subcellular distributions. However, the small number of catalytic subunit isoforms and their similar substrate specificities would not allow for the highly diverse array of activities of PP-1 to be carried out with a high degree of specificity if not for the interaction with the regulatory subunits (Cohen 2002).

Many regulatory subunits of PP-1 have been identified and they can be broadly classified as either targeting or modulator proteins. Modulator proteins include inhibitor proteins, chaperones, and activator proteins that do not target PP-1 to particular subcellular locations (e.g. DARPP-32 and Inhibitor-3). Sorting of PP-1 via the wide array of targeting proteins allows the enzyme to participate in an variety of critical processes, including cell division, vesicle formation, ion channel function, and glycogen metabolism (Cohen 2002) . Scaffolding proteins that organize glycogen metabolism, known collectively as glycogen-targeting subunits of protein phosphatase 1 (hereafter referred to generally as glycogen-targeting subunits), are among the family of PP-1 binding proteins. PP-1, the enzymes of glycogen metabolism, and the glycogen particle are juxtaposed by glycogen-targeting subunits allowing for the efficient regulation of glycogen metabolism (Newgard, Brady et al. 2000).

Several distinct glycogen-targeting subunits have been identified (Figure 1.2). G_M, also named R_{GL} or PPP1R3, is a 124-kDa protein that is expressed in skeletal and cardiac muscle (Tang, Bondor et al. 1991; Chen, Hansen et al. 1994). G_L is a 35-kDa protein expressed

preferentially in the liver (Doherty, Moorhead et al. 1995). Protein targeted to glycogen (PTG), also known as PPP1R5, and PPP1R6 are similar in size to G_L, but are expressed in a wide variety of tissues (Doherty, Young et al. 1996; Armstrong, Browne et al. 1997; Printen, Brady et al. 1997). PPP1R3E is the most newly discovered member of the family, and is expressed at highest levels in liver and heart in rats, but in humans, is preferentially expressed in skeletal muscle and heart (Munro, Ceulemans et al. 2005). Multiple glycogen targeting subunit isoforms are expressed in skeletal muscle and liver in rats; G_M, PTG, and PPP1R6 in skeletal muscle (Delibegovic, Armstrong et al. 2003) and G_L, PTG, PPP1R6, and PPP1R3E in liver (Browne, Delibegovic et al. 2001; Green, Aiston et al. 2004; Munro, Ceulemans et al. 2005). All of the glycogen-targeting subunits are able to bind to glycogen and PP-1, and appear to interact with glycogen synthase, glycogen phosphorylase, and phosphoryalse kinase with differential capacities (Newgard, Brady et al. 2000).

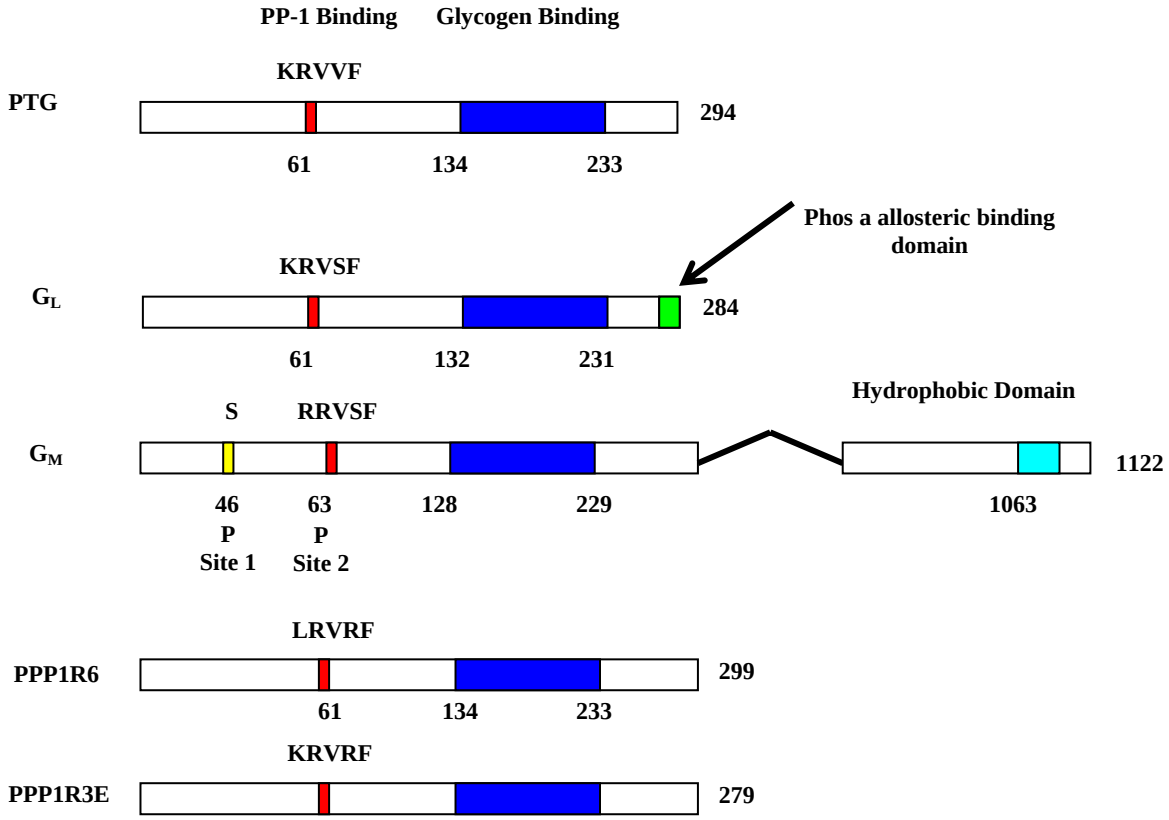


Figure 1.2. Schematic alignment of the mammalian glycogen targeting subunits of PP-1.

The five known members of the family are shown. G_M is expressed primarily in striated skeletal muscle, G_L is expressed primarily in liver, PTG and PPP1R6 are expressed in many tissues, and PPP1R3E has species specific expression. G_M is approximately three times larger than the other isoforms and its COOH-terminal extension contains a hydrophobic sarcoplasmic binding domain that has no homology with the other family members. G_M also contains two serine residues (designated Site 1 and Site 2) that are phosphorylated in response to glycogenolytic agents. Site 1 is absent from the other glycogen-targeting subunits and site 2 has variable sequences as shown for each form. Also indicated is a conserved glycogen binding motif. G_L is the only glycogen-targeting subunit that contains an allosteric phosphorylase a binding domain at the COOH-terminal tail. Numbers under each targeting subunit refer to the amino acid sequence of each protein. (adapted from (Newgard, Brady et al. 2000).

The PP-1 binding motif and putative-glycogen-binding regions in the various glycogen-targeting subunits exhibit sequence homology. G_M is different from the other glycogen-targeting subunits in that it contains a large COOH-terminal domain. This unique domain contains a hydrophobic region that mediates binding of G_M to the sarcoplasmic reticulum in skeletal muscle (Tang, Bondor et al. 1991). In addition, G_M contains two potential phosphorylation sites. Site 1 has the sequence RRGS (serine 46 in the human G_M sequence) and is not found in other glycogen-targeting subunit isoforms, whereas site 2 contains a consensus PKA-mediated phosphorylation site in the middle of the PP-1 binding motif (serine 65 in the human G_M sequence). Phosphorylation of this site leads to dissociation of the PP-1-G_M complex and ultimately decreased glycogen synthesis.

G_L is expressed in liver (Doherty, Moorhead et al. 1995). Levels of G_L are increased in the presence of insulin and decreased in starvation (Doherty, Cadefau et al. 1998). Phosphorylase a also plays a role in the regulation of the activity of G_L and its binding to the C-terminus of G_L decreases the affinity of G_L for PP-1, thereby decreasing PP-1 enzymatic activity and glycogen synthase activation leading to decreased glycogenesis (Moorhead, MacKintosh et al. 1995). This phosphorylase a binding site is unique to G_L and allows G_L to preferentially regulate glycogen synthase activity.

The expression pattern of PTG is different from G_M and G_L in that it has a wide tissue distribution, although highest levels of expression are seen in skeletal muscle, liver, and heart (Printen, Brady et al. 1997). PTG regulates glycogen metabolism through deactivation of phosphorylase a and activation of glycogen synthase via directed glycogen synthase phosphatase activity of PP-1 (Green, Aiston et al. 2004). Levels of PTG have been shown to

be altered in response to insulin and starvation in a similar fashion as G_L (Doherty, Cadefau et al. 1998).

PPP1R6, like PTG, is expressed in a wide variety of tissues (Armstrong, Browne et al. 1997). Insulin has not been shown to impact levels of PPP1R6 (Browne, Delibegovic et al. 2001). In liver, PPP1R6 is primarily in the soluble fraction as opposed to the glycogen pellet suggesting a relatively low glycogen-binding affinity (Arden, Green et al. 2006).

The newest member of the glycogen targeting subunit family is PPP13E. It shows a distinct expression pattern in different species with the highest expression found in rat liver and heart muscle and human skeletal muscle and heart. Like PTG and G_L, insulin increases expression of hepatic PPP13E. No evidence for regulation of this glycogen targeting subunit by phosphorylase a binding was reported (Munro, Ceulemans et al. 2005). Moreover, inspection of the sequence of the C-terminal tail of PPP1R3E reveals no homology with the C-terminal tail of G_L.

Changes in concentration of glycogen-targeting subunits can exert a regulatory effect on glycogen metabolism. This concept came to light when overexpression of PTG was shown to stimulate glycogen accumulation in 3T3-L1 cells or rat hepatocytes with a concomitant stimulation of G6P-independent glycogen synthase activity (Printen, Brady et al. 1997; Berman, O'Doherty et al. 1998). Overexpression of PTG caused dramatic enhancement of glycogen synthesis even when carbohydrates and insulin were absent from the culture media (amino acids were the main carbon source), suggesting that PTG can function to stimulate the gluconeogenic pathway of glycogen synthesis. Morover, PTG overexpression impaired the glycogenolytic action of forskolin and glucagon (Berman, O'Doherty et al. 1998).

Subsequent studies in rat hepatocytes compared the metabolic effects of PTG to those of other glycogen targeting subunit isoforms. Adenoviral overexpression of G_L, G_M, and a truncated form of G_M that lacked its C-terminal 700 amino acids, termed $G_M\Delta C$, demonstrated that the isoforms had different capacities for stimulating glycogen accumulation. The glycogenic potency of these glycogen targeting subunits was found to be G_L>PTG>$G_M\Delta C$>G_M (Gasa, Jensen et al. 2000; Yang, Cao et al. 2002). In response to decreased glucose concentrations in the media, stimulation with forskolin, or a combination of both, only hepatocytes overexpressing $G_M\Delta C$ were able to degrade glycogen (Yang, Cao et al. 2002).

Levels of glycogen targeting subunits have been found to be altered in diabetic states. Insulin-dependent diabetic rats were found to have decreased levels of G_L in the liver and administration of insulin resulted in restoration of G_L levels (Doherty, Cadefau et al. 1998). More recent experiments also found that livers from insulin-dependent diabetic rats and starved rats have decreased levels of G_L (Browne, Delibegovic et al. 2001). However, these experiments also showed that the levels of PTG are decreased in these rats. Interestingly, treatment with insulin restored the levels and activities of both G_L and PTG. PPP1R6 was not altered by these manipulations.

Studies involving the use of hepatocytes from Zucker fa/fa rats, a model of type 2 diabetes, also suggest that glycogen targeting subunits play a role in abnormal glycogen metabolism. Isolated hepatocytes from Zucker rats had normal expression of both G_L and PTG, but had decreased levels of PPP1R6. The results of these studies suggest that leptin in concert with insulin plays a role in the regulation of PPP1R6 (Arden, Green et al. 2006).

Knockout of the G_M isoform in mice leads to a 90% reduction in glycogen stores of skeletal muscles compared to wild-type mice (Suzuki, Lanner et al. 2001). This decrease in tissue glycogen was accompanied by decreased glycogen synthase and PP-1 activities and increased phosphorylase activity. Despite the large decrease in muscle glycogen content, the animals remained normoglycemic and insulin responsive. The lack of changes in glycemia is not due to increased glucose accumulation in fat or increased hepatic glycogen accumulation. Weight gain, fat deposition, glucose tolerance, and insulin responsiveness remained similar to wild-type mice up to twelve months of age. These results suggest that other glycogen targeting subunits and/or glycogen synthase phosphatases may play a role in the regulation of skeletal muscle glycogen metabolism.

A more recent study that also knocked out G_M substantiated the above results with a 90% reduction in skeletal muscle glycogen content with a concomitant decrease in glycogen synthase activity and increase in phosphorylase activity (Delibegovic, Armstrong et al. 2003). However, in this study the knockout mice exhibited a greater weight gain compared to wild-type mice starting at three months of age. In addition, by eleven months of age the mice lacking G_M exhibited glucose intolerance and insulin resistance most likely due to increased adipose stores. Surprisingly, the G_M knockout mice exhibited a compensatory increase in insulin stimulated activity of PTG. The apparent discrepancies between these two knock out studies may be due to differences in the 129 substrains and/or the method used to achieve the knockout of G_M. Further studies will be needed to reconcile these differential responses to the deletion of G_M.

PTG is expressed in many tissues and heterozygous deletion of the PTG gene in mice leads to decreased PTG protein levels and glycogen stores in liver, skeletal muscle, heart, and adipose tissue (Crosson, Khan et al. 2003). Homozygous deletion of PTG is embryonic lethal. PTG heterozygous mice have decreased glycogen synthase activity and a decreased rate of glycogen synthesis in fat, liver, and heart. Despite alterations in glycogen content in several tissues, young heterozygous PTG mice exhibit normal glucose tolerance. However, glucose intolerance, hyperinsulinemia, and insulin resistance all develop with age in concert with increased intramyocellular triglyceride, fasting serum triglyceride, and fasting free fatty acid levels. Thus, PTG plays an important role in glycogen synthesis, and in the long term, deficiencies in expression of this protein impact metabolic fuel homeostasis.

Specific Aims

It is clear that the liver produces abnormally high levels of glucose in diabetes mellitus, but the contribution of dysregulated hepatic glycogen metabolism to the development of hyperglycemia is still incompletely understood, as is the potential of therapeutic strategies aimed at improving hepatic glycogen storage. This dissertation is focused on trying to understand the role that glycogen-targeting subunits of PP-1 play in fuel homeostasis. The ability of overexpressed glycogen-targeting subunits to improve glucose tolerance in high fat fed rats was first examined. Since the results indicated that it was possible to improve glucose tolerance by increasing hepatic glycogen content, the remainder of the dissertation focused on determining the mechanism of action of a dominant-negative glycogen-targeting subunit and its impact on glucose homeostasis. The results of these studies are summarized in the following three chapters of this dissertation, and in aggregate, provide new information about the role of glycogen-targeting subunits in glycogen metabolism.

CHAPTER TWO

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

INTRODCTION

Hepatic glycogen storage is impaired in all major forms of diabetes, suggesting a possible contribution to the development of hyperglycemia (Magnuson, Rothman et al. 1992; Cline, Rothman et al. 1994; Velho, Petersen et al. 1996). 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 (O'Doherty, Lehman et al. 1999) or mice (Hariharan, Farrelly et al. 1997; Niswender, Shiota et al. 1997) 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 (O'Doherty, Lehman et al. 1999), consistent with the large increase in glycolytic flux caused by overexpression of glucokinase in hepatocytes or hepatoma cells (Valera and Bosch 1994; O'Doherty, Lehman et al. 1996).

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 (Newgard, Brady et al. 2000). Prominent members of this gene family include G_M or R_{GL} (hereafter referred to as G_M), expressed primarily in striated skeletal muscle (Tang, Bondor et al. 1991), G_L, expressed primarily in liver (Doherty, Moorhead et al. 1995), and protein targeting to glycogen (PTG) (Doherty, Young et al. 1996; Printen, Brady et al. 1997) and PPP1R6 (Armstrong, Browne et al. 1997), 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 (Tang, Bondor et al. 1991; Doherty, Young et al. 1996; Armstrong, Browne et al. 1997; Printen, Brady et al. 1997; Newgard, Brady et al. 2000).

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 $G_L > PTG > G_M$ (Gasa, Jensen et al. 2000). 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 $G_M > G_L \approx PTG$ (Gasa, Jensen et al. 2000; Yang, Cao et al. 2002). Hepatic overexpression of PTG in normal rats was shown to improve glucose tolerance without perturbation of lipid homeostasis (O'Doherty, Jensen et al. 2000). 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 to the design and testing of a novel form of glycogen-targeting subunit derived from G_M (Yang, Cao et al. 2002). Native G_M is distinct from other members of its gene family in that it contains two consensus sequences for protein kinase A-mediated serine phosphorylation. One of these sites resides within the PP-1 binding site of G_M, and its phosphorylation leads to dissociation of the phosphatase, contributing to inactivation of glycogen synthesis (Dent, Lavoinne et al. 1990; Tang, Bondor et al. 1991; Wu, Kleiner et al. 1996). G_M 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 (Hubbard, Dent et al. 1990; Tang, Bondor et al. 1991). Removal of 735 C-terminal amino acids from native G_M 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 in hepatocytes reveals that the former protein is more effective at stimulating glycogen synthesis (Yang, Cao et al. 2002), but is less glycogenic than PTG or G_L. Cells that overexpressed either PTG or G_L were unable to decrease glycogen stores in response to glycogenolytic signals such as forskolin or decreased media glucose concentration. Interestingly, hepatocytes with overexpressed G_M or $G_M\Delta C$ were able to decrease glycogen content in response to these same glycogenolytic signals. Although both G_M and $G_M\Delta C$ were able to appropriately degrade glycogen, the fact that

$G_M\Delta C$ was more glycogenic than G_M suggests that $G_M\Delta C$ would be more effective at regulating hepatic glycogen metabolism when overexpressed *in vivo*.

The studies described in this chapter were designed to compare the metabolic impact of $G_M\Delta C$, G_L, and G_M overexpression in whole animals. These studies were performed in rats fed on a high fat diet for seven weeks to cause a syndrome of insulin resistance and glucose intolerance such as seen in early stage type 2 diabetes. $G_M\Delta C$ is unique among the molecules tested in its capacity to reverse diet-induced glucose intolerance.

MATERIALS AND METHODS

Animal Maintanence

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, Winfield, IA) 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 seven weeks before adenovirus administration.

Administration of Recombinant Adenoviruses

Rats were treated with cyclosporine (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, Kalamazoo, MI) on the day of adenoviral treatment. The preparation and testing of recombinant adenoviruses containing the cDNAs encoding G_L (AdCMV-G_L), G_M (AdCMV- G_M), a truncated form of G_M with its 735 C-terminal amino acids deleted (AdCMV- $G_M\Delta C$), or β-galactosidase (AdCMV-βGal) have been described previously (Herz and Gerard 1993; Gasa, Jensen et al. 2000; O'Doherty, Jensen et al. 2000; Yang, Cao et al. 2002). Aliquots of these viruses were amplified and purified for the current study as described previously (Becker TC 1994). Between 0.5 and 1.5 x 10^{12} recombinant adenovirus particles were administered via tail vein injection to rats anesthetized with Nembutal (50 μg/g of body weight intraperitoneally; Abbott Laboratories, North Chicago, IL) or a 50:5:1 mixture of ketamine (Avoco, Fort Dodge, IA), Rompun (Avoco), and acepromazine (Haver, Shawnee, KS) as described elsewhere (O'Doherty, Lehman et al. 1999; Buettner, Newgard et al. 2000). 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, AdCMV-$G_M\Delta C$, 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 of body weight intraperitoneally) and administration of a bolus of 2 g of glucose/kg of body weight by gavage of a 45% solution of glucose in water. Blood samples (~ 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-$G_M\Delta C$, 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 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 used was based on methods described previously (Gasa, Jensen et al. 2000; O'Doherty, Jensen et al. 2000). Total RNA was extracted from powdered liver or muscle tissue using RNeasy 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 mixture containing *Taq* DNA polymerase (2.5 units) and buffer (Promega Corp., Madison, WI), dNTP mix (final concentrations of 40 mM of each dNTP except dCTP, which was present at 20 mM; Invitrogen) and with or without 1.25 μCi of [α-^{33}P]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 181-bp fragment from the G_L transgene and did not amplify endogenous rat G_L because the upstream primer hybridizes to 5′ untranslated sequence derived from the adenovirus vector (5′ primer, CGAGCTCGGTACCAACTTC; 3′ primer, GAAGGTGAAGCGCTCTCTG). The second primer set amplified a 162-bp product from either full-length endogenous G_M or $G_M\Delta C$ transgene (5′ primer , CTCAAAGGAAGATCTTATGCAAC; 3′ primer, GTTTGCCAGTCATCTAAGGAC) as described previously (Gasa, Jensen et al. 2000). The third oligonucleotide pair specifically amplified a 900-bp fragment of $G_M\Delta C$ derived by expression from the AdCMV- $G_M\Delta C$ adenovirus because the upstream primer hybridizes to sequence within the $G_M\Delta C$ sequence, while the 3′ primer hybridizes to the 3′ untranslated region derived from the adenovirus vector (5′ primer, CTCAAAGGAAGATCTTATGCAAC; 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 transcript, a 201-bp fragment of the elongation factor-1α (EF-1 α) mRNA (O'Doherty, Jensen et al. 2000), or a 250-bp fragment of the α -tubulin gene (5′ primer,

GCGTGAGTGTATCTCCATCCA; 3′ primer, GGTAGGTGCCAGTGCGAACTT). In experiments involving the inclusion of [α-^{33}P]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) of 95 °C for 45 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. The final PCR products were mixed with 98% formamide denaturing loading buffer and separated on a 6% (w/v) polyacrylamide gel containing 7 M urea. The gel was subsequently dried and exposed to a PhosphorImager screen, and the resulting scan was analyzed using ImageQuant from Molecular Dynamics (Sunnyvale, CA). In the experiments designed to assess expression of the $G_M\Delta C$ transgene in extrahepatic tissues, PCRs were carried out in the absence of [α-^{33}P]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. Five ml of the PCR product was resolved on an agarose gel, and products were visualized by incubation of the gel with 0.6 μg/ml ethidium bromide.

Plasma 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).

Tissue Analysis

Glycogen content was assayed by homogenizing 150 mg powdered liver in 1 ml 30% potassium hydroxide, boiling the extract for 15 minutes, and centrifuging at 3,000 rpm for 10 minutes. Glycogen in the cleared supernatant was measured as described (Newgard, Hirsch et al. 1983). Protein content in the samples was determined by method of Bradford (Bradford 1976), using the Bio-Rad assay kit.

Statistical Analysis

Data are expressed as the mean ± S.E.M. Statistical significance was determined by unpaired Student's *t* test using the statistics module of Microsoft Excel. Statistical significance was assumed at $p \leq 0.05$.

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 is Figure 2.1A. Animals treated with

AdCMV-βGal exhibited either no signal or background at the positions expected for the reverse-transcribed and amplified fragments of G_L, $G_M\Delta C$, or G_M. Rats infused with AdCMV- G_L, AdCMV- G_M,or AdCMV- $G_M\Delta C$ showed clear expression of the respective trangene mRNAs. When normalized to the internal control, TATA-binding protein, G_L and $G_M\Delta C$ mRNA levels were found to be indistinguishable and ~ 3-fold greater than the levels of G_M transgene RNA (Figure 2.1B). The lower apparent efficiency of G_M overexpression relative to the other two targeting subunits is consistent with our previous findings in isolated hepatocytes (Gasa, Jensen et al. 2000; Yang, Cao et al. 2002). No attempt could be made to correct for the clearly lower level of G_M 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 G_L with our novel construct $G_M\Delta C$.

OGTT in Wistar Rats with Adenovirus-mediated G_L, $G_M\Delta C$, or G_M Overexpression in Liver

To test the capacity of the various glycogen-targeting subunits to improve glucose homeostasis, we performed oral glucose tolerance tests (OGTTs) in the HF animals evaluated for targeting subunit expression in Figure 2.1, as well as a group of rats that were fed on normal chow and infused with AdCMV-βGal. As shown in Figure 2.2, 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 or AdCMV- G_L exhibited no significant improvement in glucose tolerance during OGTT. In contrast, HF animals treated with AdCMV- $G_M\Delta C$ 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 with the standard chow controls but sill lower than that in the other three treatment groups.

Effects of G_L, $G_M\Delta C$, or G_M Overexpression on Liver Glycogen following OGTT

To determine whether the differential effects of the various glycogen-targeting subunits of 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 2.2. Figure 2.3 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 did not enhance glycogen accumulation compared with either control group. However, treatment of HF animals with AdCMV- G_L or AdCMV- $G_M\Delta C$ resulted in 108 and 138% increases in liver glycogen, respectively, relative to the AdCMV-βGal-treated HF controls. Thus, both G_L - and $G_M\Delta C$ -

overexpressing animals had higher liver glycogen levels following OGTT, but only the AdCMV- $G_M\Delta C$-treated animals 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 2.2 (180-minute time point), allowing several plasma variables to be measured. As summarized in Table 2.1, 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 HF controls. Treatment of animals with AdCMV- G_L or AdCMV- G_M also did not alter circulating triglyceride (TG) levels. However, AdCMV- $G_M\Delta C$ 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- $G_M\Delta C$-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 (Trinh, O'Doherty et al. 1998; O'Doherty, Lehman et al. 1999).

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 $G_M\Delta C$ transcript in liver and skeletal muscle of AdCMV-βGal- and AdCMV- $G_M\Delta C$-infused animals. This assay used an oligonucleotide pair that specifically amplifies the transcript derived from the adenovirus construct and not endogenous G_M. As a positive control, treatment of 293 cells with AdCMV- $G_M\Delta C$ and RT-PCR analysis of RNA derived from such cells resulted in amplification of a band of the predicted size of 900 nucleotides (Figure 2.4). RT-PCR analysis was also performed on RNA isolated from liver and muscle samples taken from three AdCMV-βGal- or three AdCMV- $G_M\Delta C$-treated rats subjected to OGTT. As shown in Figure 2.4, a band of the same size as that in AdCMV- $G_M\Delta C$-treated 293 cells was clearly detected in liver samples of AdCMV- $G_M\Delta C$-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- $G_M\Delta C$. These findings clearly demonstrate that the improved glucose tolerance reported in Figure 2.2 is due to expression of $G_M\Delta C$ 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 $G_M\Delta C$ and G_L overexpression on glucose tolerance (Figure 2.2), we next studied liver glycogen levels in fed and fasted HF

animals treated with AdCMV- $G_M\Delta C$ and AdCMV- G_L. Multiplex RT-PCR analysis of transgene expression levels in these animals is summarized in Figure 2.5. In both AdCMV- G_L- and AdCMV- $G_M\Delta C$-treated groups, transgene expression tended to be lower in fasted animals, but this difference was not significant in either group. Comparison of $G_M\Delta C$ to G_L mRNA levels in fed *versus* fed or fasted *versus* fasted groups also revealed no significant differences.

As shown in Figure 2.6, livers of 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-hour fast. Interestingly, fed AdCMV- G_L-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- $G_M\Delta C$-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-hour 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- G_L-treated rats was 80% of that in AdCMV- G_L-treated rats following OGTT. In contrast, liver glycogen content in fasted AdCMV- $G_M\Delta C$-treated rats was only 29% of that in AdCMV- $G_M\Delta C$-treated rats following OGTT. In other words, AdCMV- $G_M\Delta C$-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- G_L-treated animals (values obtained by subtracting the glycogen levels

in fasted rats shown in Figure 2.6 from the glycogen levels after OGTT shown in Figure 2.3; note that the animals were fasted for 20 hours prior to OGTT). This suggests that the differential potency of $G_M\Delta C$ and G_L for lowering of blood glucose in glucose-intolerant HF rats may have been due in part to the high basal glycogen levels in G_L-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 the OGTT (Table 2.1) was measured in fed and 20-hour fasted HF rats treated with the various recombinant adenoviruses (Table 2.2). Animals treated with AdCMV-βGal, AdCMV- G_L, or AdCMV- $G_M\Delta C$ 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 (Buettner, Newgard et al. 2000). Circulating TGs were more than twice as high in fed compared with fasted AdCMV-βGal- or AdCMV- $G_M\Delta C$-treated rats. However, in AdCMV- G_L-treated animals, TG remained low in the fed state and was indistinguishable from fasted values. Lactate levels in fasted AdCMV- G_L-infused fats were also lower than in AdCMV- $G_M\Delta C$- 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 (Ferre, Pujol et al. 1996; Hariharan, Farrelly et al. 1997; Niswender, Shiota et al. 1997; O'Doherty, Lehman et al. 1999) or 6-phosphofructo-2kinase/fructose-2,6-bisphosphatase (Wu, Okar et al. 2001) has been shown to lower blood glucose levels in normal or diabetic animals. Furthermore, overexpression of phosphoenolpyruvate carboxykinase (Valera, Pujol et al. 1994), the glucose-6-phosphatase catalytic subunit (Trinh, O'Doherty et al. 1998), or the transcriptional co-activator PGC-1, which stimulates expression of the genes encoding several gluconeogenic enzymes (Yoon, Puigserver et al. 2001), all result in hyperglycemia. However, overexpression of glucokinase or 6-phosphofructo-2kinase/fructose-2,6-bisphosphatase results in increases in the levels of circulating lipids (O'Doherty, Lehman et al. 1999; Wu, Okar et al. 2001), 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 (She, Shiota et al. 2000), 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 (O'Doherty, Jensen et al. 2000). 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 disease.

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 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 (Gasa, Jensen et al. 2000). These findings led us to investigate the possibility that the glycogenic impact of G_M could be improved by deletion of its unique C-terminal tail that includes a putative sarcoplasmic association domain. To this end, we prepared a truncated form of G_M ($G_M\Delta C$) and demonstrated that its overexpression in

hepatocytes had a more potent glycogenic effect than native G_M but with retention of glycogenolytic responsiveness to forskolin, a fall in media glucose, or the combination of both glycogenolytic signals (Yang, Cao et al. 2002).

These *in vitro* findings led us to compare, in the current study, the metabolic effects of hepatic overexpression of $G_M\Delta C$, native G_M, and the most glycogenic of all the targeting subunits, G_L. These studies were performed in Wistar rats fed on a high fat diet for a period of seven 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 (Buettner, Newgard et al. 2000). This study reveals that at similar levels of overexpression in liver, $G_M\Delta C$ but not G_L lowers blood glucose levels toward normal during OGTT in insulin-resistant, glucose-intolerant, HF rats. Native G_M, which consistent with our previous findings cold not be overexpressed as efficiently as the other targeting isoforms (Gasa, Jensen et al. 2000; Yang, Cao et al. 2002), also did not improve glucose tolerance.

The explanation for the difference in effect of $G_M\Delta C$ and G_L appears to be that animals with overexpressed $G_M\Delta C$ experience a larger increment in hepatic glycogen storage during OGTT than animals with overexpressed G_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_L-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 $G_M\Delta C$-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 $G_M\Delta C$-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 $G_M\Delta C$ 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, free fatty acids, and TG were normal in *ad libitum* fed and 20-hour fasted $G_M\Delta C$-overexpressing rats. Thus, the overexpressed $G_M\Delta C$ 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 disposal 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 overxpression 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 2.2). 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 (Buettner, Newgard et al. 2000). 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 issues such as muscle and fat. Given the correlation between intramyocellular lipid stores and insulin resistance (Stein, Dobbins et al. 1997; Krssak M 1999), 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, and 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 libitum* 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 (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 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 (Newgard, Brady et al. 2000). 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 (Martin, Hoover et al. 1998) 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.

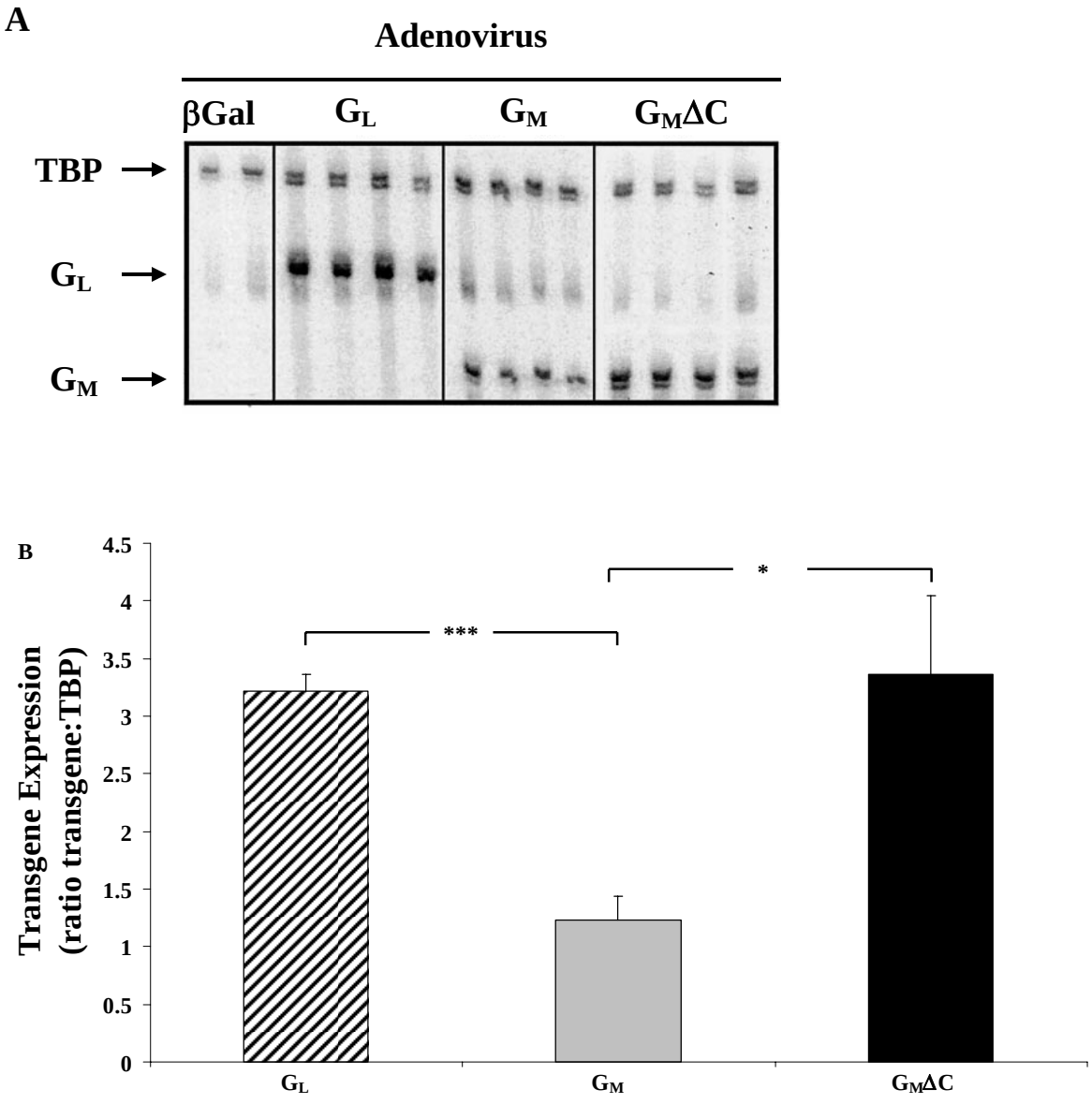

A
Adenovirus
βGal
GL
GM
GMΔC
TBP
GL
GM
B
Transgene Expression
(ratio transgene:TBP)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
*
GL
GM
GMΔC

Figure 2.1. Expression of the G_L, G_M, and $G_M\Delta C$ transgenes in liver after OGTT. Male Wistar rats were fed a high fat diet for seven weeks. At the end of this period, animals were treated with the Ad-CMV-βGal, AdCMV- G_L, AdCMV- G_M, or AdCMV- $G_M\Delta C$ 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 2 g/kg body weight. Animals were sacrificed 180 minutes 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 under "Materials and Methods." A, representative gel displaying RT-PCR results for two to four 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:TATA-binding protein (TBP) for all animals included in the OGTT protocol. Data represent mean $\pm$ S.E.M. for a total of six G_L-, seven G_M-, and seven $G_M\Delta C$-overexpressing animals. Symbols * and *** indicate significant differences between the $G_M\Delta C$- and G_L-overexpressing groups relative to the G_M-overexpressing group with levels of significance of $p<0.05$ and $p<0.0001$, respectively. Expression levels in the G_L- and $G_M\Delta C$-overexpressing groups were not significantly different.

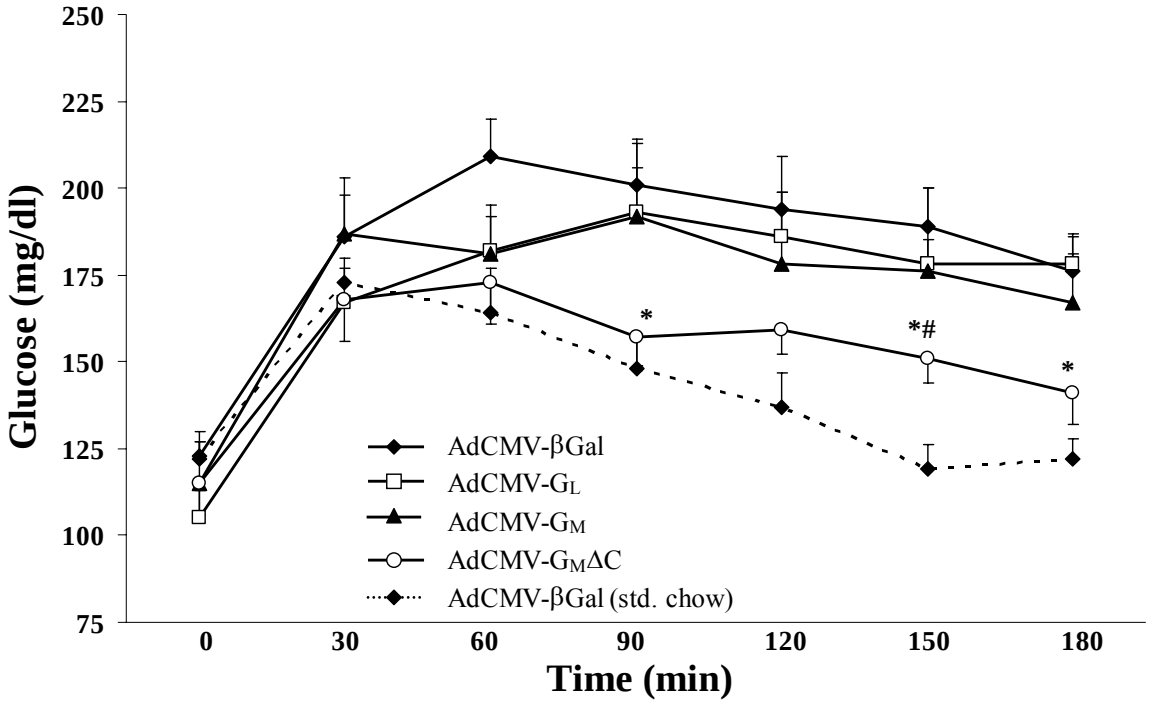


Figure 2.2. Oral glucose tolerance test. Animals were treated as described in the legend for Figure 2.1. Tail vein blood samples were taken, and glucose levels were measured at the indicated times after the glucose bolus. Data are mean $\pm$ S.E.M. for 12 β-galactosidase (βGal)-, six G_L-, seven G_M-, and seven $G_M\Delta C$-overexpressing animals. The symbol * indicates those time points at which blood glucose levels were significantly lower in HF AdCMV- $G_M\Delta C$-treated rats versus HF AdCMV- βGal-treated controls with $p<0.05$. A Second control group of animals fed a standard chow diet for seven 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 (#, $p<0.01$).

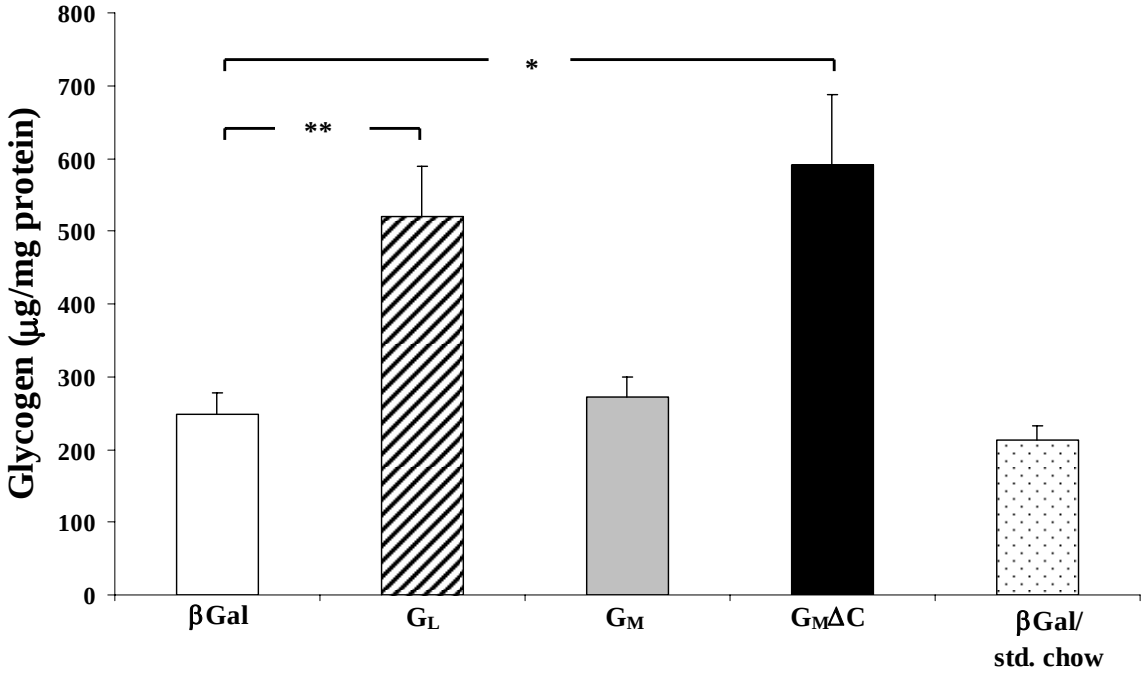


Figure 2.3. Liver glycogen levels after the OGTT. Animals were sacrificed for collection of liver samples at the 180-mintue point of the OGTT shown in Figure 2.2. Data represent mean $\pm$ S.E.M. for six G_L-, seven G_M-, and seven $G_M\Delta C$-overexpressing HF rats and 12 β-galactosidase-overexpressing HF (βGal) and eight β-galactosidase-overexpressing standard chow-fed (βGal/std. chow) rats. The symbols * and ** indicate that G_L- and $G_M\Delta C$-overexpressing animals stored more glycogen than HF β-galactosidase controls with levels of significance of $p<0.001$ and $p<0.05$, respectively.

	AdCMV-βGal (n = 12)	AdCMV-G_L (n = 6)	AdCMV-G_M (n = 7)	AdCMV-$G_M\Delta C$ (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

Table 1. Plasma variables in AdCMV-βGal-, AdCMV-G_L-, AdCMV-G_M-, and AdCMV-$G_M\Delta C$-treated rats after the OGTT protocol. Male Wistar rats were fed a high fat diet for seven weeks. At the end of this period, animals received the AdCMV-βGal, AdCMV-G_L, AdCMV-G_M, and AdCMV-$G_M\Delta C$ 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 (2 g/kg). Blood samples were collected after the 180-minute time point of the OGTT for analysis of the indicated plasma variables. Data are mean ± S.E.M. 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.

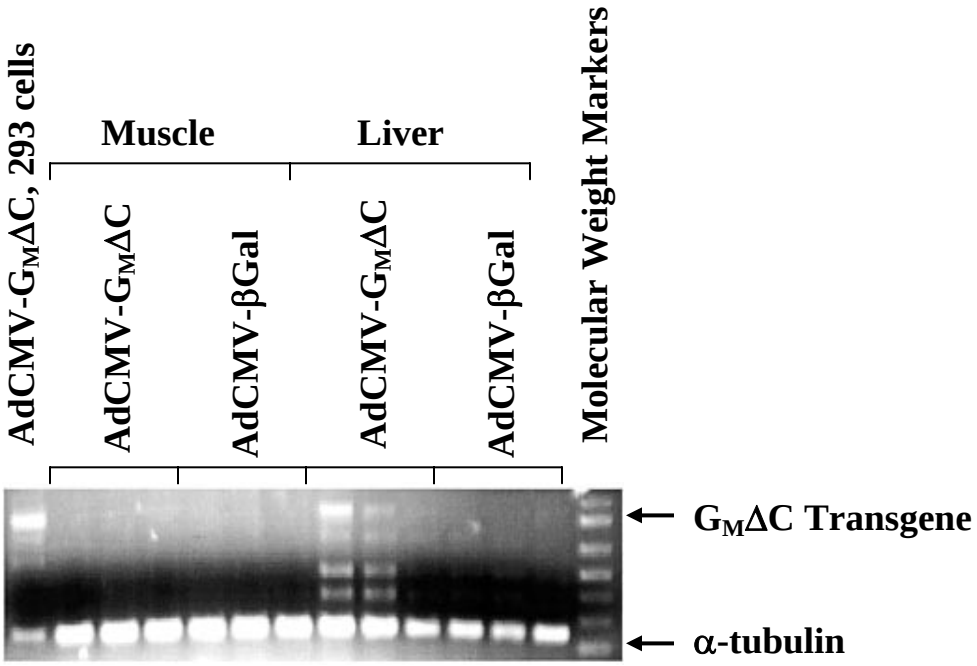


Figure 2.4. RT-PCR analysis of $G_M\Delta C$ transgene expression in liver and muscle. Oligonucleotides specific to the $G_M\Delta C$ gene product expressed for the AdCMV- $G_M\Delta C$ adenovirus or, as an internal control, to α-tubulin were used for multiplex PCR analysis of liver and muscle samples from a subset or the animals used for OGTT as described in Figures 2.1-2.3. 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.

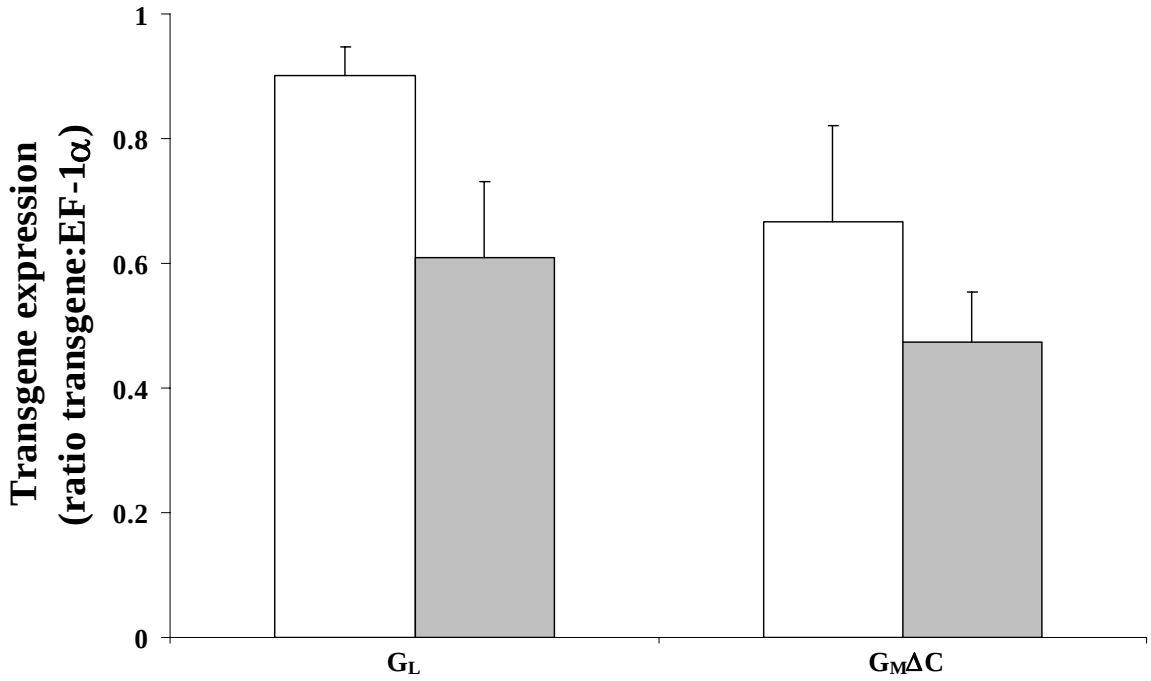


Figure 2.5. Expression of the G_L and $G_M\Delta C$ transgenes in liver of fasted and *ad libitum* fed rats. Animals were treated as described in the legend to Figure 2.1 with either the AdCMV- G_L or AdCMV- $G_M\Delta C$ adenoviruses. Ninety hours after virus infusion, animals were either allowed to continue feeding *ad libitum* on the high fat diet (white bars) or were fasted for 20 hours (dark bars). Livers were collected, and transgene expression was measured by multiplex RT-PCR. Band intensities were normalized to EF-1α as the internal control Results represent mean $\pm$ S.E.M. 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 with a virus treatment or when comparing fed to fed or fasted to fasted animals between viral treatment groups.

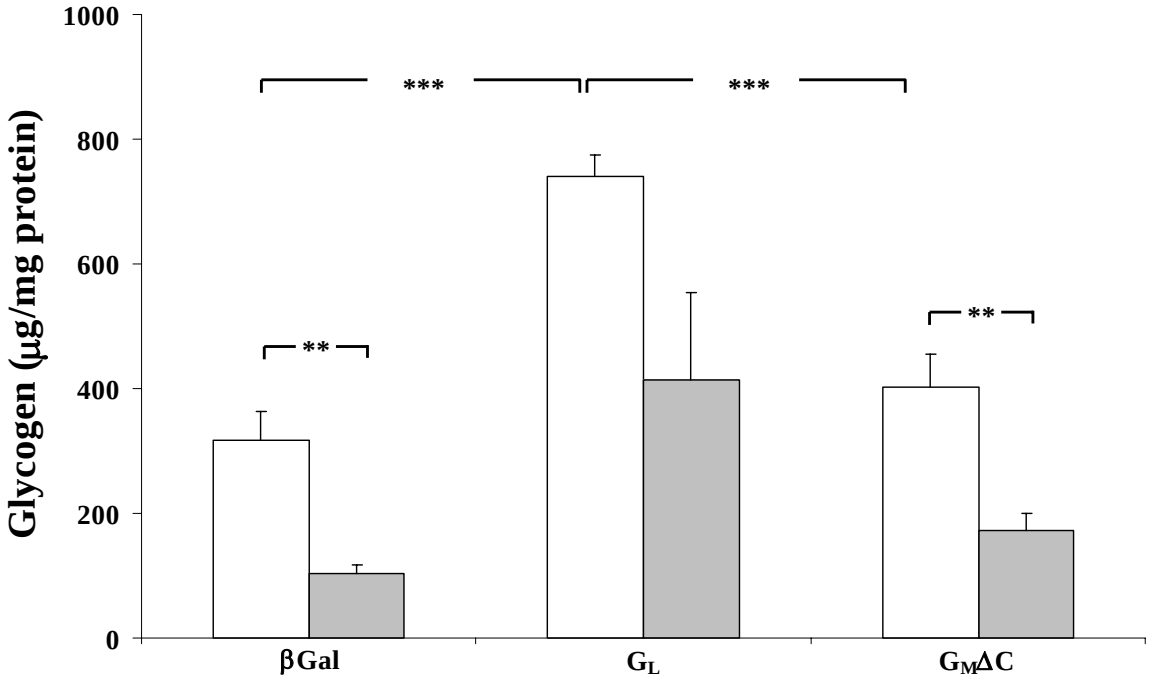


Figure 2.6. Liver glycogen levels in fasted and *ad libitum* fed rats. Animals were treated as described in the legend to Figure 2.1. Ninety hours after virus administration, animals were either allowed to continue feeding *ad libitum* (white bars) or were fasted for 20 hours (dark bars). Liver samples were taken for measurement of glycogen content. Results are mean $\pm$ S.E.M. 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.

	AdCMV-βGal		AdCMV-G_L		AdCMV-$G_M\Delta C$	
	Fed (n = 8)	Fast (n = 9)	Fed (n = 8)	Fast (n = 5)	Fed (n = 10)	Fast (n = 11)
Glucose (mg/dl)	141 ± 3	112 ± 3	130 ± 5	115 ± 7	141 ± 8	114 ± 5
Triglycerides (mg/dl)	136.1 ± 28.9	56.0 ± 5.4	67.5 ± 7.9*	68.1 ± 17.1	138.3 ± 27.0	65.3 ± 18.2
FFAs (mmol/l)	0.33 ± 0.01	0.34 ± 0.01	0.29 ± 0.01	0.46 ± 0.10	0.34 ± 0.10	0.44 ± 0.01
Ketones (mg.dl)	3.94 ± 0.83	7.75 ± 1.25	2.87 ± 0.42	8.43 ± 1.76	3.06 ± 0.97	7.24 ± 1.05
Lactate (mg/dl)	11.3 ± 1.3	10.8 ± 1.2	11.8 ± 1.3	7.6 ± 1.1*	15.7 ± 1.7	11.2 ± 1.6
Insulin (ng/ml)	4.90 ± 0.6	3.71 ± 0.4	4.91 ± 0.4	2.95 ± 0.5	4.18 ± 0.4	4.22 ± 0.9

Table 2.2. Plasma variables in fed and fasted AdCMV-βGal-, AdCMV-G_L-, and AdCMV-$G_M\Delta C$-treated rats. Animals were treated as described in the legend to Table 2.1. Ninety hours 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 ± S.E.M. 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 $p \leq 0.05$. FFAs, free fatty acids.

CHAPTER THREE

Development and Testing of a Dominant-Negative Glycogen Targeting Subunit of Protein Phosphatase 1

INTRODUCTION

Protein phosphatase 1 (PP-1) is ubiquitously expressed and plays a role in the regulation of a variety of cellular processes. Interaction of PP-1 with diverse substrates is facilitated by proteins that target the phosphatase to various subcellular locations. In glycogen metabolism, these proteins are known collectively as glycogen targeting subunits of PP-1.

Several distinct glycogen targeting subunits have been identified. G_M, also named R_{GL} or PPP1R3, is a 124-kDa protein that is expressed in skeletal and cardiac muscle (Stralfors, Hiraga et al. 1985; Hubbard and Cohen 1989; Tang, Bondor et al. 1991). G_L is a 35-kDa protein expressed preferentially in the liver (Doherty, Moorhead et al. 1995). Protein targeted to glycogen (PTG), also known as PP1R5, and PPP1R6 are similar in size to G_L, but are expressed in a wide variety of tissues (Doherty, Young et al. 1996; Armstrong, Browne et al. 1997; Printen, Brady et al. 1997). PPP1R3E is a glycogen targeting subunit which is most abundant in liver and hearts in rats, but most highly expressed in skeletal muscle and heart in humans (Munro, Ceulemans et al. 2005).

Knockout mice lacking G_M (R_{GL}) exhibited a 90% reduction of skeletal muscle glycogen stores compared to wild-type mice (Suzuki, Lanner et al. 2001). This decrease in tissue glycogen was accompanied by decreases in PP-1 and basal glycogen synthase activities. Remarkably, despite the large decrease in muscle glycogen deposition, the animals remained

normoglycemic. Since stimulation of glycogen synthase by insulin remained intact in muscle of G_M / R_{GL} knock out mice, other glycogen targeting subunits and/or glycogen synthase phosphatases may participate in regulation of glycogen metabolism in skeletal muscle.

Another line of GM knockout mice also exhibited a 90% reduction in skeletal muscle glycogen content (Delibegovic, Armstrong et al. 2003). However, in sharp contrast to the above studies, the mice lacking GM in these studies exhibited increased weight gain after three months of age and became obese by twelve months of age. In addition, the knockout mice developed impaired glucose tolerance and insulin resistance. Surprisingly these animals exhibited a compensatory increase in insulin stimulated activity of PTG that is absent from wild-type mice. The discrepancy between these two studies may arise from differences in genetic backgrounds or methods of gene disruption and will require further analysis.

PTG is expressed in many tissues and heterozygous deletion of the PTG gene in mice leads to decreased glycogen stores in liver, skeletal muscle, heart, and adipose tissue (Crosson, Khan et al. 2003). In addition to decreased glycogen stores, PTG heterozygous mice have decreased glycogen synthase activity and a decreased rate of glycogen synthesis in fat, liver, and heart. Young mice exhibit normal glucose tolerance, however glucose intolerance, hyperinsulinemia, and insulin resistance all develop with age. Thus, PTG plays an important role in glycogen synthesis, and in the long term, deficiencies in expression of this protein impact metabolic fuel homeostasis.

While these studies provide some insight into the elimination of the single glycogen targeting subunit isoforms G_M and PTG in glycogen metabolism, most tissues express more

than one of the family members. Thus, knock out of single isoforms does not provide complete insight into the essentiality of glycogen targeting subunits in control of glycogen metabolism. In an effort to address this issue, we sought to create a pan-selective dominant-negative mutant of PTG, PTG-VF, that would compete with the activities of any of the endogenous glycogen targeting subunits expressed in mammalian cells. The encoded dominant-negative PTG protein contains alanine residues engineered in place of the naturally occurring VF residues in the PP-1 binding site of native mouse PTG. Mutation of these two residues is predicted to prevent PP-1 binding, but in the context of a protein that can still continue to interact with enzymes of glycogen metabolism, thus forming complexes in which the regulatory effect of PP-1 on these enzymes is not properly engaged. We show that overexpression of PTG-VF in primary rat hepatocytes results in an 83% decrease in glycogen content suggesting efficient suppression of PTG, G_L, PPP1R6, and PPP1R3E activities (these are the glycogen targeting subunits that are naturally expressed in rat liver cells). However, in futher studies, we show that PTG-VF preferentially suppressed G_L activity relative to PTG, with this effect explained by a differential ability of PTG and G_L to interact with phosphorylase. These studies shed new light on the dynamics of interactions between specific glycogen targeting subunits and the proteins that they regulate, and also provide new information about the relative roles of G_L and PTG in control of liver glycogen metabolism.

MATERIALS AND METHODS

Construction of Recombinant Adenoviruses

A plasmid containing the cDNA encoding mouse PTG with valine 62 and phenylalanine 64 mutated to alanine was obtained from the lab of Dr. Alan Saltiel (Fong, Jensen et al. 2000). This was used to prepare a recombinant adenovirus (AdCMV-PTG-VF-FLAG) by following the methods previously described (Gomez-Foix, Coats et al. 1992; Becker, Noel et al. 1994). PCR was used to prepare a fragment of G_L encoding amino acids 1-268 and lacking the 16 C-terminal amino acids. The oligonucleotides used were 5'-ATGGCCGTGGACATAGAATACAGC-3' (forward) and 5'-CTACTTGTCATCGTCGTCCTTGTAGTCCATCAGGCCGAAGGAAACCG-3' (reverse). This was used to prepare a recombinant adenovirus (AdCMV-$G_L\Delta$C-FLAG) by following the methods previously described (Gomez-Foix, Coats et al. 1992; Becker, Noel et al. 1994). Preparation of recombinant adenoviruses expressing rat G_L (AdCMV-G_L-FLAG), mouse PTG (AdCMV-PTG-FLAG), and the *Escherichia coli* β-galactosidase gene (AdCMV- β Gal) have been described elsewhere (Herz and Gerard 1993; Becker, Noel et al. 1996; Printen, Brady et al. 1997; Berman, O'Doherty et al. 1998; Gasa, Jensen et al. 2000; O'Doherty, Jensen et al. 2000).

Hepatocyte Isolation and Viral Treatment

Primary hepatocytes were prepared from overnight fasted male Wistar rats by collagenase perfusion (Massague and Guinovart 1978). Cells were suspended in attachment medium (Dulbecco's modified Eagle's medium with 25 mM glucose, 10% fetal bovine serum, 100 nM insulin, 100 nM dexamethasone, 2 mM sodium pyruvate, and antibiotics). Cells were

seeded onto collagen-coated plates at a cell density of 2.4 X 10^6 cells/60-mm culture dish or 8 X 10^6 cells/100-mm culture dish and allowed to attach for 90 minutes at 37° C. Hepatocytes were washed twice with phosphate-buffered saline (PBS) and treated with adenovirus for 90 minutes at 37° C. Adenoviruses were diluted in 2 ml (60-mm dishes) or 5 ml (100-mm dishes) of "culture medium" consisting of DMEM supplemented with 1 mM glucose, 0.07% bovine serum albumin, 1 nM insulin, 10 nM dexamethesone, 2 mM sodium pyruvate, and antibiotics. Following viral treatment, cells were washed twice with PBS and incubated for 24 hours in culture medium containing 20 mM glucose.

Measurement of Expression of Glycogen Targeting Subunits in Hepatocytes by RT-PCR

Total RNA was isolated using RNeasy (Qiagen) and quantified using RiboGreen RNA quantitation kit (Molecular Probes). cDNA was synthesized from 1.0 μg RNA using the IScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed with 20 ng of cDNA by using the Applied Biosystems Prism 7000 sequence detection system, software, and reagents (An, Muoio et al. 2004). Primers for PCR reactions (Applied Biosystems) had the following sequences: G_L and $G_L\Delta C$, 5'-TCCTTCGCCGACAACCA-3' (forward), 5'-TCGAATTCCGAGAACACTTTCA-3' (reverse); PTG and PTG-VF, 5'-CCACACAACCAAGCCAAGAAG-3' (forward), and 5'-TCTGGAAGGTCGGAGAAGACA-3' (reverse). Probes for PCR reactions had the following sequences: G_L and $G_L\Delta C$, 5'-6FAM-TGGCCCTAACAATG-MGB; PTG, 5'-

6FAM-CGGGTCGTGTTTGCGGACTC-MGB; and PTG-VF, 5'-6FAM-CGGGCCGTGGCAGCGGACTC-MGB (Applied Biosystems).

Glycogen Measurements

Glycogen content was measured by scraping cells into 30% KOH, boiling the extract for 15 minutes, and centrifuging at 3,000 rpm for 10 minutes. Glycogen in the cleared supernatant was measured as described (Newgard, Hirsch et al. 1983). Protein content of the samples was determined by method of Bradford (Bradford 1976), using Bio-Rad reagents.

Immunoprecipitation

Hepatocytes were lysed on ice in a buffer consisting of 50 mM Hepes (pH 7.4), 135 mM NaCl, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, and 0.1 mM leupeptin). Protein content of the samples was determined by method of Bradford (Bradford 1976), using Bio-Rad reagents. Cells lysates were incubated with protein A beads (Sigma) for 30 minutes at 4° C. An aliquot was saved as "start" and the remaining lysate was incubated with anti-FLAG monoclonal M2 antibodies coupled to agarose beads (Sigma) for 1 hour at 4° C. An aliquot was saved as "flow through" and the beads were washed 3 times with homogenization buffer representing the "IP" fractions. Samples were resolved on a 4-15% SDS-polyacrylamide gel and transferred onto

polyvinylidene difluoride (PVDF) membranes. Following blocking of the membranes for 1 hour in Tris-buffered saline containing 5% nonfat dry milk, the blots were incubated with anti-FLAG M2 antibody (Stratagene) at 1:1000 dilution or anti-PP-1 antibody (Santa Cruz Biotechnology) at a 1:250 dilution at 4° C overnight. Membranes were washed and subsequently treated with a secondary antibody conjugated to horseradish peroxidase and visualized using an enhanced chemiluminescence (ECL) detection kit.

Immunoblot Analyses

Hepatocytes were lysed on ice in a buffer consisting of 50 mM Hepes (pH 7.4), 135 mM NaCl, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors (2 mM PMSF, 5 μg/ml aprotinin, and 0.1 mM leupeptin). Cell lysates were centrifuged at 2,000 rpm for 10 minutes and the supernatants were collected. Protein content was determined by method of Bradford (Bradford 1976), using Bio-Rad reagents. Alliquots containing 75 mg protein were resolved on a 4-15% SDS-polyacrylamide gel and transferred onto PVDF membranes. Following blocking of the membranes for 1 hour in Tris-buffered saline containing 5% nonfat dry milk, the blots were incubated with an affinity-purified anti-phosphorylase a antibody at a 1:500 dilution (Gasa, Jensen et al. 2000), a crude antiserum that recognizes total phosphorylase levels at 1:1000 dilution (Gasa, Jensen et al. 2000), anti-glycogen synthase antibody (Cell Signaling Technology) at 1:500 dilution, or anti-γ-tubulin antibody (Sigma) at 1:1000 dilution at 4° C overnight. Membranes were washed and subsequently treated with a secondary antibody

conjugated to horseradish peroxidase and visualized using an ECL detection kit. Band areas were determined using ImageQuant TL 1D gel analysis program (Amersham Biosciences).

Enzyme Activity Assays

Hepatoctyes were lysed in homogenization buffer consisting of 10 mM Tris (pH 7.0), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mM benzamidine, and 1 mM PMSF. Protein content was determined by method of Bradford (Bradford 1976), using Bio-Rad reagents. Total glycogen synthase activity was assayed as described previously by measuring the incorporation of [^{14}C]UDP-glucose into glycogen I the presence of 6.6 mM glucose-6-phosphate (Seoane, Gomez-Foix et al. 1996). Activated glycogen phosphorylase (phosphorylase a) activity was assayed by measuring incorporation of [^{14}C]glucose-1-phosphate into glycogen as described (Coats, Browner et al. 1991).

Statistical Analysis

Data are expressed as the mean $\pm$ S.E.M. Statistical significance was determined by unpaired Student's *t* test using the statistics module of Microsoft Excel. Statistical significance was assumed at $p \leq 0.05$.

RESULTS

Expression of PTG-VF in Hepatocytes

We constructed a recombinant adenovirus containing the cDNA encoding a mutant form of PTG with alanine residues in place of V62 and F64 in the PP-1 binding site; this cDNA was cloned downstream of a FLAG sequence to facilitate specific immunoblotting of the expressed protein. Treatment of hepatocytes from fasted Wistar rats with increasing doses of AdCMV-PTG-VF resulted in a dose-dependent increase in PTG-VF mRNA levels (Figure 3.1A). Since the probe used only recognizes PTG-VF and not endogenous PTG, the lowest level of overexpression was used as the baseline measurement of mRNA levels. In separate samples of the same cells examined for mRNA expression in Figure 3.1A, anti-FLAG immunoprecipitation followed by anti-FLAG immunoblotting revealed a similar dose-dependent increase in PTG-VF protein expression (Figure 3.1B). In the absence of viral treatment, hepatocytes from fasted rats accumulated 132 $\pm$ 7 μg glycogen/mg protein during a 24 hour incubation in 20 mM glucose. Treatment with a control adenovirus, AdCMV-β-Galactosidase (AdCMV- βGal), had no effect on glycogen content compared to untreated control cells. However, expression of AdCMV-PTG-VF resulted in a dose-dependent decrease in glycogen content (Figure 3.1C) with a maximal reduction in glycogen content of 83% compared to untreated control hepatocytes at the highest viral dose.

PTG-VF is Unable to Bind PP-1

In order to determine if mutation of V62 and F64 to alanine prevents the binding of PP-1 to PTG-VF as anticipated, co-immunoprecipitation studies were performed in extracts from hepatocytes treated with AdCMV-PTG-VF-FLAG, AdCMV-PTG-FLAG, or AdCMV-βGal, using anti-FLAG M2 agarose beads followed by immunoblotting with either anti-FLAG or anti-PP-1 antibodies. As can be seen in Figure 3.2, anti-FLAG immunoblotting of the total cell lysates (start), the supernatant following IP (FT), and the immunoprecipitate (IP) samples clearly detects expression of the PTG-VF-FLAG and PTG-FLAG proteins. The anti-FLAG antibody does not detect β–galactosidase protein in any of these samples due to the lack of the FLAG epitope in these viral vectors. Levels of PP-1 in both the start and FT fractions are not altered due to expression of any of the viruses. In the anti-FLAG immunoprecipitated samples, treatment with AdCMV-PGT-FLAG, but not AdCMV-PTG-VF-FLAG resulted in co-immunoprecipitaiton of PP-1, providing a direct demonstration the PTG-VF is unable to bind PP-1.

PTG-VF Preferentially Decreases the Glycogenic Potential of G_L Compared to PTG

To further investigate the mechanism of action of PTG-VF, co-overexpression studies with PTG and G_L were performed. Treatment of hepatocytes with either AdCMV-PTG-FLAG or AdCMV-G_L-FLAG led to a dose-dependent increase in glycogen content (Figure 3.3A) with similar amount of glycogen accumulation in the two treatment groups. Using the highest viral dose characterized in Figure 3.1 (2.5×10^{12} particles) AdCMV-PTG-VF was

able to decrease the glycogenic potency of AdCMV-PTG at the lowest titer of AdCMV-PTG used by 40% (Figures 3.3A and 3.3B). However, the ability of AdCMV-PTG-VF to block glycogen accumulation in cells co-overexpressing AdCMV-PTG decreased as the titer of AdCMV-PTG increased such that only a 17% suppression of glycogen accumulation was seen at the highest titer of PTG. Interestingly, AdCMV-PTG-VF was more effective in blocking the glycogenic potential of AdCMV-G_L at all titers studies with the degree of suppression ranging from 73% to 44% (Figures 3.3A and 3.3B).

To eliminate the possibility that the differential effects of PTG-VF expression in AdCMV-PTG- and AdCMV-G_L-treated cells was due to different levels of expression of the adenovirus-encoded mRNAs, we performed real-time PCR studies. The primer/probe sets were designed to amplify both the endogenous and overexpressed PTG and G_L transcripts. Since endogenous levels of G_L are higher than those for PTG, it was necessary to use the lowest titer of virus as the baseline in order to accurately assess the extent of adenovirus-mediated overexpression. Figure 3.3C demonstrates that PTG mRNA levels were increased to a greater extent than G_L mRNA levels by adenovirus-mediated expression in hepatocytes, especially at the higher viral doses. The finding that these levels of overexpression of PTG and G_L resulted in similar levels of glycogen deposition are consistent with our prior work showing that G_L overexpression has a higher glycogenic potency in liver cells than PTG when expression levels are more closely matched (Gasa, Jensen et al. 2000; Yang, Cao et al. 2002). Nevertheless, our data still indicates a preferential suppression of glycogen synthesis by PTG-VF in G_L expressing cells, since comparison of cells with similar amounts of PTG and G_L expression (e.g. AdCMV-PTG-FLAG 5.0 x 10^{10} versus AdCMV-G_L-FLAG 1.0 x

10^{11} or AdCMV-PTG-FLAG 7.5 x 10^{10} versus AdCMV-G_L-FLAG 1.25 x 10^{11}), still indicates that PTG-VF is less effective in blocking the action of AdCMV-PTG-FLAG than AdCMV-G_L-FLAG (17% versus 44% or 29% versus 44% respectively).

Deletion of Phosphorylase a Binding Site on AdCMC-G_L Renders the Molecule Less Susceptible to Inhibition by AdCMV-PTG-VF

G_L is reported to contain a glycogen phosphorylase a binding motif at its C-terminus that allows this targeting subunit isoform to be uniquely regulated in response to levels of phosphorylase a within cells (Moorhead, MacKintosh et al. 1995). This raises the possibility that the enhanced capacity of PTG-VF to suppress glycogen synthesis in G_L expressing cells may be related to the binding of phosphorylase a to the G_L C-terminus. To test this idea, we created a recombinant adenovirus containing a cDNA encoding a truncated version of G_L in which 16 amino acids were deleted from the C-terminus, thus removing the phoshporylase a binding site (AdCMV- $G_L\Delta C$). Our hypothesis was that if allosteric regulation by phosphorylase a is responsible for the greater susceptibility of G_L to inhibition by PTG-VF, deletion of the phosphorylase a binding site should result in lesser inhibition of G_L by PTG-VF. Conversely, if the differential effects are independent of phosphorylase a binding, removal of the binding site would have little impact on the ability of AdCMV-PTG-VF to compete with AdCMV- $G_L\Delta C$. Treatment of hepatocytes with AdCMV-$G_L\Delta C$ led to a dose-dependent increase in glycogen content (Figure 3.4A). Clearly, cells treated with AdCMV-$G_L\Delta C$ are less susceptible to the effects of AdCMV-PTG-VF than cells treated with

AdCMV-G_L (Figure 3.4A and 3.4B). In fact, as is clearly illustrated in Figure 3.4B, AdCMV-$G_L\Delta C$ behaves very similar to AdCMV-PTG when co-overexpressed with AdCMV-PTG-VF suggesting that allosteric regulation of AdCMV-G_L plays an important role in the ability of AdCMV-PTG-VF to suppress its glycogenic activity.

Real time PCR was performed to determine levels of overexpression. As in the studies summarized in Figures 3.1 and 3.3, the lowest titer of virus was used as the baseline. The results indicate that AdCMV-$G_L\Delta C$ and AdCMV-G_L were expressed at similar levels (Figure 3.4C). Thus, the decreased susceptibility of $G_L\Delta C$ to PTG-VF suppression is due to the deletion of the phosphorylase a binding site and not different levels of expression.

PTG-VF Expression Increases Glycogen Phosphorylase Activity

Since allosteric regulation of AdCMV-G_L by phosphorylase a appears to play a major role in the action of AdCMV-PTG-VF, we next determined if AdCMV-PTG-VF altered phosphorylase protein or activity levels. Total phosphorylase protein levels were not impacted by any of the viral treatments as determined by immunoblot (Figures 3.5A and 3.5B). In contrast, expression of PTG or $G_L\Delta C$, but not native G_L, led to a decrease in immunodetectable phosphorylase a levels (Figure 3.5C). Interestingly, treatment of cells with AdCMV-PTG-VF, either alone or in combination with any of the other adenoviruses, caused a trend towards an increase in phosphorylase a levels. These data are supported by assays of glycogen phosphorylase activity in extracts form the same cells (Figure 3.5D). Thus, AdCMV-PTG and AdCMV- $G_L\Delta C$ treatment led to a significant decrease in

phosphorylase activity compared to untreated control cells (1.35 $\pm$ 0.05 or 1.24 $\pm$ 0.05 versus 1.68 $\pm$ 0.09 p=0.003 and p=0.0001 respectively) which would favor glycogen synthesis, whereas AdCMV-G_L expression did not alter phosphorylase activity. Overexpression of AdCMV-PTG-VF, either alone or in combination with any of the other adenoviruses, led to an increase in glycogen phosphorylase activity, which would favor glycogen degradation. Taken together, these data indicate that overexpressed G_L is less effective at deactivating phosphorylase a and decreasing glycogen phosphorylase activity that PTG. However, deletion of the C-terminus of G_L, thought to constitute a phosphorylase a binding domain, produces a truncated molecule with a capacity for inactivation of phosphorylase approximately equal to that of PTG. Finally, PTG-VF appears to cause increases in phosphorylase a levels and phosphorylase enzyme activity in all of the cells groups summarized in Figure 3.5.

PTG-VF Has Little Impact on Glycogen Synthase Activity

Net glycogen depostition or degradation is determined by the balance between glycogen phosphorylase and glycogen synthase activities. Thus, we next sought to determine if AdCMV-PTG-VF altered glycogen synthase protein levels or enzymatic activity. Immunoblotting with total glycogen synthase antibody shows that expression of AdCMV-PTG, AdCMV-G_L, or AdCMV-$G_L\Delta C$ all increased glycogen synthase protein levels (Figures 3.6A and 3.6B) although this increase did not quite achieve statistical significance (p=0.065) in cells treated with AdCMV-PTG. In contrast, overexpression of AdCMV-PTG-VF had no

significant impact on glycogen synthase protein levels. These results were again supported by measurement of glycogen synthase activity (Figure 3.6C). Thus, hepatocytes treated with AdCMV-PTG, AdCMV-G_L, or AdCMV-$G_L\Delta C$ all contained increased synthase activity compared to untreated control hepatocytes (0.82 ± 0.03, 1.08 ± 0.03, and 1.06 ± 0.05 versus 0.71 ± 0.03 respectively) which would favor glycogen synthesis. Co-treatment of cells treated with AdCMV-PTG, AdCMV-G_L, or AdCMV-$G_L\Delta C$ with AdCMV-PTG-VF had no impact on glycogen synthase activity. Taken together, the data summarized in Figures 3.5 and 3.6 suggest that the main mechanism by which PTG-VF inhibits glycogen synthesis is via an increase in glycogen phosphorylase activity rather than by a decrease in glycogen synthase activity.

DISCUSSION

The liver plays a key role in control of fuel homestasis. During the fasted-to-fed transition the liver converts glucose and other precursors to glycogen for storage, whereas in the fed-to-fasted transition, glycogen is degraded to produce free glucose. The key enzymes of glycogen metabolism, glycogen synthase and glycogen phosphorylase, are reciprocally regulated in response to hormonal and metabolic signals. Phosphorylation of glycogen synthase under fasted conditions occurs at a cluster of serine residues, primarily catalyzed by PKA and glycogen synthase kinase-3, leading to progressive inactivation (Lawrence and Roach 1997). Under the same conditions, glycogen phosphorylase is phosphorylated on ser 14 by phosphorylase kinase, resulting in its activation and net glycogen degradation

(Newgard, Hwang et al. 1989). Conversely, during the fed-to-fasted transition, both glycogen synthase and phosphorylase are dephosphorylated, primarily by protein phosphatase 1, resulting in the activation of the former and inactivation of the latter, with a net stimulation of glycogen synthesis (Newgard, Hwang et al. 1989; Ferrer, Favre et al. 2003).

Glycogen targeting subunits not only bring the enzymes of glycogen metabolism together in one location, but they also exert some control over glycogen metabolism. PTG has been found to inactivate glycogen phosphorylase, stimulate the translocation of glycogen synthase to the glycogen particle, and activate glycogen synthase through the localization of PP-1 (Green, Aiston et al. 2004). G_L appears to function primarily as a facilitator of synthase phosphatase activity (Browne, Delibegovic et al. 2001) and is negatively regulated in this function through allosteric binding of phosphorylase a to its C-terminus (Moorhead, MacKintosh et al. 1995). Since the various glycogen targeting subunits appear to contribute differentially to glycogen metabolism and several tissues contain more than one glycogen targeting subunit, it is unlikely that knock out of one isoform (Suzuki, Lanner et al. 2001; Crosson, Khan et al. 2003) can definitively determine the importance of glycogen targeting subunits in the control of fuel homeostasis.

In an attempt to address this issue, we created a dominant-negative glycogen targeting subunit, PTG-VF, which is unable to bind to PP-1 yet retains its ability to interact with glycogen synthase and glycogen phosphorylase. Overexpression of PTG-VF in isolated hepatocytes dramatically reduced glycogen content, with a maximal reduction of 83%. This suggests that the activity of endogenous glycogen targeting subunits is essential for the

synthesis of glycogen and that PTG-VF functions as a pan-specific dominant-negative glycogen targeting subunit. However, further studies involving co-expression of PTG-VF with PTG or G_L indicated that PTG-VF was more effective at blocking glycogenesis stimulated by G_L than PTG. Since PTG has been shown to decrease phosphorylase a levels (Green, Aiston et al. 2004), we hypothesized that PTG-VF may increase phosphorylase a levels and therefore, preferentially impact G_L due to its C-terminal phosphorylase a binding site (Moorhead, MacKintosh et al. 1995) that is absent from PTG (Figure 3.7). To test this idea, we created a truncated version of G_L lacking its 16 C-terminal amino acids ($G_L\Delta C$), where the phosphorylase a binding site was deleted. $G_L\Delta C$ was less susceptible to inhibition by PTG-VF than native G_L, suggesting that the allosteric regulation of G_L played a critical role in the differential impact of PTG-VF on PTG and G_L.

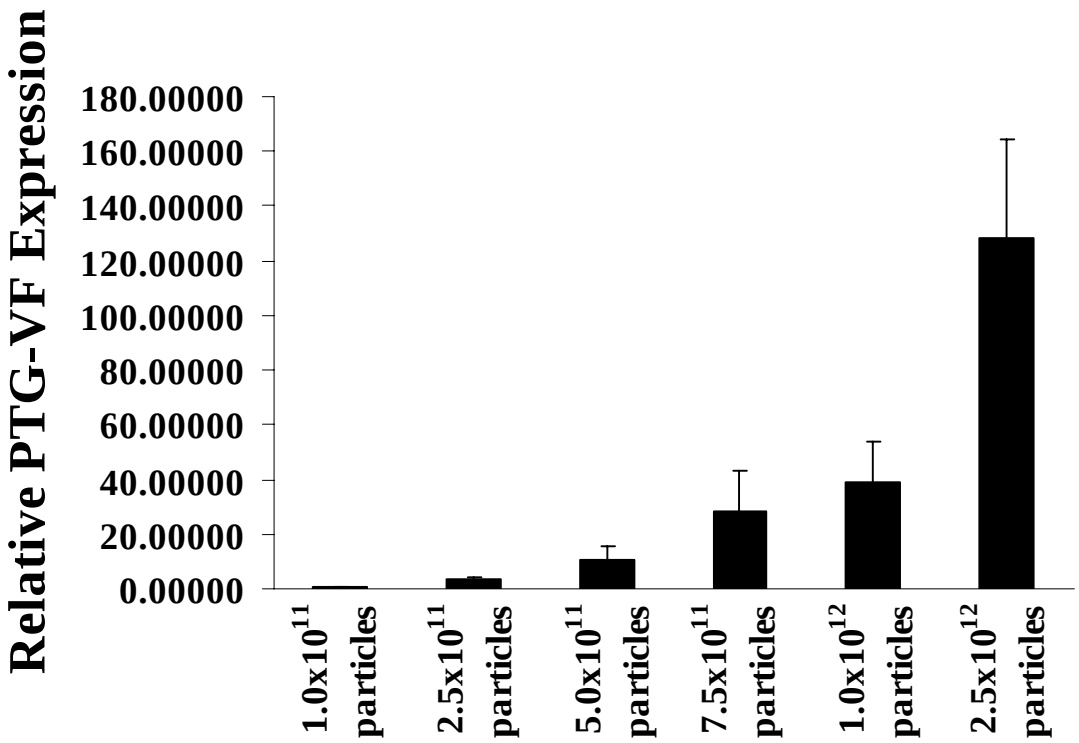
Since the above hypothesis is dependent on PTG-VF increasing phosphorylase a levels and/or activity, we measured both protein levels and activity of phosphorylase in cell lysates. We found that PTG-VF overexpression modestly increased phosphorylase a protein levels, but significantly increased the activity of this enzyme even when PTG or G_L are co-overexpressed. Increased phosphorylase activity would shift the balance of glycogen metabolism towards glycogen degradation in response to PTG-VF overexpression.

We also studied the impact of PTG-VF expression on glycogen synthase protein and activity levels. While PTG, G_L, and $G_L\Delta C$ all increased glycogen synthase protein and activity levels, PTG-VF had little impact on glycogen synthase. Thus, it is unlikely that glycogen synthase plays a large role in the activity of PTG-VF.

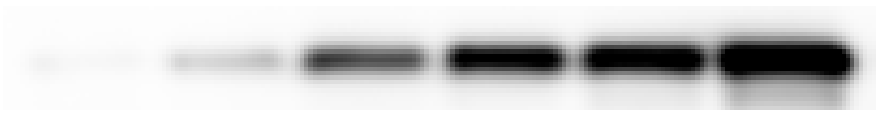
In summary, we have created a dominant-negative glycogen targeting subunit that drastically decreases glycogen content of hepatocytes. While this mutant appeared to be a pan-specific molecule, further studies found that PTG-VF preferentially suppressed glycogen synthesis stimulated by G_L compared to PTG. This is due to the fact that the main mechanism of action of PTG-VF is via an increase in glycogen phosphorylase activity rather than by a decrease in glycogen sythase activity. These studies demonstrate that glycogen targeting subunits play a critical role in control of glycogen metabolism. They also provide a potential rationale for expression of multiple glycogen targeting subunits within individual tissues, as this appears to afford differential capacities to interact with specific glycogenolytic versus glycogenic enzymes.

FIGURES

A


Relative PTG-VF Expression
180.00000
160.00000
140.00000
120.00000
100.00000
80.00000
60.00000
40.00000
20.00000
0.00000
1.0x10^11 particles
2.5x10^11 particles
5.0x10^11 particles
7.5x10^11 particles
1.0x10^12 particles
2.5x10^12 particles

B



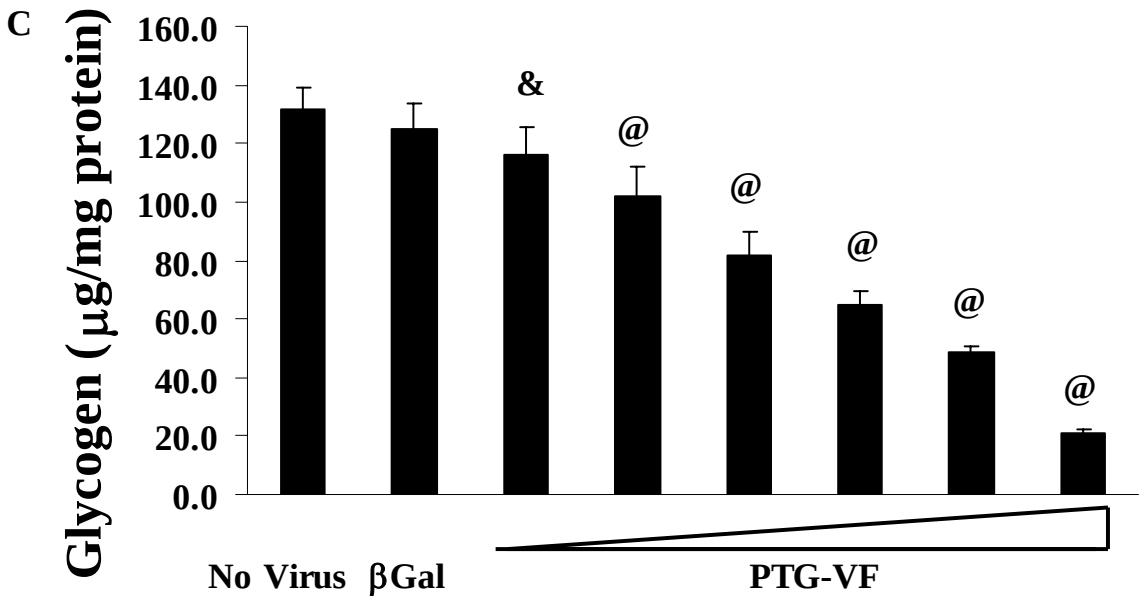


Figure 3.1. PTG-VF overexpression leads to decreased glycogen content in primary hepatocytes. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal or PTG-VF. After exposure to the virus for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Thereafter, cells were collected for measurement of glycogen content, RNA isolation for real time PCR, or anti-FLAG immunoprecipitation followed by anti-FLAG immunoblotting. Control hepatocytes were cultured under the same conditions without adenovirus treatment. A. Glycogen content of primary hepatocytes. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbols & and @ denote differences between no virus control hepatocytes and treatment with PTG-VF at levels of significance of $p \leq 0.05$ and $p \leq 0.001$ respectively. B. Relative PTG-VF mRNA expression in primary hepatoctyes. All data are compared to the amount of mRNA detected for the lowest titer of PTG-VF expressed. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbol & denotes differences between baseline (1.0×10^{11}) and the current titer at a level of significance of $p \leq 0.05$. C. Representative anti-FLAG immunoblot following anti-FLAG immunoprecipitation.

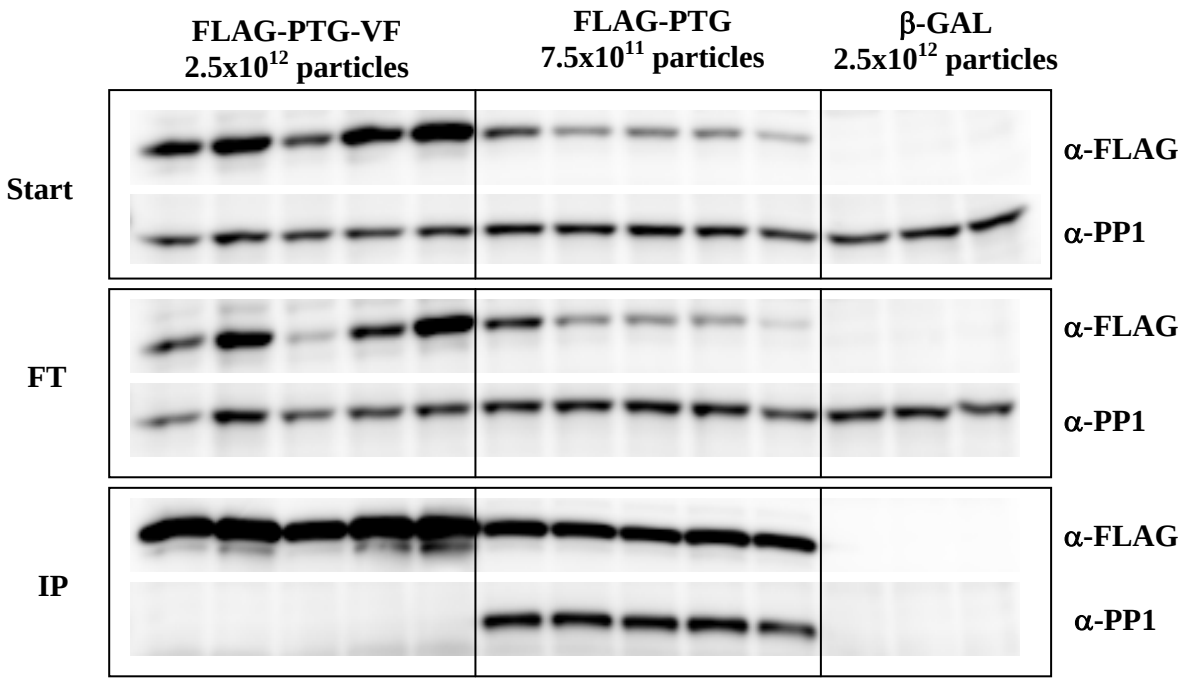
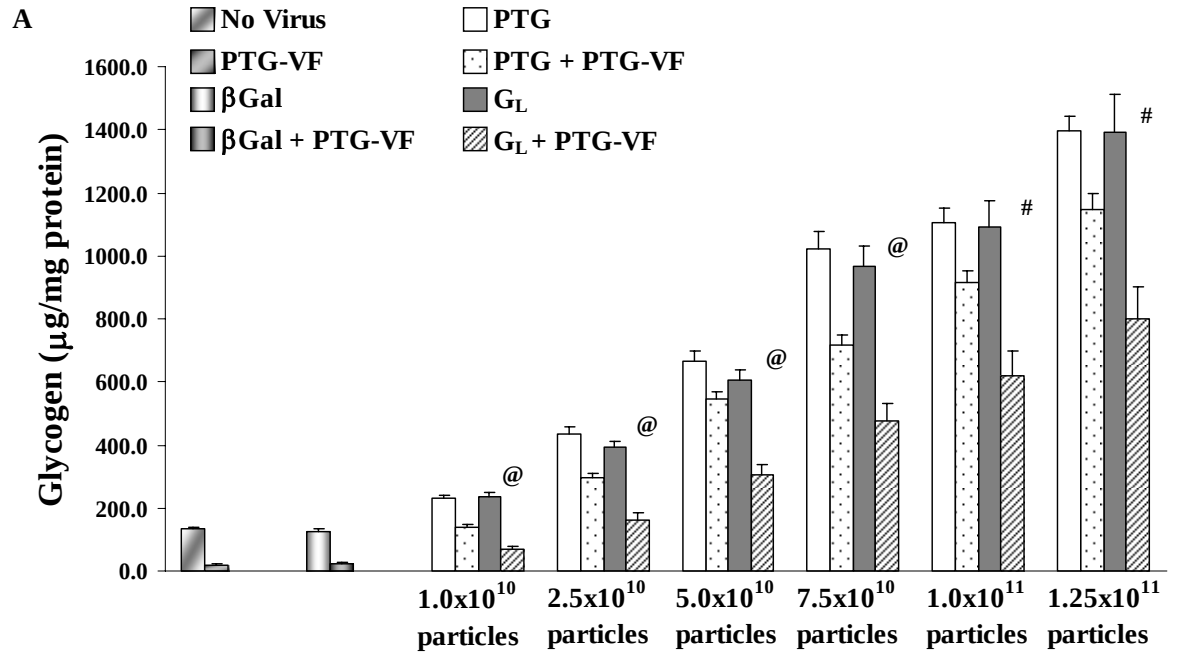
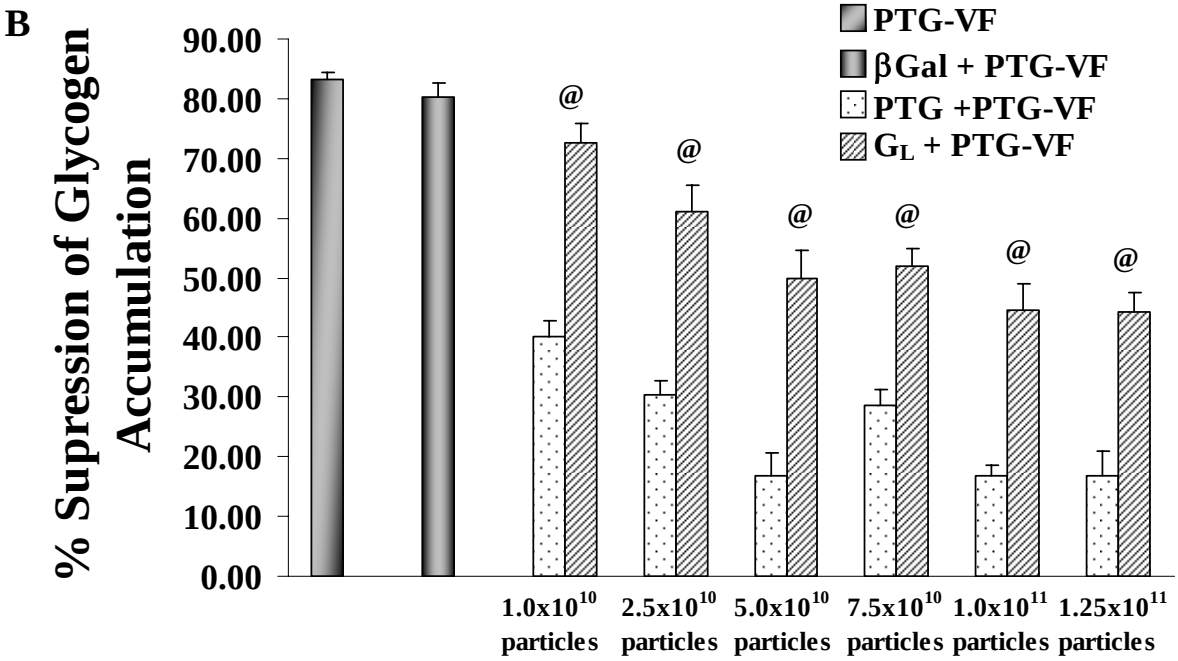


Figure 3.2. PTG-VF does not bind PP-1. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal, FLAG-PTG, or FLAG-PTG-VF. After exposure to these viruses for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Cells were harvested and anti-FLAG immunoprecipitations (IP) were performed. Samples were then analyzed by anti-FLAG and anti-PP-1 immunoblotting. Start, total cell lysate; FT, supernatant following pull down with anti-FLAG beads (Flow Through); IP, proteins that bound to the anti-FLAG beads; α-FLAG, lanes subjected to immunoblot with anti-FLAG antibody; α-PP-1, lanes subjected to immunoblot with anti-PP-1 antibody.


A
No Virus
PTG
PTG-VF
PTG + PTG-VF
βGal
G_L
βGal + PTG-VF
G_L + PTG-VF
Glycogen (μg/mg protein)
1600.0
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
@
#
1.0x10^10 particles
2.5x10^10 particles
5.0x10^10 particles
7.5x10^10 particles
1.0x10^11 particles
1.25x10^11 particles


B
% Supression of Glycogen Accumulation
PTG-VF
βGal + PTG-VF
PTG +PTG-VF
G_L + PTG-VF
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
@
1.0x10^10 particles
2.5x10^10 particles
5.0x10^10 particles
7.5x10^10 particles
1.0x10^11 particles
1.25x10^11 particles

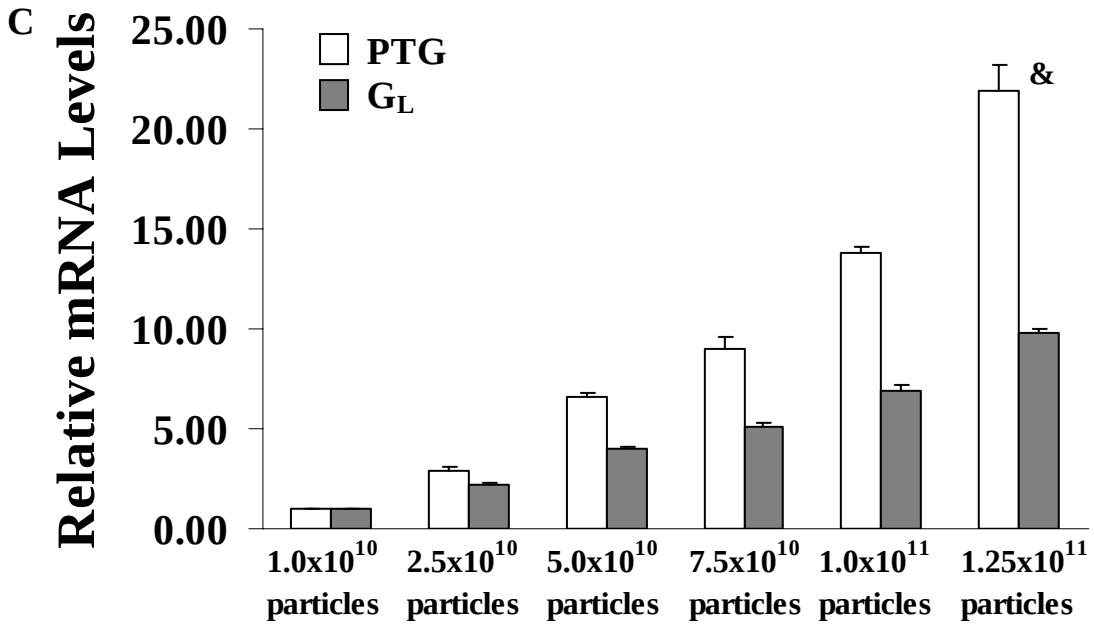
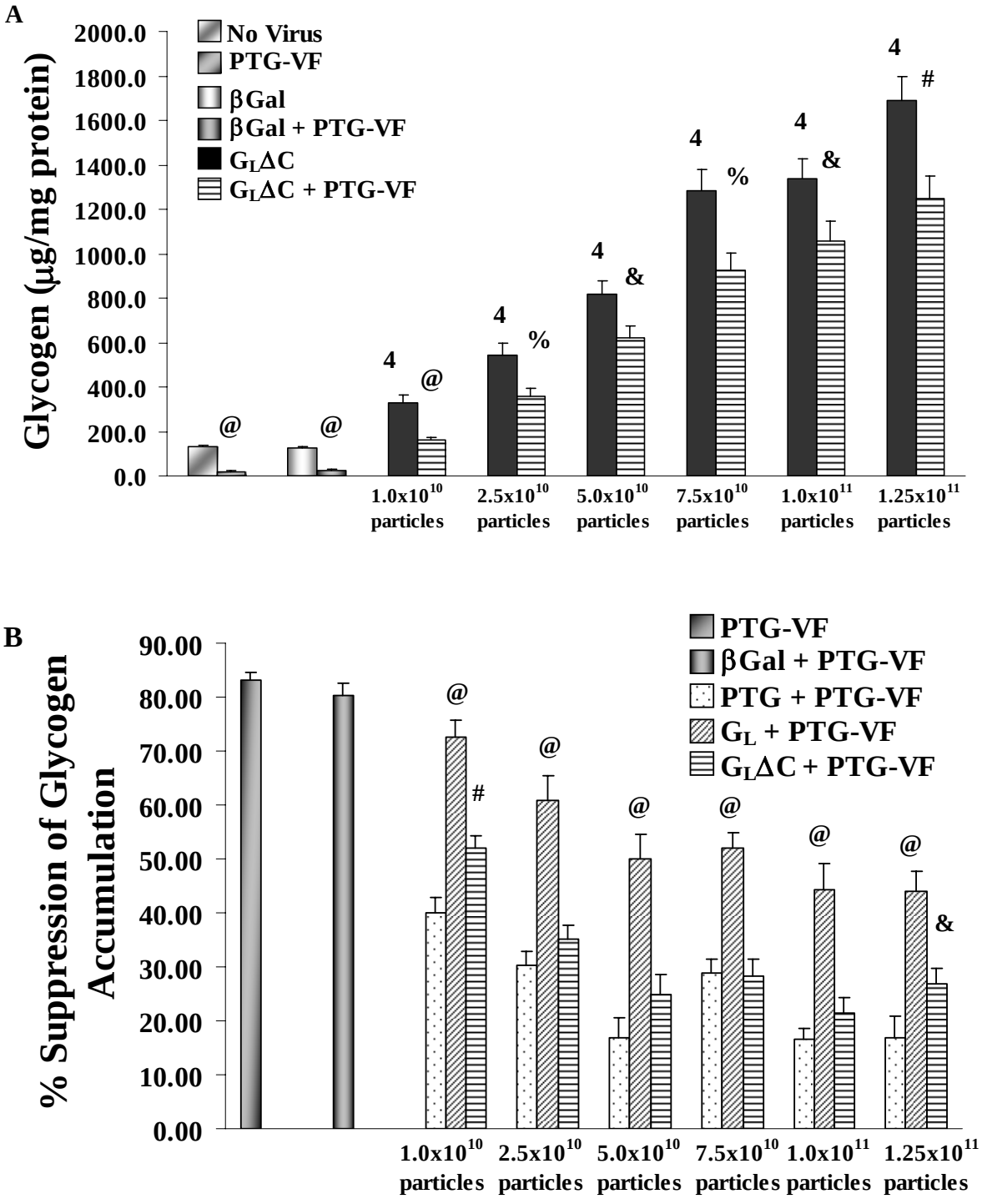


Figure 3.3. PTG-VF is more effective in blocking the glycogenic potential of G_L than PTG. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal, PTG, G_L, and/or PTG-VF. After exposure to the virus for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Thereafter, cells were collected for measurement of glycogen content or isolation of RNA for real time PCR. Control hepatocytes were cultured under the same conditions without adenovirus treatment. A. Glycogen content of primary hepatocytes. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbols # and @ denote differences between hepatocytes treated with PTG + PTG-VF and cells treated with G_L + PTG-VF at levels of significance of $p \leq 0.005$ and $p \leq 0.001$ respectively. B. Percent suppression of glycogen accumulation. The data are presented as the difference between cells treated with PTG alone and PTG + PTG-VF or G_L alone and G_L + PTG-VF. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbol @ denotes differences between hepatocytes treated with PTG + PTG-VF and cells treated with G_L + PTG-VF at levels of significance of $p \leq 0.001$. C. Relative expression of PTG and G_L. All data are compared to the amount of mRNA detected for the lowest titer of virus expressed. Data represent the mean $\pm$ SEM of 5 experiments, each performed in triplicate. The symbol & denotes differences between PTG and G_L treated hepatocytes at a level of significance of $p \leq 0.05$.


A
Glycogen (μg/mg protein)
2000.0
1800.0
1600.0
1400.0
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
No Virus
PTG-VF
βGal
βGal + PTG-VF
GLΔC
GLΔC + PTG-VF
1.0x10^10 particles
2.5x10^10 particles
5.0x10^10 particles
7.5x10^10 particles
1.0x10^11 particles
1.25x10^11 particles
B
% Suppression of Glycogen Accumulation
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
PTG-VF
βGal + PTG-VF
PTG + PTG-VF
GL + PTG-VF
GLΔC + PTG-VF
1.0x10^10 particles
2.5x10^10 particles
5.0x10^10 particles
7.5x10^10 particles
1.0x10^11 particles
1.25x10^11 particles

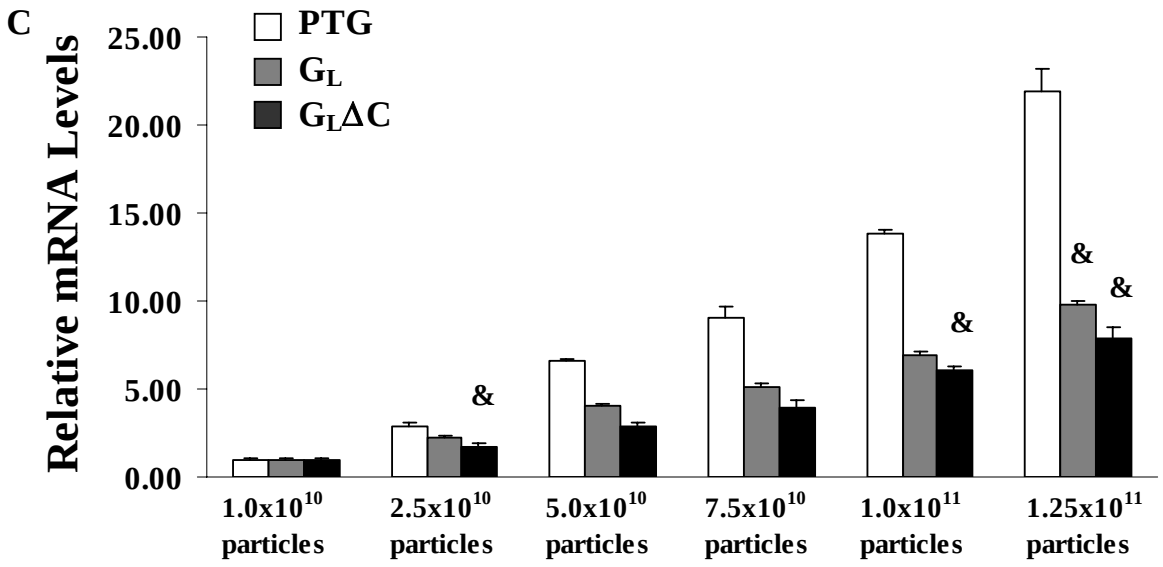
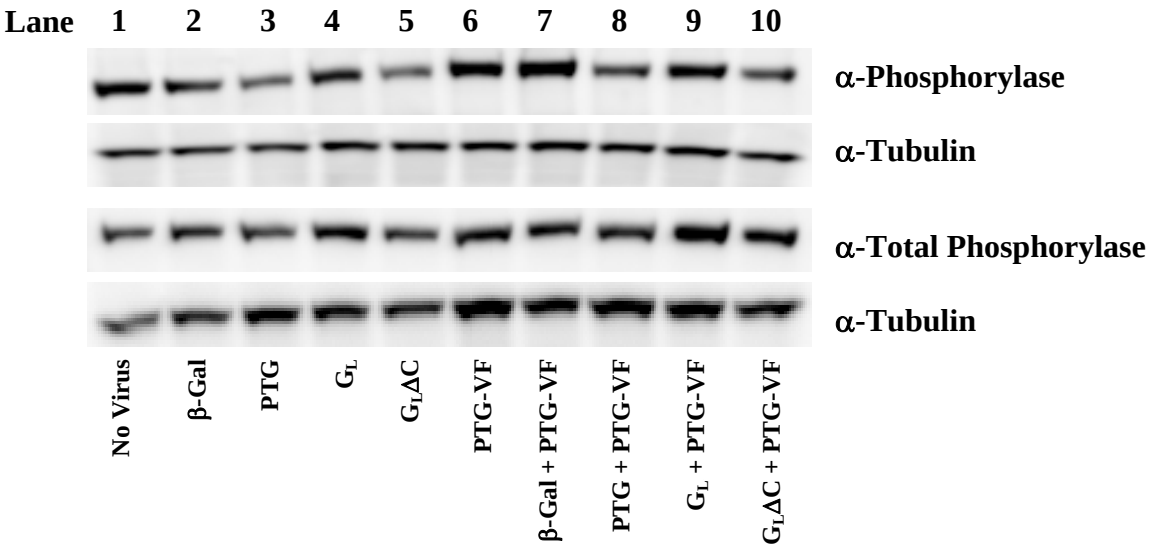
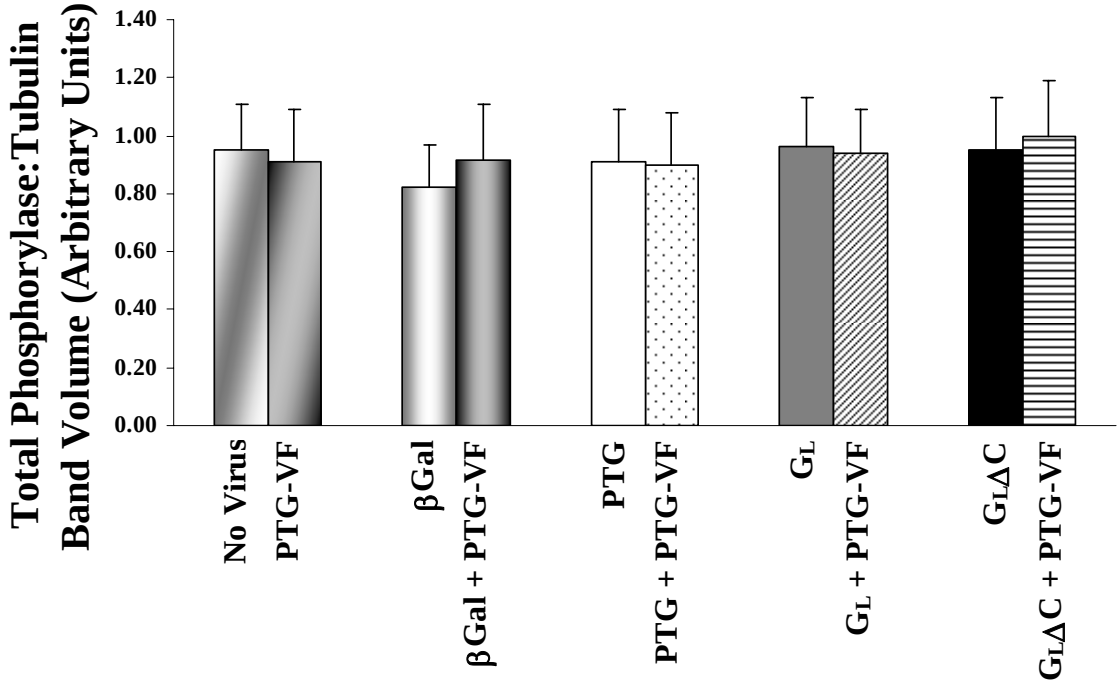


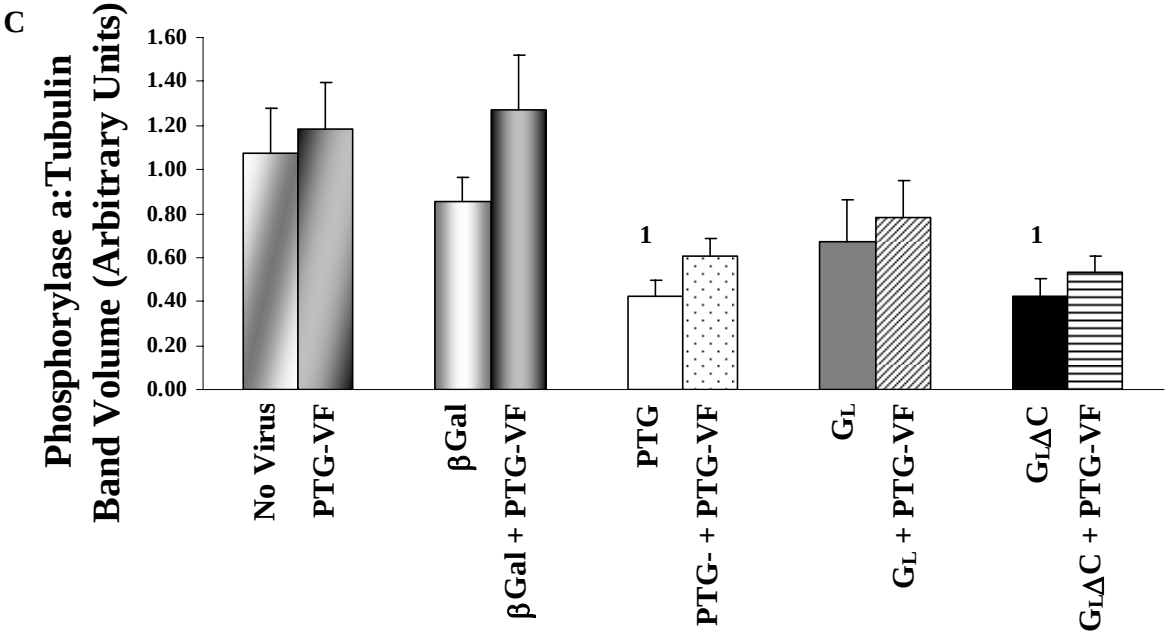
Figure 3.4. Deletion of phosphorylase a binding site on G_L renders the glycogen targeting subunit less susceptible to inhibition by PTG-VF. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal, PTG, G_L, $G_L\Delta C$, and/or PTG-VF. After exposure to the virus for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Thereafter, cells were collected for measurement of glycogen content or isolation of RNA for real time PCR. Control hepatocytes were cultured under the same conditions without adenovirus treatment. A. Glycogen content of primary hepatocytes. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbol 4 denotes differences between no virus control cells and GLDC treated cells at levels of significance of $p \leq 0.001$. The symbols @, #, %, and & represent differences between cells treated with $G_L\Delta C$ and cells treated with $G_L\Delta C$ + PTG-VF at a levels of significance of $p \leq 0.001$, $p \leq 0.005$, $p \leq 0.01$, and $p \leq 0.05$ respectively. B. Percent suppression of glycogen accumulation. The data are presented as the difference between cells treated with PTG alone and PTG + PTG-VF, G_L alone and G_L + PTG-VF, and $G_L\Delta C$ alone and $G_L\Delta C$ + PTG-VF. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbol @ denotes differences between hepatocytes treated with GL versus PTG and $G_L\Delta C$ treated cells at a level of significance of $p \leq 0.001$. The symbols # and & denote differences between PTG and $G_L\Delta C$ treated cells at a level of significance of $p \leq 0.005$ and $p \leq 0.05$ respectively. C. Relative expression of PTG, GL, and GLΔC. All data are compared to the amount of mRNA detected for the lowest titer of virus expressed. Data represent the mean $\pm$ SEM of 5 independent experiments, each performed in triplicate. The symbol & denotes differences between PTG and G_L or PTG and $G_L\Delta C$ treated hepatocytes at a level of significance of $p \leq 0.05$.

A


Lane 1 2 3 4 5 6 7 8 9 10
α-Phosphorylase
α-Tubulin
α-Total Phosphorylase
α-Tubulin
No Virus
β-Gal
PTG
GL
GLΔC
PTG-VF
β-Gal + PTG-VF
PTG + PTG-VF
GL + PTG-VF
GLΔC + PTG-VF

B


Total Phosphorylase:Tubulin Band Volume (Arbitrary Units)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
No Virus
PTG-VF
βGal
βGal + PTG-VF
PTG
PTG + PTG-VF
GL
GL + PTG-VF
GLΔC
GLΔC + PTG-VF


C
Phosphorylase a:Tubulin
Band Volume (Arbitrary Units)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
1
No Virus
PTG-VF
βGal
βGal + PTG-VF
PTG
PTG- + PTG-VF
GL
GL + PTG-VF
GLΔC
GLΔC + PTG-VF

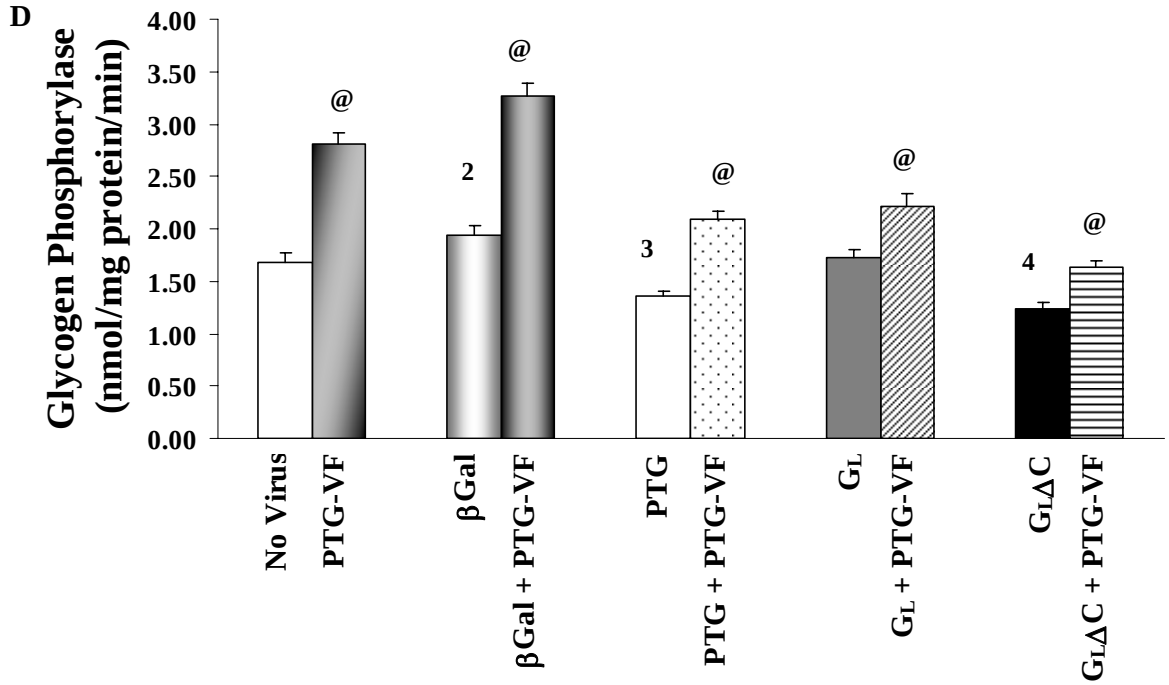
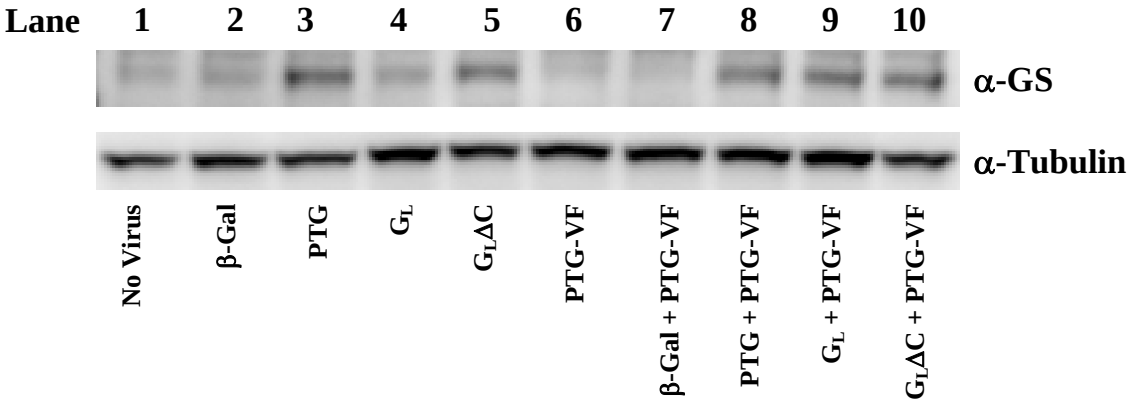
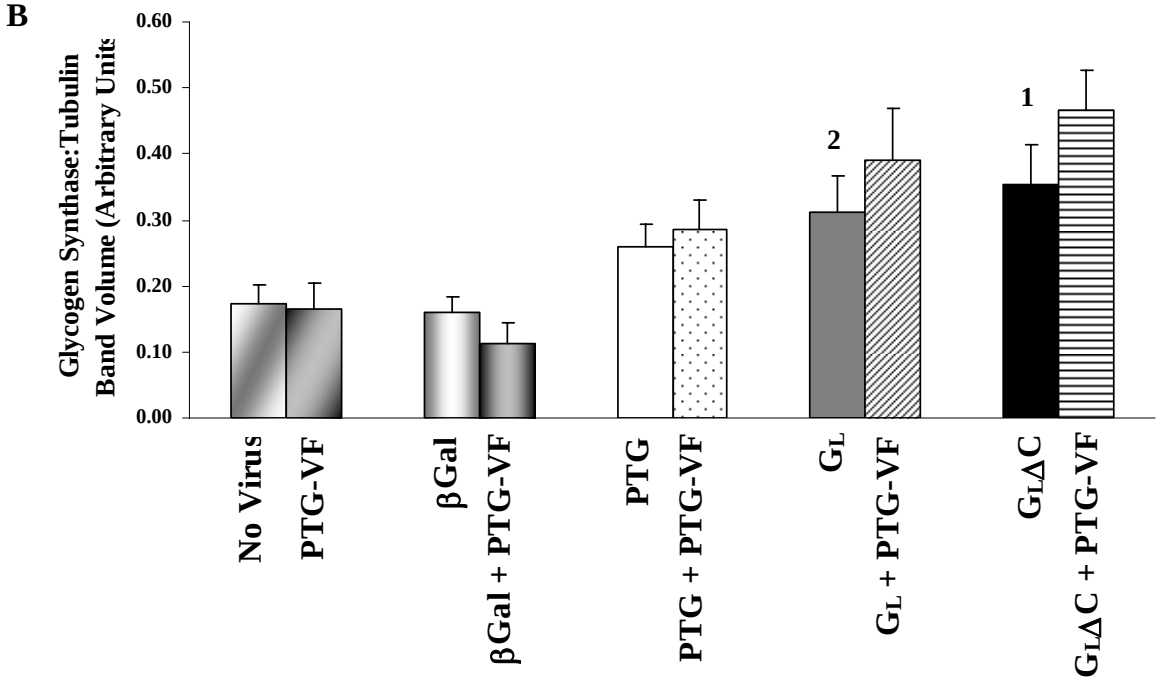

D
Glycogen Phosphorylase
(nmol/mg protein/min)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
@
@
@
@
@
2
3
4
No Virus
PTG-VF
βGal
βGal + PTG-VF
PTG
PTG + PTG-VF
GL
GL + PTG-VF
GLΔC
GLΔC + PTG-VF

Figure 3.5. PTG-VF increases phosphorylase a activity. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal, PTG, G_L, $G_L\Delta C$, and/or PTG-VF. After exposure to the virus for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Thereafter, cells were collected for immunoblotting or measurement of glycogen phosphorylase activity. A. Representative immonoblots. α-phosphorylase a, samples subjected to immunoblot with anti-phosphorylase a antibody; α - total phosphorylase, samples subjected to immunoblot with anti-total phosphorylase antibody (recognizes phosphorylase a and b); α -tubulin, samples subjected to immunoblot with anti-tubulin antibody. B. Volumes of bands on immunoblots from 5 independent experiments run in duplicate were obtained and are represented graphically as the ratio of total phosphorylase to tubulin. The data are presented as mean $\pm$ SEM. C. Volumes of bands on immunoblots from 5 independent experiments run in duplicate were obtained and are represented graphically as the ratio of phosphorylase a to tubulin. The data are presented as mean $\pm$ SEM. The symbol 1 denotes differences between untreated control hepatocytes and cells treated with either PTG or $G_L\Delta C$ at a level of significance of $p \leq 0.01$. D. Glycogen phosphorylase enzyme activity. The data are presented as mean $\pm$ SEM of 5 independent experiments, each run in triplicate. The symbols 2, 3, and 4 denote differences between untreated control hepatocytes and cells treated with β-Gal, PTG, or $G_L\Delta C$ at a level of significance of $p \leq 0.05$, $p \leq 0.005$, $p \leq 0.001$ respectively. The symbol @ denotes differences between no virus control cells or cells treated with a single adenovirus versus cells treated with PTG-VF alone or in combination at a level of significance of $p \leq 0.001$.

A


Lane 1 2 3 4 5 6 7 8 9 10
α-GS
α-Tubulin
No Virus
β-Gal
PTG
GL
GLΔC
PTG-VF
β-Gal + PTG-VF
PTG + PTG-VF
GL + PTG-VF
GLΔC + PTG-VF

B


Glycogen Synthase:Tubulin
Band Volume (Arbitrary Units)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
2
No Virus
PTG-VF
βGal
βGal + PTG-VF
PTG
PTG + PTG-VF
GL
GL + PTG-VF
GLΔC
GLΔC + PTG-VF

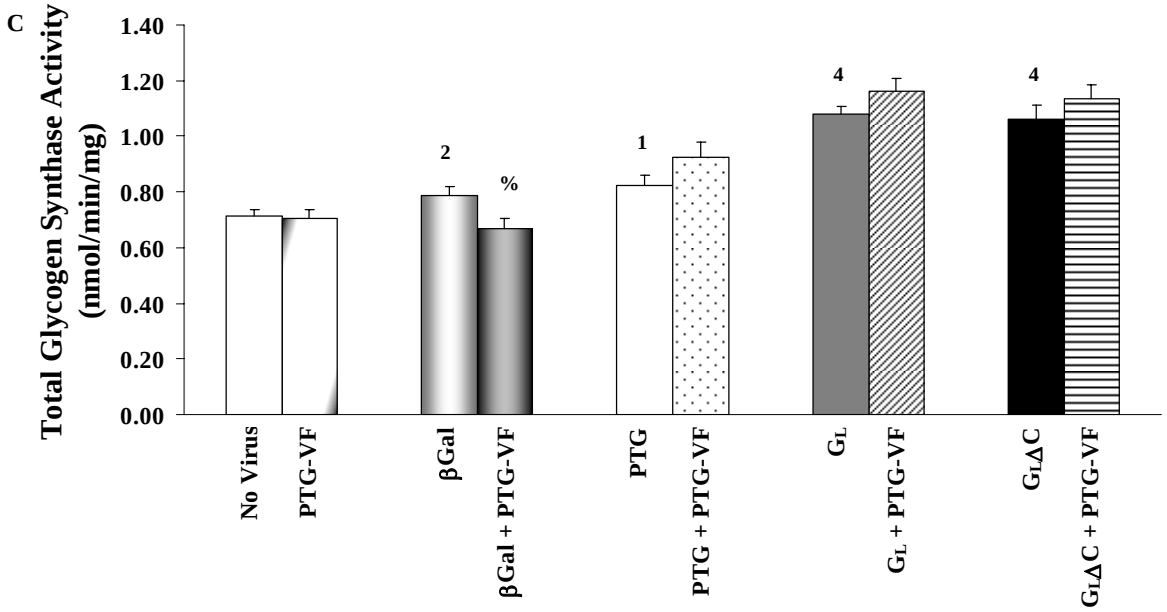


Figure 3.6. PTG-VF overexpression has little impact on glycogen synthase activity. Hepatocytes were prepared from fasted male Wistar rats and treated with adenoviruses encoding β-Gal, PTG, G_L, $G_L\Delta C$, and/or PTG-VF. After exposure to the virus for 90 minutes, cells were cultured for 24 hours in the presence of 20 mM glucose. Thereafter, cells were collected for immunoblotting or measurement of glycogen phosphorylase activity. A. Representative immunoblots. α-GS, samples subjected to immunoblot with anti-glycogen synthase antibody; α -tubulin, samples subjected to immunoblot with anti-tubulin antibody. B. Volumes of bands on immunoblots from 5 independent experiments run in duplicate were obtained and are represented graphically as the ratio of glycogen synthase to tubulin. The data are presented as mean $\pm$ SEM. The symbol 1 denotes differences between untreated control hepatocytes and cells treated with $G_L\Delta C$ at a level of significance of $p \leq 0.01$. The symbol 2 denotes differences between untreated control hepatocytes and cells treated with G_L at a level of significance of $p \leq 0.05$. C. Total glycogen synthase enzyme activity. The data are presented as mean $\pm$ SEM of 5 independent experiments, each run in triplicate. The symbols 1, 2, and 4 denote differences between untreated control hepatocytes and cells treated with PTG, β-Gal, G_L, or $G_L\Delta C$ at a level of significance of $p \leq 0.01$, $p \leq 0.05$, $p \leq 0.001$ respectively. The symbol % denotes differences between β-Gal treated control cells and cells treated with β-Gal + PTG-VF at a level of significance of $p \leq 0.01$.

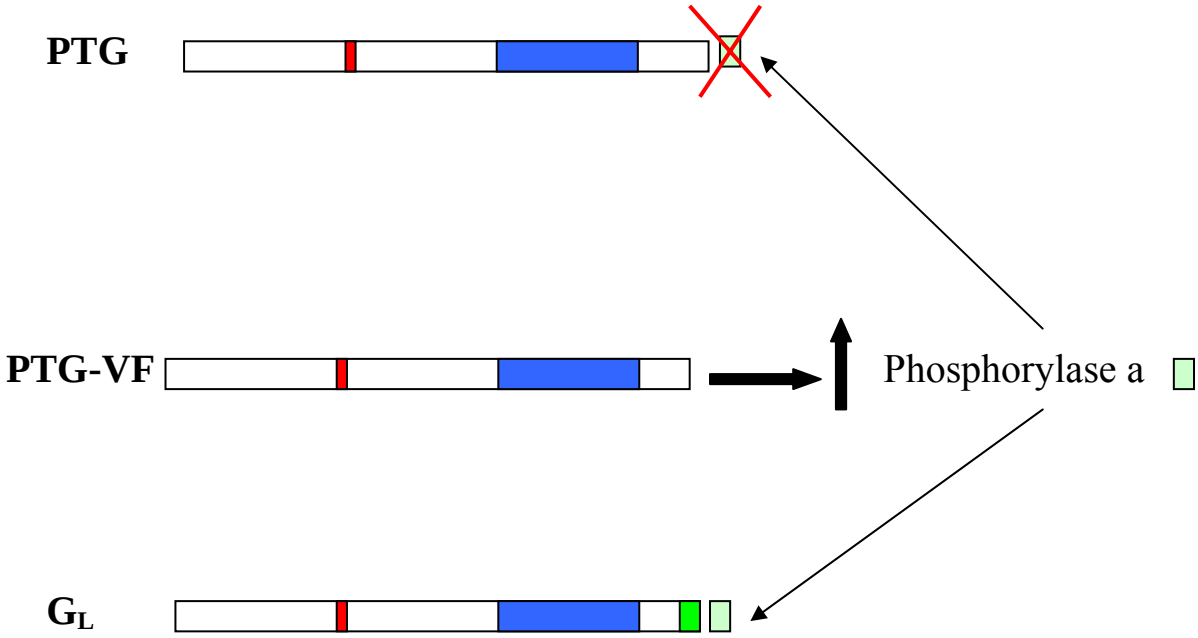


Figure 3.7. Model of PTG-VF mechanism of action. PTG-VF increases phosphorylase a levels which can bind to and allosterically regulate G_L. Increased phosphorylase a content does not impact the activity of PTG due to the lack of a phosphorylase a binding site.

CHAPTER FOUR

Adenoviral Overexpression of PTG-VF in Rats Decreases Liver Glycogen Content

INTRODUCTION

In diabetes the highly regulated control of hepatic glucose metabolism is disrupted, leading to inappropriately high levels of hepatic glucose production. Glycogen targeting subunits of protein phosphatase 1 have been shown to play critical roles in the regulation of glycogen metabolism and levels of these subunits have been found to be altered in diabetic states. Insulin-dependent diabetic rats have been shown to have decreased levels of the liver isoform, G_L (Doherty, Cadefau et al. 1998; Browne, Delibegovic et al. 2001). Levels of PTG were also decreased in livers of insulin-dependent diabetic rats and treatment with insulin restored both G_L and PTG protein levels and activities. PPP1R6, also expressed in livers, was not found to be altered in these studies (Browne, Delibegovic et al. 2001). Interestingly, hepatocytes from Zucker fa/fa rats expressed normal amounts of liver G_L and PTG, but had decreased concentrations of PPP1R6 (Arden, Green et al. 2006). The regulation of a newly discovered isoform, PPP1R3E by insulin has been shown (Munro, Ceulemans et al. 2005).

Knockout studies have also indicated that glycogen targeting subunits exert some control over glycogen metabolism. Deletion of the skeletal muscle isoform, G_M, decreases skeletal glycogen content by 90% (Suzuki, Lanner et al. 2001; Delibegovic, Armstrong et al. 2003). In one study the reduced skeletal muscle glycogen stores did not impact glycemia (Suzuki, Lanner et al. 2001) while the results from the other study showed that the animals developed glucose intolerance with age (Delibegovic, Armstrong et al. 2003). PTG heterozygous mice

exhibited decreased glycogen stores in liver, skeletal muscle, heart, and adipose tissue (Crosson, Khan et al. 2003). Glucose intolerance, insulin resistance, and hyperinsulinemia all developed in the PTG heterozygous mice with age.

Taken together these results indicate that glycogen targeting subunits exert profound effects on glycogen metabolism and ultimately fuel homeostasis. However, elimination of a single glycogen targeting subunit isoform does not allow appreciation of the full import of these molecules since several tissues express more than one of the family members. In the previous chapter we described a dominant-negative glycogen targeting subunit, PTG-VF, which was able to decrease the glycogen content of hepatocytes by 83%. The results from this study indicate that the activity of endogenous glycogen targeting subunits is necessary for glycogen metabolism. The initial results suggested that PTG-VF was a pan-specific dominant-negative glycogen targeting subunit, however further studies indicated that PTG-VF preferentially blocked the glycogenic action of G_L relative to PTG. Nevertheless, given the effective block of glycogen accumulation in normal hepatocytes, these findings led us, in the current study, to study the impact of PTG-VF expression in whole animals. We studied animals fed on standard chow and a high fat diet. The latter studies were conducted to determine if suppression of glycogen targeting subunit activity would be sufficient to drive animals from glucose intolerant states to frank diabetes. We find that PTG-VF is able to suppress glycogen accumulation in rats fed on standard chow or a high fat diet, but to a much lesser extent than in hepatocytes. This level of suppression of glycogen accumulation was not sufficient to cause significant changes in glucose homeostasis.

MATERIALS AND METHODS

Animal Maintanence

All procedures were carried out in accordance with animal care guidelines of Duke University and the National Research Council. Male Wistar rats (Harlan Tekland Laboratory, Winfield, IA) 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 (45% fat, 35% carbohydrate, 20% protein; Research Diets Inc. diet 1245) unless otherwise specified. The rats were housed under these conditions for eleven weeks before adenovirus administration.

Administration of Recombinant Adenoviruses

The preparation and testing of recombinant adenoviruses containing the cDNAs encoding PTG-VF or β-galactosidase (AdCMV-βGal) have been described previously (Chapter 3 and Herz *et al.* 1993). Aliquots of these viruses were amplified and purified for the current study as described previously (Becker *et al.* 1994). Between 1.0 and 2.0 x 10^{12} recombinant adenovirus particles were administered via tail vein injection to rats anesthetized with Nembutal (50 μg/g of body weight intraperitoneally; Abbott Laboratories, North Chicago, IL) as described elsewhere (O'Doherty *et al.* 1999 and Buettner *et al.* 2000). 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-PTG-VF 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 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-PTG-VF 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 of body weight intraperitoneally) and administration of a bolus of 2 g of glucose/kg of body weight by gavage of a 45% solution of glucose in water. Blood samples (~ 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.

Measurement of Glycogen-targeting Subunit Expression in Liver by Immunoprecipitation

Hepatocytes were lysed on ice in a buffer consisting of 50 mM Hepes (pH 7.4), 135 mM NaCl, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors (2 mM PMSF, 5 μg/ml aprotinin, and 0.1 mM leupeptin). Protein content of the samples was determined by method of Bradford (23), using Bio-Rad reagents. Cell lysates were incubated with protein A beads (Sigma) for 30 minutes at 4° C. Samples were centrifuged at 2,000 rpm for 2 minutes and then the supernatant was incubated with anti-FLAG monoclonal M2 antibodies coupled to agarose beads (Sigma) for 1 hour at 4° C. The beads were washed 3 times with homogenization buffer. Samples were resolved on a 4-15% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes. Following blocking of the membranes for 1 hour in Tris-buffered saline containing 5% nonfat dry milk, the blots were incubated with anti-FLAG M2 antibody (Stratagene) at 1:1000 dilution at 4° C overnight. Membranes were washed and subsequently treated with a secondary antibody conjugated to horseradish peroxidase and visualized using an enhanced chemiluminescence (ECL) detection kit.

Plasma Analysis

Plasma insulin and leptin levels were measured by radioimmunoassay (Linco Research, St. Charles, MO). Plasma triglyceride, ketone, GOT (AST), and GPT (ALT) levels were measured using kits from Stanbio Laboratory (Boerne, TX). 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).

Tissue Analysis

Glycogen content was assayed by homogenizing 150 mg powdered liver in 1 ml 30% potassium hydroxide, boiling the extract for 15 minutes, and centrifuging at 3,000 rpm for 10 minutes. Glycogen in the cleared supernatant was measured as described (Newgard et al. 1983). Protein content in the samples was determined by method of Bradford (Bradford 1976), using the Bio-Rad assay kit.

Statistical Analysis

Data are expressed as the mean ± S.E.M. Statistical significance was determined by unpaired Student's *t* test using the statistics module of Microsoft Excel. Statistical significance was assumed at $p \leq 0.05$.

RESULTS

Expression of PTG-VF in Rat Liver

Adenovirus-mediated expression of PTG-VF in liver was evaluated by immunoprecipitation followed by immunoblotting. A representative immunoblot is shown in Figure 4.1. Animals treated with AdCMV-βGal had either no signal or background at the

position expected for PTG-VF. Rats infused with AdCMV-PTG-VF had variable levels of transgene expression. Since levels of expression were found to be so variable, animals were originally divided into groups that exhibited high expression of PTG-VF and those that had low expression of PTG-VF. However, no correlation was found to exist between levels of expression and degree of suppression of hepatic glycogen content (data not shown). Therefore, data are pooled for all animals that had clear levels of expression of PTG-VF for each treatment protocol studied.

Liver Glycogen Levels in Fasted and Ad Libitum Fed Rats

As shown in Figure 4.2, hepatic glycogen stores in AdCMV-βGal control rats fed on standard chow (SC) were high in the fed state (673 $\pm$ 17 μg of glycogen/mg protein) but were depleted by 61% following a twenty hour fast. Animals that received AdCMV-PTG-VF had decreased glycogen content in the fed state (513 $\pm$ 41 μg glycogen/mg protein; p=0.002) compared to AdCMV-βGal control rats and this was depleted by 82% in response to fasting, such that glycogen levels in liver of fasted AdCMV-PTG-VF-treated rats were lower than in the AdCMV-βGal-treated controls, with p=0.000001. AdCMV-βGal control rats fed on high fat (HF) diet had similar glycogen levels as SC fed control animals (726 $\pm$ 43 μg glycogen/mg protein versus 673 $\pm$ 17 μg glycogen/mg protein, respectively) and this was decreased by 69% after a twenty hour fast. The glycogen content in livers from AdCMV-PTG-VF treated HF fed rats (566 $\pm$ 69 μg glycogen/mg protein) was 22% less than HF fed control rats. A twenty hour fast of the HF AdCMV-PTG-VF treated rats caused an 82%

reduction in glycogen levels, resulting in levels that were 56% lower than HF AdCMV-βGal fasted rats. Thus, PTG-VF overexpression impacts hepatic glycogen levels to a greater extent following a twenty hour fast then in *ad libitum* fed rats, for both the SC and HF groups.

Effects of PTG-VF Overexpression on Circulating Metabolites and Hormones in Fasted and Fed Rats

To further evaluate the impact of AdCMV-PTG-VF overexpression, various plasma assays were performed. HF feeding led to the expected rise in plasma leptin levels (Table 4.1). In addition, animals that were fasted for twenty hours had decreased plasma triglyceride levels and increased ketone levels. Fasting also caused an increase in free fatty acid levels in animals treated with AdCMV-βGal, while treatment with AdCMV-PTG-VF abolished this rise in free fatty acid levels. Otherwise, while AdCMV-PTG-VF overexpression decreased hepatic liver glycogen content in all circumstances studied except for in animals fed the HF diet *ad-libitum*, plasma metabolic variables were not significantly altered by AdCMV-PTG-VF treatment when compared to AdCMV-βGal control rats.

OGTT in Wistar Rats with Adenovirus-mediated PTG-VF Overexpression in Liver

Since hepatic glycogen content was decreased by AdCMV-PTG-VF overexpression while circulating metabolites were not impacted, OGTTs were performed to see if treatment with

PTG-VF affected the metabolic response to an acute glucose challenge. AdCMV-βGal SC control animals exhibited the expected rapid rise in blood glucose levels followed by a slow decline toward starting levels (Figure 4.3). AdCMV-βGal HF control rats exhibited an even greater excursion in blood glucose levels 30 minutes after the glucose bolus (2 g/kg) and these levels remained elevated throughout the study period indicating that HF feeding impairs glucose clearance. There is a trend towards impaired glucose tolerance with the administration of AdCMV-PTG-VF however, this trend did not reach statistical significance in either the SC or HF groups except at the 150-minute time point when SC animals treated with AdCMV-PTG-VF had elevated blood glucose levels compared to SC control animals. Only those animals that had clear expression of PTG-VF as determined by immunoprecipitation (data not shown) were included in this analysis.

Effects of PTG-VF Overexpression on Liver Glycogen Following OGTT

To determine if glycogen deposition was impaired by AdCMV-PTG-VF treatment during an OGTT despite a lack of impact on glucose tolerance, liver glycogen levels were measured in animals at the conclusion of the experiment (180-minute time point) as summarized in Figure 4.3. SC fed rats treated with AdCMV-PTG-VF had a glycogen content that was 46% lower than AdCMV-βGal SC control rats following OGTT (Figure 4.4). The hepatic glycogen content of AdCMV-PTG-VF treated HF fed rats was also 40% lower than HF fed AdCMV-βGal control rats after OGTT. Thus, animals receiving AdCMV-PTG-VF were clearly less able to accumulate hepatic glycogen during an OGTT.

Effects of PTG-VF 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 4.3 (180-minute time point), allowing several plasma variables to be measured. As summarized in Table 4.2, overexpression of AdCMV-PTG-VF had no effect on circulating triglycerides, free fatty acids, ketones, insulin, leptin, or glucose relative to AdCMV-βGal controls. HF feeding led to increased plasma leptin and ketone levels. In addition, plasma triglyceride levels were elevated in HF AdCMV-PTG-VF treated rats when compared to SC AdCMV-PTG-VF treated rats.

DISCUSSION

Overexpression of PTG-VF, a dominant-negative mutant of PTG, in isolated hepatocytes resulted in an 83% reduction in glycogen stores (Chapter 3). While, adenoviral expression of PTG-VF *in vivo* also resulted in decreased glycogen stores, the effect was much less pronounced. How can this be reconciled? The simplest explanation lies in a discrepancy in viral titers. In order to achieve the 83% reduction in glycogen content in hepatocytes, a viral titer of 2.5×10^{12} particles was required. However, the *in vivo* studies used 1.0×10^{12} or 2.0×10^{12} particles per rat. The higher titer of virus resulted in a larger number of animals expressing high levels of PTG-VF protein, however this titer also resulted in a larger proportion of animals exhibiting toxicity as determined by plasma levels of the liver enzymes aspartate aminotransaminase (pGOT) and alanine aminotrasferase (pGPT). The 61%

suppression of glycogen accumulation seen in hepatocytes at the 1.0×10^{12} titer more closely approximates the degree of suppression seen *in vivo* in the fasted state. However, overall the degree of suppression achieved *in vivo* was much less than that achieved *in vitro* so differences in viral titers does not completely explain this difference.

Another potential explanation for the decreased efficacy of PTG-VF in rats is the fact that it is impossible to completely recapitulate the *in vivo* milieu in isolated cell systems. It is quite likely that one or more metabolic or hormonal regulatory factors were missing *in vitro* that counteract the activities of a dominant-negative glycogen targeting subunit *in vivo*.

Rodent liver expresses G_L, PTG, PPP1R6, and PPP1R3E. G_L is unique in that is contains a C-terminal phosphorylase a binding domain (Moorhead, MacKintosh et al. 1995) that is absent from the other glycogen targeting subunits. This phosphorylase a regulatory region renders G_L more susceptible than PTG to inhibition by PTG-VF (Chapter 3). Presumably PPP1R6 and PPP1R3E would behave more like PTG than G_L since allosteric regulation of these glycogen targeting subunit isoforms by phosphorylase a does not occur (Armstrong, Browne et al. 1997; Printen, Brady et al. 1997; Munro, Ceulemans et al. 2005). If isolated hepatocytes contain proportionately more G_L than livers *in vivo*, this would render hepatocytes more responsive to the inhibitory effects of PTG-VF. This possibility is currently being investigated (see Chapter 5).

Knockout of G_M results in a 90% reduction in skeletal muscle glycogen content. These animals exhibited a compensatory increase in insulin-stimulated activity of PTG in skeletal muscle a phenomenon not observed in wild-type mice (Delibegovic, Armstrong et al. 2003). While compensatory changes in hepatic glycogen targeting subunits have not been shown, it

is not impossible to envision increased activity of one or more glycogen targeting subunits *in vivo* in response to administration of PTG-VF which would decrease the efficacy of this dominant-negative molecule.

Figures

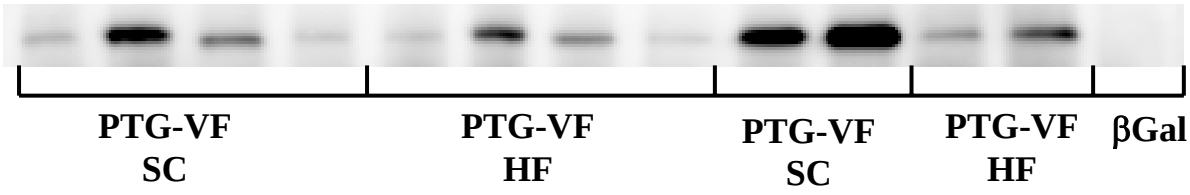


Figure 4.1. AdCMV-PTG-VF Expession in Livers as Determined by Immunoprecipitation.
Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals were treated with the AdCMV-PTG-VF or AdCMV-βGal adenoviruses and allowed to feed *ad libitum* for 90 hours after viral administration. Animals were then either allowed to continue feeding *ad libitum* or were fasted for twenty hours. Liver samples were taken for anti-FLAG immunoprecipitation followed by anti-FLAG immunoblot. Representative immunoblot.

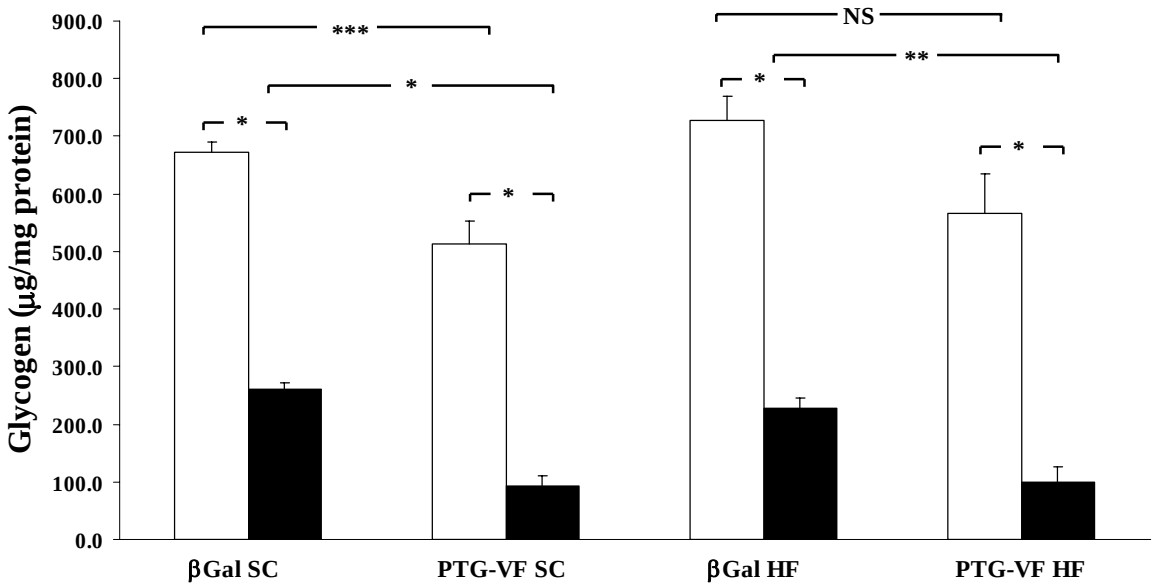


Figure 4.2. Liver Glycogen Levels Are Reduced by PTG-VF Expression in Fasted and *Ad Libitum* Fed Rats. Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals were treated with the AdCMV-PTG-VF or AdCMV-βGal adenoviruses and allowed to feed *ad libitum* for 90 hours after viral administration. Animals were then either allowed to continue feeding *ad libitum* (white bars) or were fasted for twenty hours (dark bars). Liver samples were taken for measurement of glycogen content. Results represent the mean $\pm$ S.E.M. for six βGal SC fed, six βGal SC fasted, seven βGal HF fed, ten βGal HF fasted, five PTG-VF SC fed, four PTG-VF SC fasted, five PTG-VF HF fed, and five PTG-VF HF fasted animals. The symbols *, **, and *** represent significant differences between the indicated groups with levels of significance of $p \leq 0.0001$, $p \leq 0.0005$, and $p \leq 0.005$, respectively.

	AdCMV-βGal SC		AdCMV-PTG-VF SC		AdCMV-βGal HF		AdCMV-PTG-VF HF	
	Fed (n = 6)	Fast (n = 6)	Fed (n = 5)	Fast (n = 4)	Fed (n = 7)	Fast (n = 10)	Fed (n = 6)	Fast (n = 5)
Triglycerides (mg/dl)	212.8 ± 54.0	88.7 ± 10.8*	168.9 ± 33.8	59.1 ± 13.6*	128.6 ± 20.4	100.4 ± 16.9	188.8 ± 42.9	87.3 ± 11.9*
FFAs (mmol/l)	0.13 ± 0.02	0.26 ± 0.05*	0.18 ± 0.07	0.18 ± 0.02	0.15 ± 0.02	0.23 ± 0.03*	0.17 ± 0.03	0.22 ± 0.02
Ketones (mg.dl)	2.40 ± 0.37	4.23 ± 0.87	2.32 ± 0.61	3.82 ± 0.15*	3.17 ± 0.57	8.73 ± 1.34#b	4.08 ± 0.92	9.97 ± 0.71@c
Insulin (ng/ml)	3.96 ± 0.64	1.84 ± 0.64*	3.31 ± 0.57	2.50 ± 1.52	3.29 ± 0.61	3.11 ± 0.49	3.30 ± 0.86	2.49 ± 0.68
Letpin (ng/ml)	3.42 ± 0.77	1.79 ± 0.66	1.98 ± 0.73	0.54 ± 0.10	13.51 ± 3.48a	4.21 ± 0.82*a	6.33 ± 1.44a	3.26 ± 1.00a
Glucose (mg/dl)	192 ± 5	174 ± 9	173 ± 11	174 ± 8	209 ± 5a	185 ± 8*	206 ± 15	183 ± 14
pGOT (U/L)	43.7 ± 6.0	66.1 ± 12.0	120.0 ± 39.8	95.5 ± 44.1	78.8 ± 21.6	62.4 ± 10.9	65.6 ± 19.7	76.4 ± 30.5
pGPT (U/L)	29.7 ± 6.2	35.8 ± 8.1	74.3 ± 28.1	48.7 ± 29.7	32.7 ± 7.5	25.2 ± 7.9	29.3 ± 11.3	21.7 ± 5.1

Table 4.1. Plasma Variables in Ad Libitum Fed and Fasted PTG-VF Treated Rats. Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals received the AdCMV- βGal or AdCMV-PTG-VF adenoviruses and were allowed to feed *ad libitum* for ninety hours after viral administration. Animals were then either allowed to continue feeding *ad libitum* or were fasted for twenty hours. Blood was collected for analysis of the indicated plasma variables. Results represent mean ± S.E.M. for the indicated number of animals in each group. a=p≤0.05 Standard Chow versus High Fat, b=p≤0.01 Standard Chow versus High Fat, c=p≤0.001 Standard Chow versus High Fat, *=p≤0.05 Fed versus Fast, #=p≤0.01 Fed versus Fast, @=p≤0.001 Fed versus Fast. FFAs, free fatty acids.

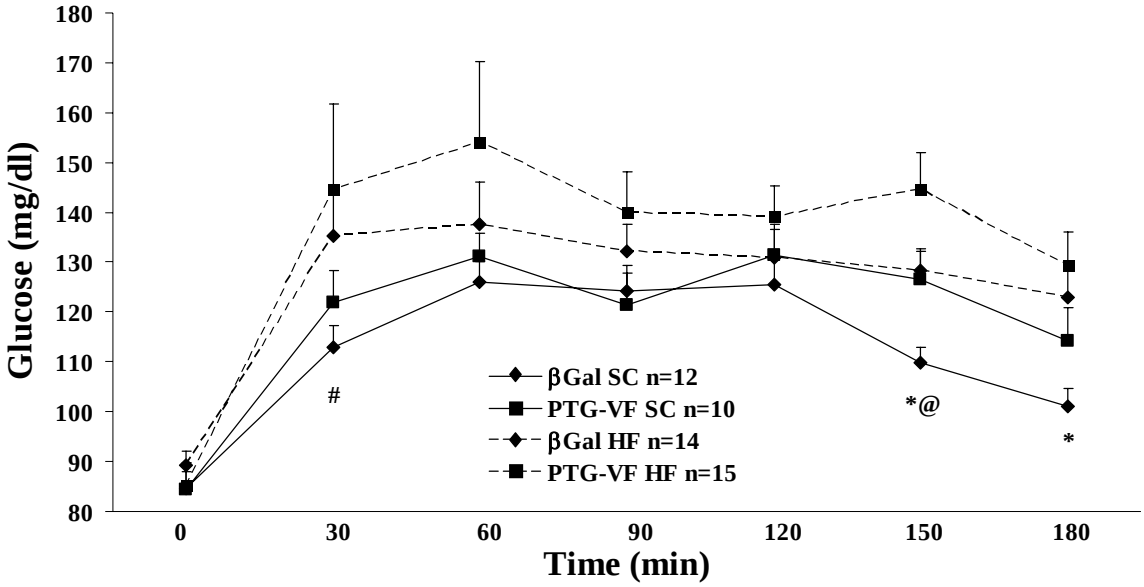


Figure 4.3. Oral Glucose Tolerance Test. Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals were treated with AdCMV- βGal or AdCMV-PTG-VF and allowed to feed *ad libitum*. Ninety hours after viral administration animals were fasted for twenty hours and subjected to an OGTT. Results represent the mean ± S.E.M. for the indicated number of animals. The symbols # and * denote differences between βGal SC and βGal HF at the corresponding time point at levels of significance of $p<0.03$ and $p<0.002$, respectively. The symbol @ denotes differences between βGal SC and PTG-VF SC at the 150-minute time point at levels of significance of $p<0.002$.

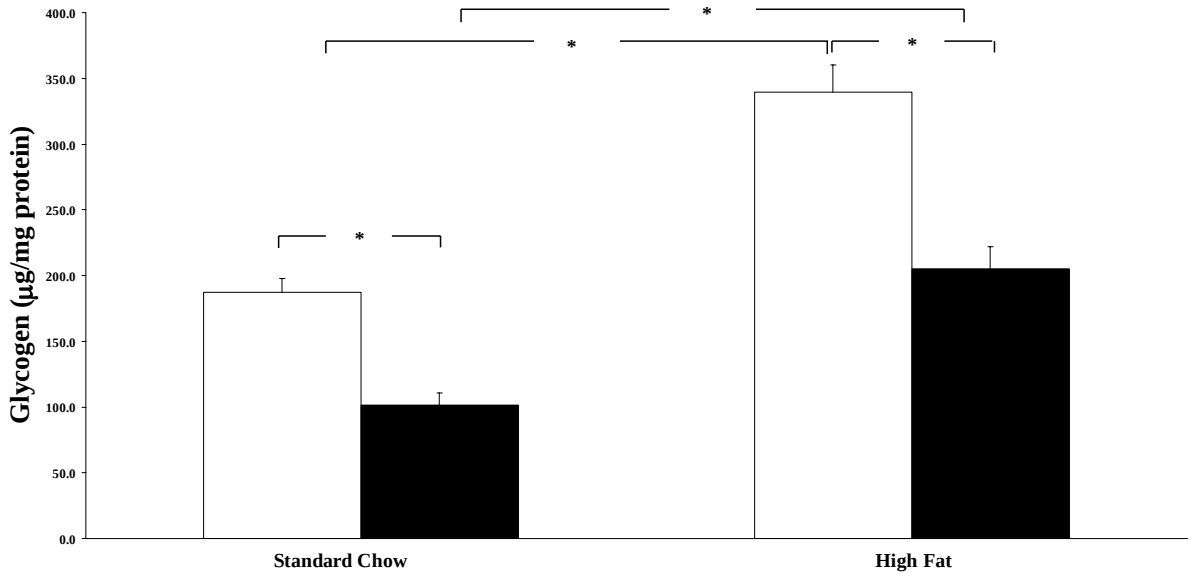


Figure 4.4. Liver Glycogen Levels After the OGTT. Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals were treated with AdCMV- βGal (white bars) or AdCMV-PTG-VF (dark bars) and allowed to feed *ad libitum*. Ninety hours after viral administration animals were fasted for twenty hours and subjected to an OGTT. Immediately following the 180-minute time point liver samples were taken for measurement of glycogen content. Results represent the mean $\pm$ S.E.M. for twelve βGal SC, fourteen βGal HF, ten PTG-VF SC, and fifteen PTG-VF HF. The symbol * indicate a significant difference between the indicated groups with levels of significance of $p \leq 0.001$.

	AdCMV-βGal SC (n = 12)	AdCMV-PTG-VF SC (n = 10)	AdCMV-βGal HF (n = 14)	AdCMV-PTG-VF HF (n = 15)
Triglycerides (mg/dl)	68.8 ± 15.9	61.2 ± 10.2	103.4 ± 13.5	91.1 ± 8.5[a]
FFAs (mmol/l)	0.15 ± 0.02	0.15 ± 0.02	0.24 ± 0.04	0.21 ± 0.02
Ketones (mg/dl)	2.62 ± 0.29	2.74 ± 0.47	4.52 ± 0.62[b]	4.74 ± 0.53[b]
Insulin (ng/ml)	2.06 ± 0.38	1.83 ± 0.55	2.87 ± 0.43	2.21 ± 0.49
Leptin (ng/ml)	1.46 ± 0.24	1.36 ± 0.24	6.67 ± 1.52[b]	8.22 ± 1.24[c]
Glucose (mg/dl)	200 ± 9	207 ± 13	214 ± 9	217 ± 14
pGOT (U/L)	66.6 ± 11.7	91.5 ± 13.2	75.1 ± 14.7	75.2 ± 6.2
pGPT (U/L)	32.9 ± 7.2	51.0 ± 8.8	31.1 ± 8.2	24.5 ± 2.4[b]

Table 4.2 Plasma Variables in AdCMV-βGal and AdCMV-PTG-VF Treated Rats After the OGTT Protocol. Male Wistar rats were fed a high fat diet for eleven weeks. At the end of this period, animals were treated with AdCMV- βGal or AdCMV-PTG-VF and allowed to feed *ad libitum*. Ninety hours after viral administration animals were fasted for twenty hours and subjected to an OGTT. Blood samples were collected after the 180-minute time point of the OGTT for analysis of the indicated plasma variables. Data are mean ± S.E.M. for the number of animals indicated in each group. a=$p \leq 0.05$ Standard Chow versus High Fat, b=$p \leq 0.01$ Standard Chow versus High Fat, c=$p \leq 0.001$ Standard Chow versus High Fat. FFAs, free fatty acids.

CHAPTER FIVE

Conclusions and Future Directions

Glycogen targeting subunits of PP-1 play a critical role in glucose homeostatsis. Although much attention has been given to the role played by individual targeting subunits in fuel homeostasis, manipulation of multiple isoforms has not been reported prior to the studies described in this dissertation. Mutation of V62 and F64 to alanine in the PP-1 binding pocket of PTG creates a molecule that is unable to bind PP-1 and yet retains its ability to interact with glycogen synthase and glycogen phosphorylase. Adenoviral overexpression of PTG-VF in hepatocytes decreased glycogen content by 83% suggesting that PTG-VF is a pan-specific dominant-negative glycogen targeting subunit (Chapter 3). Further studies found that PTG-VF preferentially blocks the glycogenic potency of G_L relative to PTG. This was found to be due to the C-terminal phosphorylase a binding domain on G_L that is absent from PTG. PTG-VF increased phosphorylase a activity without impacting glycogen synthase activity.

In order to compare levels of transgene expression in the experiments described in Chapter 3 I had to use real time PCR. While it is likely that the increase in mRNA seen with increasing viral titers corresponds to increased protein levels as is seen with PTG-VF expression, a direct measurement of protein levels would have been more accurate. In theory this should have been relatively easy since all of the adenoviral constructs of the glycogen targeting subunits contained FLAG epitopes. In fact, whereas detection of PTG-FLAG and PTG-VF-FLAG proteins was consistently achieved by immunoblot analysis, G_L-FLAG and $G_L\Delta$C-FLAG were not detectable (data not shown). The FLAG tag was located on the N-terminus in both PTG constructs and C-terminus in both G_L constructs. It is possible that the

tertiary structure of G_L is such that the FLAG epitope is less exposed and therefore less likely to bind the FLAG antibody. To test this hypothesis new adenoviruses should be constructed where the FLAG epitope is located on the N-terminus of G_L and $G_L\Delta C$.

PTG-VF was able to decrease hepatic glycogen content in standard chow and high fat fed rats (Chapter 4). The degree of suppression was greater in fasted animals than in *ad libitum* fed animals. Despite impaired glycogen accumulation, glucose homeostasis was not altered. The efficacy of PTG-VF was clearly lower *in vivo* than *in vitro*. This could be due to a difference in titer used in the two studies ($2.5x10^{12}$ particles in hepatocytes versus $1.0x10^{12}$ or $2.ox10^{12}$ particles per rat). However, due to viral toxicity, the titer used *in vivo* could not be matched with the titer used *in vitro*.

Rodent liver expresses four glycogen targeting subunits; G_L, PTG, PPP1R6, and PPP1R3E (Browne, Delibegovic et al. 2001; Green, Aiston et al. 2004; Munro, Ceulemans et al. 2005). Only G_L contains a C-terminal phosphorylase a binding site that renders it more susceptible to inhibition by PTG-VF. Since allosteric regulation of PPP1R6 and PPP1R3E by phosphorylase a does not occur, it is likely that these two subunits would behave more like PTG than G_L making them less susceptible to inhibition by PTG-VF. The proportion of the four targeting subunits in liver *in vivo* versus *in vitro* is not known. If isolated hepatocytes contain proportionately more G_L than livers *in vivo*, this would allow PTG-VF to suppress glycogen accumulation in hepatocytes than *in vivo*. In order to address this question, real time PCR is being performed to compare mRNA levels of G_L, PTG, PPP1R6, and PPP1R3E in freshly collected livers versus hepatocytes following 24 hours in culture with 20 mM glucose. Analysis of AdCMV-βGal-treated hepatocytes versus livers from

AdCMV-βGal-treated animals will allow us to determine if the isolation procedure itself alters the proportion of glycogen targeting subunits.

Another issue that can be addressed in the aforementioned real time PCR studies is if PTG-VF overexpression itself alters levels of endogenous glycogen targeting subunits. The 24 hour incubation period in the in vitro studies may not be long enough to see alterations in glycogen targeting subunit protein levels, while the 5 day *in vivo* study period could be long enough for PTG-VF to cause compensatory increases in expression of one or several of the native glycogen targeting subunit isoforms. Results from RT-PCR studies in AdCMV-G_L versus AdCMV-G_L + AdCMV-PTG-VF treated hepatocytes suggest that this may, in fact, be a contributing factor (Figure 5.1). The primer/probe set used was designed to recognize both endogenous and exogenous G_L and did not recognize PTG-VF. Therefore, we are unable to distinguish if the increased levels of G_L detected are due to increased endogenous G_L, virally expressed G_L, or a combination of both. The primer/probe set used in Chapter 3 did not allow a similar analysis of PTG because both PTG and PTG-VF were recognized.

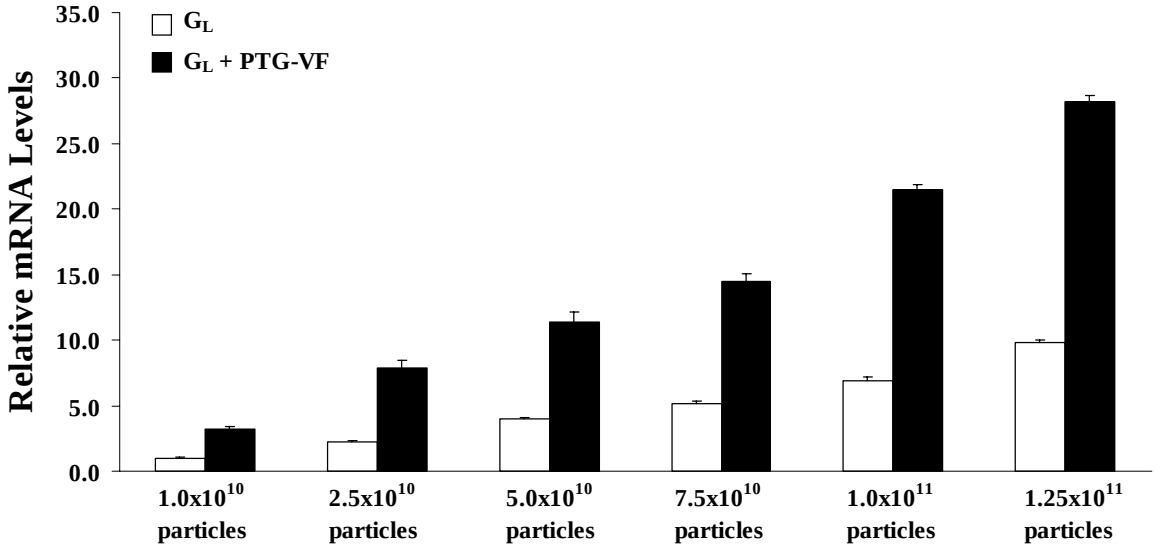


Figure 5.1. mRNA Levels of G_L in Hepatocytes Overexpressing either G_L alone or G_L + PTG-VF. Relative expression of G_L. All data are compared to the amount of mRNA detected for the lowest titer of G_L expressed. Data represent the mean + SEM of 5 independent experiments, each performed in triplicate.

Compensatory increases in activity of PTG have been shown to occur in G_M knockout mice (Delibegovic, Armstrong et al. 2003). Similar compensatory responses may play a role in the decreased efficacy of PTG-VF *in vivo* versus *in vitro*. As with changes in levels of glycogen targeting subunit expression, the limited study period in the hepatocyte protocol may not allow any compensatory activity levels in response to PTG-VF expression to be manifested. However, the extended study period in the *in vivo* protocol could provide ample time for alterations in activity of glycogen targeting subunits to limit the impact of PTG-VF. Analysis of glycogen phosphorylase and glycogen synthase activity levels in liver samples from the animals represented in Chapter 4 will provide some insight into the impact of PTG-VF on these variables.

Mutation of the PP-1 binding site in PTG, a glycogen targeting subunit known to decrease phosphorylase activity (Green, Aiston et al. 2004), results in a dominant-negative subunit that preferentially increases phosphorylase activity over decreasing glycogen synthase activity. Therefore, PTG-VF is more effective in blocking the glycogenic action of G_L than PTG. It would be interesting to discover if mutation of the PP-1 binding region in G_L would also produce a dominant-negative subunit. Furthermore, since G_L appears to function primarily as a facilitator of synthase phosphatase activity, it is possible that this would create a dominant-negative glycogen targeting subunit that preferentially impacted PTG over G_L. PPP1R6 and PPP1RE3, the other glycogen targeting subunit isoforms located in rat liver, may also be more susceptible to inhibition by a dominant-negative G_L subunit since, like PTG, these isoforms are not allosterically regulated by phosphorylase.

An alternate approach to using dominant-negative glycogen targeting subunits to decrease the function of endogenous subunits would be to design adenoviruses expressing small interfering RNA sequences. This approach can be used to direct the degradation of transcripts complementary to the siRNA thereby suppressing expression of the protein of interest (Fire, Xu et al. 1998). Work in our laboratory has shown that the toxicity of Ad-siRNAs is less than that seen with overexpression adenoviruses. Therefore, it is possible to overexpress more than one Ad-siRNA at one time. This would allow the simultaneous knock down of two or more glycogen targeting subunits and reveal the relative abilities of the remaining endogenous glycogen targeting subunits to compensate for this loss in targeting subunit activity.

While deletion of the muscle isoform, G_M, has been reported (Suzuki, Lanner et al. 2001; Delibegovic, Armstrong et al. 2003), liver specific knockout of glycogen targeting subunits has not been reported. Specific deletion of a glycogen targeting subunit in the liver would allow a more critical investigation in the capability of endogenous glycogen targeting subunits to compensate for the loss of one of the isoforms.

The results presented in this dissertation underscore the importance of glycogen targeting subunits in fuel homeostasis. Overexpression of functional glycogen targeting subunits stimulates glycogen accumulation and, in the case of $G_M\Delta C$, improves glucose tolerance. This variant glycogen targeting subunit embodies appropriate regulatory properties. Overexpression of a dominant-negative glycogen targeting subunit, PTG-VF, suppresses all targeting subunit activity and strongly impairs glycogen accumulation in isolated hepatocytes. Studies with PTG-VF provide a direct demonstration of the ability of different glycogen targeting subunit isoforms to respond differently to cellular conditions. The example provided here is that G_L responds directly to changes in glycogen phosphorylase a levels, whereas PTG does not. These results suggest that hepatic glycogen targeting subunits are a potential drug target for treatment of diabetes. Manipulation of the subunits that enhance their glycogenic potential could enhance hepatic glucose disposal, however, it is imperative that the glycogen targeting subunit retain the appropriate regulatory control. In addition, these studies have shown that expression of multiple glycogen targeting subunit isoforms in the same tissue, in this case the liver, provides a level of redundancy that can, at least partially, compensate for decreased activity of one or more subunits *in vivo* and highlights the importance of glycogen targeting subunits in fuel homeostasis.

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VITAE

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