Central Role for Protein Targeting to Glycogen in the Maintenance of Cellular Glycogen Stores in 3T3-L1 Adipocytes

Cynthia C. Greenberg, Arpad M. Danos, and Matthew J. Brady*

Department of Medicine, Committee on Molecular Metabolism and Nutrition, University of Chicago, Chicago, Illinois

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Overexpression of the protein phosphatase 1 (PP1) subunit protein targeting to glycogen (PTG) markedly enhances cellular glycogen levels. In order to disrupt the endogenous PTG-PP1 complex, small interfering RNA (siRNA) constructs against PTG were identified. Infection of 3T3-L1 adipocytes with PTG siRNA adenovirus decreased PTG mRNA and protein levels by >90%. In parallel, PTG reduction resulted in a >85% decrease in glycogen levels 4 days after infection, supporting a critical role for PTG in glycogen metabolism. Total PP1, glycogen synthase, and GLUT4 levels, as well as insulin-stimulated signaling cascades, were unaffected. However, PTG knockdown reduced glycogen-targeted PP1 protein levels, corresponding to decreased cellular glycogen synthase- and phosphorylase-directed PP1 activity. Interestingly, GLUT1 levels and acute insulin-stimulated glycogen synthesis rates were increased two- to threefold, and glycogen synthase activation in the presence of extracellular glucose was maintained. In contrast, glycogenolysis rates were markedly increased, suggesting that PTG primarily acts to suppress glycogen breakdown. Cumulatively, these data indicate that disruption of PTG expression resulted in the uncoupling of PP1 activity from glycogen metabolizing enzymes, the enhancement of glycogenolysis, and a dramatic decrease in cellular glycogen levels. Further, they suggest that reduction of glycogen stores induced cellular compensation by several mechanisms, but ultimately these changes could not overcome the loss of PTG expression.

The proper organization of intracellular effectors and downstream targets plays a critical role in the efficient transmission of a variety of extracellular signals. A growing number of targeting proteins, which bind to enzymes and specific cellular structures, and scaffolding proteins, which assemble sequential enzymatic mediators, have been identified in recent years. In mammals, scaffolding proteins have been identified in the regulation of multiple cellular processes, including apoptosis, cardiac contraction, and epithelial trafficking pathways (1, 5, 39). Additionally, mitogen-activated protein kinase isoforms in Saccharomyces cerevisiae and mammals are organized with immediate upstream activating kinases through a variety of scaffolding proteins such as Ste5 and JNK-interacting protein (29, 35). Also, the family of A-kinase anchoring proteins acts as multivalent scaffolding proteins, assembling protein kinase A and a variety of kinases, phosphatases, and other signaling elements in specific subcellular locations in a wide array of cell types (40).

Targeting proteins also play a critical role in the regulation of glycogen metabolism by insulin (32). The two opposing enzymes controlling glycogen synthetic rates, glycogen synthase and phosphorylase, are both regulated by protein phosphorylation. Insulin markedly enhances glycogen accumulation through the coordinated dephosphorylation of these enzymes, resulting in the activation of glycogen synthase and the inactivation of glycogen phosphorylase. Although inactivation of upstream kinases such as glycogen synthase kinase 3 (GSK3) and phosphorylase kinase partially mediates the regulation of glycogen metabolism, the activation of protein phosphatase 1 (PP1) by insulin plays a critical role in the enhancement of glucose storage as glycogen (8). However, insulin treatment of cells results in the enhanced phosphorylation of a large number of proteins while simultaneously promoting the dephosphorylation of a limited number of enzymes primarily involved in lipid and glucose storage. Additionally, PP1 is involved in the regulation of numerous cellular processes that are not regulated by insulin, such as RNA processing and cell cycle progression (11). Thus, mechanisms must exist for the discrete activation of specific pools of PP1.

For regulation of glycogen metabolism, the PP1-targeting subunit protein targeting to glycogen (PTG or R5) is one of five proteins that localize PP1 to the glycogen particles (15, 34). This family of proteins has been shown to potently regulate glycogen levels through both cellular overexpression studies and transgenic and knockout animal models (4, 12, 13, 18, 20, 28, 33, 37). In addition to targeting PP1 to glycogen, PTG also directly binds to glycogen synthase and phosphorylase, thus enabling the efficient regulation of glycogen metabolism (17, 34). Overexpression of PTG in a variety of cell types and in rodent livers in vivo resulted in the dephosphorylation of glycogen synthase and phosphorylase and alteration of their enzymatic activities, which resulted in a dramatic increase in cellular glycogen accumulation (17–20, 28, 33, 34, 42). Conversely, heterozygous disruption of PTG in mice resulted in decreased tissue glycogen stores, corresponding with reduced glycogen synthase activity and glycogen synthesis (12). However, the precise role of the endogenous PTG-PP1 complex in the control of glycogen metabolism has not been elucidated. Theoretically, PTG-PP1 could act via regulation of glycogen synthase, glycogen phosphorylase, or both enzymes. In addition, the contribution of the PTG-PP1 complex versus the hormonal reg-
ulation of upstream kinases and other phosphatases to glycogen metabolism remains controversial. In particular, the inactivation of GSK3 by insulin has been proposed to be a principal regulator of glycogen synthase activity (10). Therefore, RNA interference (RNAi) against PTG was used to examine the impact of reducing PTG levels on glycogen metabolism in the highly insulin responsive and metabolically active 3T3-L1 adipocyte line. PTG knock-down resulted in decreased targeting of PP1 to glycogen, corresponding to reduced phosphatase activity against glycogen synthase and phosphorylase, increased glycogenolysis, and a marked decrease in glycogen levels. These results indicate that the PTG-PP1 complex occupies a critical role in the regulation of glycogen metabolism in 3T3-L1 adipocytes.

MATERIALS AND METHODS

Generation and screening of siRNA constructs. Four short (19-nucleotide) target sequences proposed to specifically silence gene expression of PTG were identified online using siRNA Target Finder (Ambion). Potential PTG siRNAs were termed siRNA 1 to siRNA 4, with PTG siRNA 1 corresponding to the murine PTG gene sequence (34) starting at nucleotide (nt) 391, PTG siRNA 2 starting at nt 457, PTG siRNA 3 starting at nt 823, and PTG siRNA 4 starting at nt 748. A scrambled oligonucleotide that did not correspond to any known murine gene sequence was also identified for use as a control. Oligonucleotides containing the sense sequence, a hairpin loop region, and the antisense sequence was annealed to its complement and ligated into pSilencer-Shuttle vector (BD Biosciences Clontech). Target sequences were then tested for gene silencing of transiently overexpressed PTG in 293T cells. 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, and 0.29 mg/ml glutamine. 293T cells in 12-well dishes were transiently transfected with 0.5 μg of pcDNA3.1 vector encoding PTG and 0.5 μg of pSIREN-PTG-siRNA or pSIREN-scrambled siRNA using Lipofectamine PLUS (Invitrogen) according to the supplier’s instructions. After a 24-h recovery, cell lysates were prepared, and PTG levels were analyzed by immunoblotting to identify constructs that reduced exogenous PTG expression. PTG siRNA constructs 2 and 3 reduced endogenous PTG expression to undetectable levels. Scrambled and PTG siRNA constructs 2 and 3 were subcloned into Adeno-X viral DNA (BD Biosciences Clontech) for incorporation into adenoviral genome. In preliminary experiments, infection of 3T3-L1 adipocytes with either PTG siRNA 2 or 3 adenovirus produced similar repression of endogenous PTG protein and cellular glycogen levels, so PTG siRNA 2 was used for all experiments reported here.

3T3-L1 adipocytes were cultured and infected as previously described (20), with the following modifications: cells plated in 12-well dishes were infected 1 or 2 days following replacement of insulin-containing growth medium with growth medium in the standard differentiation protocol. A titer of 2 × 10^7 viral particles per cell was diluted with 0.5 μg/ml poly-t-lysine hydrobromide (Sigma) in DMEM supplemented with 2% FBS as described. Unless otherwise indicated, cells were used 4 or 5 days following infection, with fresh growth medium provided every 48 h.

RNA and protein analyses. Total RNA was prepared from 12-well plates of scrambled or PTG siRNA-infected 3T3-L1 adipocytes using 3 ml/plate of Trizol (Gibco-BRL). Reverse transcription-PCR (RT-PCR) was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. For real-time quantitative RT-PCR, relative expression levels were determined using iQ SYBR Green Supermix (Bio-Rad) in the iCycler system (Bio-Rad). Ten microliters of diluted cDNA was added to 12.5 μl of iQ SYBR Green Supermix (Bio-Rad), and primers were added at a concentration of 10 pM (sense, TTTCCAGAAGAACCAGCT; antisense, TTCAGGTTGAATGAC ACG). Water was added to bring the final volume to 25 μl. In parallel, standard curves for PTG and the 18S ribosomal subunit were generated by running reactions on a series of serial 1:10 dilutions, initiated by the addition of 5 μl of cDNA from control cells (one-fourth volume of RT reaction mixture) to 45 μl of water. The difference between PTG and 18S threshold cycles was assessed for each sample, and these differences were compared between scrambled and PTG-siRNA-treated samples to quantify the relative difference in PTG mRNA between the samples. Thermal cycling parameters were as follows: 3 min at 95°C, followed by 40 cycles of 30 s at 60°C, 30 s at 72°C, and 60 s at 95°C. The comparative cycle threshold method was used to quantify PTG mRNA copy number in scrambled and PTG-siRNA-infected 3T3-L1 adipocytes, relative to that of the 18S ribosomal subunit.

For immunofluorescence, lysates were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose (Schleicher and Schuell). Western blots were probed with antibodies against PTG (20), glycogen phosphorylase and phospho-phosphorylase (Ser 14) (18), glycogen synthase (Chemicon), pan-PP1 (sc-7482; Santa Cruz Biotechnology), phosphorytose (Upstate Cell Signaling Solutions), Akt, phospho-GSK3β (Ser 9), and phospho-glycogen synthase (Ser 64) (Cell Signaling Technologies) or anti-GLUT1 and anti-GLUT4 antibodies (Alpha Diagnostic International). Blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (Bio-Rad) and developed using ECL reagent (Amersham Pharmacia Biotech).

Cell fractionation. Cells were washed three times with cold phosphate-buffered saline and harvested in homogenization buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% glycerol, and protease inhibitors added just before use). Cells were lysed using a glass Dounce homogenizer and centrifuged at 1,000 × g at 4°C to pellet nuclei. The resulting postnuclear supernatant fraction was then subjected to sequential centrifugation at 4°C, first at 10,000 × g for 10 min and then at 100,000 × g for 30 min, to obtain the cytosolic and glycogen-enriched pellet fractions. The pellet fractions were resuspended in homogenization buffer by Trituration using a 23-gauge needle. To determine levels of glycogen-targeted PP1, the pellets resulting from centrifugation at 100,000 × g were resuspended in homogenization buffer plus 40 μg/ml amylase (Sigma) and incubated at 37°C for 15 min. Samples were then centrifuged at 100,000 × g for 30 min, and PP1 levels released into the supernatant were determined by immunoblotting.

For examination of GLUT1 and GLUT4 subcellular distribution, cells were first serum starved for 2.5 h in DMEM-0.5% FBS-5 mM glucose and then lysed and processed as above. The pellets resulting from centrifugation at 10,000 × g were resuspended in homogenization buffer by Trituration using a 23-gauge needle. These fractions were then combined with Laemmli sample buffer lacking β-mercaptoethanol, heated for 10 min at 37°C, and analyzed by immunoblotting.

Metabolic and enzymatic assays. For measurement of glycogen synthase activity due to covalent modification, following serum starvation for 2.25 h, cells were washed three times with medium lacking glucose and incubated in the same medium for 15 min. Cells were then either unstimulated or stimulated with 100 nM insulin for 15 min. Lysates were prepared and reactions were carried out as previously described (27). Cellular glycogen levels, PTG activity assays, and glycogen synthetic and lipogenic rates were determined as previously described (20). For determination of ATP levels, cells were washed three times with cold phosphate-buffered saline and scraped into 1 ml of assay buffer (100 mM Tris [pH 7.75], 4 mM EDTA). Lysates were transferred to microcentrifuge tubes, immediately quick-frozen in a dry ice-ethanol bath, and stored at −20°C. For the assay, lysates were boiled for 5 min, incubated on ice for 5 min, and then spun at 1,000 × g for 60 s at 4°C. The supernatant was transferred to a new tube and then diluted 1:100 in assay buffer. Fifty microliters of each diluted sample was assayed according to the supplier’s instructions using an ATP BioLuminescence Assay Kit (Promega). Phosphorylase and phospho-phosphorylase (Ser 14) and glycogen synthase (Chemicon) were determined by immunoblotting.

Glycogen pulse-chase for determination of glycogenolysis. Following infection and recovery, cells were serum starved for 2.5 h in DMEM-0.5% FBS-5 mM glucose and then stimulated for 15 min with 100 nM insulin. For glycogen labeling, 2 μCi of [14C]glucose (ICN Biomedicals) was added to each well and allowed to incorporate for 30 min. After removal of glucose, cells were collected for determination of baseline labeling of glycogen. Replica wells were incubated in growth medium for 24 h at 37°C and then collected for determination of the remaining labeled glycogen (20).

Statistical analysis. Data comparisons were analyzed by a Student’s t test. Analysis was performed using Microsoft Excel XP and considered statistically significant at a P value of <0.05.

RESULTS

Gene silencing of PTG by siRNA treatment of 3T3-L1 adipocytes. The technique of RNAi was chosen to specifically reduce PTG levels in 3T3-L1 adipocytes. First, four different siRNA constructs were screened for their ability to induce RNAi against PTG transiently overexpressed in 293T cells
responded to a cycles in cells infected with PTG siRNA. This difference cor-

4-day recovery, PTG transcript levels were determined by

siRNA at a titer of 2

adenoviral vector. After adenoviruses were packaged and pu-

PTG siRNA constructs were then subcloned into pAdeno-X

scrambled or PTG siRNA at a titer of 2

3T3-L1 adipocytes plated in 12-well dishes were infected with

(A) 3T3-L1 adipocytes infected with PTG siRNA (Fig. 1A), indi-

FIG. 1. PTG siRNA reduces PTG transcript and protein levels. (A) 3T3-L1 adipocytes plated in 12-well dishes were infected with scrambled or PTG siRNA at a titer of 2 \times 10^5 viral particles per cell. Following recovery, total RNA was prepared from cells. PTG tran-

(B) Mock-, scrambled siRNA-, or PTG siRNA-infected cells were fractionated to obtain postnuclear spin supernatant and glycogen-enriched pellet fractions. Pellets were resuspended in homogenization buffer, and samples were

(Fig. 1B, GB-PP1). These results are also consistent with pre-

previous findings in 3T3-L1 adipocytes that PTG-bound or glyco-

glycerol-phosphorylase (α-GP) immunoblotting. To determine levels of glycogen-bound PP1 (GB-

lysates from scrambled and PTG siRNA-infected cells were pre-

following incubation, samples were centrifuged at 100,000 \times g for 30 min, and PP1 levels released into the supernatant were determined by anti-PP1

Materials and Methods). The scrambled control siRNA and

PTG transcript levels were analyzed by quantitative RT-PCR and normalization to ribosomal 18S. Results are representative of two independent de-

cellular protein expression

(Fig. 1B, solid line). Glycogen levels remained low compared

in vitro against [32P]-labeled glycogen phosphorylase or myelin

previous results in 3T3-L1 adipocytes that PTG-bound or glyco-

glycerol phosphorylase levels (Fig. 1B) and total enzymatic activity (data not shown) were reduced by approximately 50% in cells infected with the PTG siRNA con-

PP1 activity against myelin basic protein. Infection of cells with scrambled siRNA showed a

The PTG-PP1 complex is required for the maintenance of

glycogen stores. Previous work showed that PTG overexpres-

3T3-L1 adipocytes specifically enhanced PP1 activity against enzymes involved in glycogen metabolism (20). To de-

to determine the effect of reducing PTG expression on PP1 activity, cyt-

lysates from scrambled and PTG siRNA-infected cells were pre-

PP1 activity against myelin basic protein was unchanged (Fig. 2). Together, these results indicated that infection of

To address the physiological significance of reducing levels of glycogen-bound PP1 activity, 3T3-L1 adipocytes were infected with scrambled or PTG siRNA, and total glycogen levels were determined. At baseline, both scrambled and PTG

siRNA-infected cells contained 1.8 mg of glycogen/mg of pro-

At days 2, 4, 6, and 9, cells infected with the scrambled siRNA construct maintained the starting level of glycogen, indicating that adenoviral-mediated siRNA delivery alone did not interfere with glucose uptake and storage as glycogen (Fig. 3A, dashed line). In contrast, infection of cells with PTG siRNA adenovirus markedly inhibited glycogen ac-

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to baseline and scrambled siRNA levels for up to 9 days following infection (Fig. 3A). Interestingly, despite the marked decrease in glycogen levels 4 days after infection, there was no apparent redistribution of glycogen synthase or phosphorylase (Fig. 3B), indicating that the enzymes remained bound to the residual glycogen. Further, adenoviral-mediated PTG overexpression in the PTG siRNA-treated cells enhanced glycogen levels to above those found in the mock- and \(\beta\)-galactosidase-infected cells (Fig. 3C), indicating that restoration of PTG expression was sufficient to rescue glycogen stores. Together, these data demonstrate that PTG plays a central role in the maintenance of glycogen stores in 3T3-L1 adipocytes by specifically targeting and directing PP1 activity against enzymes involved in glycogen metabolism.

Reduction of PTG expression increases glycogen breakdown. Cellular glycogen levels reflect the relative rates of glycogen formation and breakdown. Since reduction of PTG expression dramatically decreased glycogen storage, the phosphorylation state of glycogen phosphorylase and rates of glycogen degradation were initially examined. After infection with scrambled or PTG siRNA adenovirus and a 4-day recovery in growth medium, 3T3-L1 adipocytes were collected, glycogen-enriched pellets were prepared by ultracentrifugation, and phospho-phosphorylase levels were determined by immunoblotting (18). Reduction of PTG expression with the PTG siRNA construct strongly enhanced phospho-phosphorylase levels (Fig. 4A) and the enzyme activity ratio (data not shown). The marked increase in glycogen phosphorylase phosphorylation occurred despite a decrease in total cellular enzyme levels (Fig. 1B and 3B). In parallel, to determine glycogen degradation rates, scrambled siRNA- and PTG siRNA-infected cells were pulsed with \[^{14}C\]glucose for 30 min in the presence of insulin in order to label glycogen stores. All cells were then washed three times with medium, and baseline levels of glucose incorporated into glycogen were determined in half of the wells (Fig. 4B, Day 1). After 24 h, the amount of labeled glycogen remaining in the

FIG. 2. PTG siRNA reduces PP1 activity against glycogen phosphorylase. 3T3-L1 adipocytes were mock-infected or infected with scrambled or PTG siRNA. Following 4 days of recovery, cell lysates were prepared. PP1 activity in cell lysates was measured for 4 min, using \(^{32P}\)-labeled glycogen phosphorylase \(a\) (Phos \(a\)) or \(^{32P}\)-labeled myelin basic protein (MBP) as a substrate. A Student’s \(t\) test analysis of data from PTG siRNA versus mock and versus scrambled siRNA was used. *, \(P < 0.05\). Results are representative of three independent determinations, each performed in triplicate.

FIG. 3. PTG is required for maintenance of glycogen stores. (A) 3T3-L1 adipocytes were infected with scrambled or PTG siRNA as described. At each time point, fresh growth medium was added to cells or lysates were prepared, and glycogen and protein levels were determined. Day 0 indicates the day of infection. A Student’s \(t\) test analysis of data from PTG siRNA versus scrambled siRNA was used. *, \(P < 0.05\); **, \(P < 0.01\); +, \(P < 0.06\). Results are representative of three independent experiments, each performed in triplicate. (B) Four days after infection, mock-, scrambled siRNA-, or PTG siRNA-treated cells were fractionated by ultracentrifugation to obtain cytosolic and glycogen-enriched pellet (GEP) fractions. The samples were analyzed by immunoblotting using anti-glycogen synthase (\(\alpha\)-GS) or anti-glycogen phosphorylase (\(\alpha\)-GP) antibodies. Results are representative of three independent determinations. (C) On day 0, replicate wells of 3T3-L1 adipocytes were either mock infected (−) or infected with PTG siRNA adenovirus (+). Three days later, the indicated wells were mock infected (−) or infected with adenovirus encoding \(\beta\)-galactosidase (\(\beta\)Gal) or PTG. Two days later, cell lysates were prepared, and cellular glycogen and protein were levels determined. Results are representative of five independent determinations from two experiments. A Student’s \(t\) test analysis of data from samples versus mock-infected PTG siRNA samples was used. *, \(P < 0.03\); **, \(P < 0.01\); n/s, not significant.
resulted in a

with the scrambled siRNA was low, and insulin stimulation

determined. The basal rate of glycogen synthesis in cells infected

amounts of radiolabeled glucose incorporation were deter-

mined by scintillation counting. A Student’s t test analysis of data from PTG

siRNA versus scrambled siRNA was used. ***, P < 0.001. Results are

representative of three independent determinations, each performed in triplicate.

FIG. 4. Glycogenolysis is increased upon reduction of PTG expres-

sion. Cells were infected with scrambled (Scr) or PTG siRNA and

allowed to recover for 4 days. (A) Glycogen-enriched pellets were

prepared, and levels of phosphorylated glycogen phosphorylase (GPa)

were analyzed by phospho-specific immunoblotting. (B) Cells infected

with scrambled or PTG siRNA were serum starved for 2.25 h and then

stimulated with 100 nM insulin for 15 min. A total of 2 μCi of [14C]glu-
cose was added to each well to label glycogen. After 30 min, cells were

washed and either used to determine glucose incorporation into gly-
cogen (Day 1) or placed in growth medium and incubated for 24 h

(Day 2), after which remaining glucose incorporated into glycogen was
determined by scintillation counting. A Student’s t test was used for
data analysis: n/s, not significant; *, P < 0.03. Results are representa-
tive of three experiments, each performed in triplicate.

other half of the wells was determined (Fig. 4B, Day 2). There

was no significant change in amounts of radiolabeled glycogen

after 24 h in cells infected with scrambled siRNA. In contrast,
infection of cells with PTG siRNA reduced glycogen stores by

40% after 24 h, reflecting significantly increased glycogenolytic
rates (Fig. 4B), which paralleled the changes measured in total
glycogen levels (Fig. 3A). Cumulatively, these data suggest that

the reduction of PTG expression uncoupled PP1 activity from
phosphorylase, resulting in decreased glycogen stores due to
enhanced glycogen breakdown.

Stimulation of glucose transport and glycogen synthesis upon
depletion of cellular glycogen stores. The marked reduction of

cellular glycogen stores following ablation of PTG expression
could also occur via inhibition of glycogen synthesis. To ad-
dress this possibility, basal and insulin-stimulated glycogen syn-
thetic rates were compared in 3T3-L1 adipocytes following
infection with scrambled or PTG siRNA adeno

virus. After a

4-day recovery, cells were treated in the absence and presence
of 100 nM insulin for 15 min, followed by a 30-min incubation
with [14C]glucose. Cellular glycogen was then isolated, and the
amounts of radiolabeled glucose incorporation were deter-
mined. The basal rate of glycogen synthesis in cells infected
with the scrambled siRNA was low, and insulin stimulation
resulted in a >10-fold increase (Fig. 5). Unexpectedly, in cells

infected with PTG siRNA, the basal rate of glycogen synthesis

was increased fourfold compared to the scrambled control,
while insulin stimulation was doubled (Fig. 5). Thus, despite
the marked reduction in total glycogen stores, acute glycogen
synthesis rates were significantly augmented.

Insulin-stimulated glycogen synthesis rates are a summation of
the degree of insulin-stimulated signaling cascades resulting in
increased GLUT4 translocation and glucose uptake concomi-
tantly with glycogen synthase activation. To identify the mecha-
nism(s) underlying the increased glycogen synthesis rates, these
parameters were compared in 3T3-L1 adipocytes infected with
scrambled or PTG siRNA. After infection, 4-day recovery, and
serum starvation, cells were untreated or treated with insulin
for 15 min, and initially cell lysates were analyzed by phospho-specific
immunoblotting. Basal and insulin-stimulated phosphorylation of
the insulin receptor, GSK3β, and dephosphorylation of glycogen
synthase site 3α (Ser 640) did not differ between mock-infected
cells or cells infected with the scrambled or PTG siRNA adeno-

virus (Fig. 6). Thus, data suggest that the insulin-IRS1-GSK3
signaling pathway was unaffected by the loss of PTG expression.

Next, to determine if altered glucose uptake contributed to
the enhancement of glycogen synthesis in cells lacking PTG,
3T3-L1 adipocytes were infected with scrambled or PTG
siRNA, allowed to recover, and then treated exactly as during
the glycogen synthesis measurements, except that total glucose
uptake into the cells was determined by scintillation counting.
The rate of glucose transport in cells infected with the scram-
bled siRNA was low in the basal state and increased more than
eightfold upon insulin treatment (Fig. 7A). In cells infected
with PTG siRNA, the rates of both basal and insulin-stimu-
lated glucose uptake were significantly elevated. These data

indicate that increased glucose uptake may contribute to aug-
mented rates of glycogen synthesis upon PTG knockdown.
To examine the levels of facilitative glucose transporters GLUT1 and GLUT4, cells were infected with scrambled or PTG siRNA, and whole-cell lysates were prepared. Immunoblotting revealed that total GLUT1 levels were increased in cells infected with PTG siRNA compared to cells infected with scrambled siRNA (Fig. 7B, left). In contrast, there was no change in total GLUT4 levels (Fig. 7B, left). Next, to determine the ability of insulin to promote translocation of the glucose transporters to the cell surface, infected cells were serum starved and then untreated or stimulated with insulin for 15 min. Lysates were prepared and fractionated by centrifugation at 10,000 g in order to obtain plasma membrane-enriched fractions (pellet resulting from centrifugation at 10,000 × g) that were then analyzed by immunoblotting. In cells infected with scrambled siRNA, insulin stimulation resulted in a robust increase in GLUT4 protein levels in the pellet fraction and also stimulated GLUT1 translocation (Fig. 7B, right). In cells infected with PTG siRNA, basal and insulin-stimulated GLUT1 expression in the pellet fraction was increased compared to control cells (Fig. 7B, right), while GLUT4 levels were comparable under both treatment conditions. These data suggest that upregulation of total GLUT1 expressed and increased GLUT1 levels at the cell surface may contribute to the increased glucose transport and glycogen synthetic rates seen in 3T3-L1 adipocytes infected with PTG siRNA adenovirus.

Glycogen synthase catalyzes the rate-limiting step of glyco-
gen synthesis. To determine the regulation of glycogen synthase by insulin in the PTG-deficient cells, enzymatic activity was assayed in vitro. Cells were infected with scrambled or PTG siRNA adenovirus, allowed to recover, and then stimulated in the absence and presence of 100 nM insulin in medium lacking glucose. Under these conditions, changes in glycogen synthase activity reflect enzymatic regulation due to covalent modification independent of the effects of increased intracellular levels of glucose metabolites, particularly glucose-6-phosphate (G6P). In cells infected with scrambled siRNA, the basal glycogen synthase activity ratio was 0.10, which doubled upon insulin stimulation (Fig. 8A). In contrast, insulin stimulation did not significantly increase the glycogen synthase activity ratio in cells lacking PTG (Fig. 8A). Despite the marked re-
duction in cellular glycogen levels upon infection with PTG siRNA adenovirus (Fig. 3), there was no change in total glyco-
gen synthase levels, as measured by immunoblotting (Fig. 1B and 3B) or activity assay (data not shown). Thus, these results suggest that the PTG-PP1 complex forms the principal glyco-
gen synthase- and phosphorylase-directed phosphatase activity in 3T3-L1 adipocytes.

However, use of glucose-free medium does not recapitulate the physiological conditions used to measure glycogen synthesis rates, so glycogen synthase activity was also determined in cells incubated in medium containing 5 mM glucose. In cells infected with scrambled siRNA, the basal glycogen synthase activity ratio was low, and insulin stimulation increased the activity ratio to 0.17. In contrast to results obtained in the absence of extracellular glucose, the basal and insulin-stimulated glycogen synthase activity ratios in cells infected with PTG siRNA were similar to ratios in control cells (Fig. 8B). The insulin-stimulated dephosphorylation of site 3a on glyco-
Depletion of cellular glycogen stores does not impact glucose utilization. Glucose transported into the cell is rapidly phosphorylated to form G6P, which then may be utilized to synthesize glycogen, metabolized to generate ATP, or used to generate the glycerol backbone for triglyceride synthesis. Reduction of PTG expression dramatically decreased glycogen synthase activity, and the development of age-dependent insulin resistance (Fig. 5). To determine the effects of reduced cellular glycogen stores on glucose utilization, G6P and ATP levels and glucose incorporation into lipid were determined in cells infected with the scrambled or PTG siRNA adenovirus. Interestingly, G6P and ATP levels and basal and insulin-stimulated lipogenic rates were not altered upon depletion of cellular glycogen levels (data not shown). These results are consistent with previous work in 3T3-L1 adipocytes that indicated that a dramatic enhancement of cellular glycogen synthetic rates and total glycogen stores upon PTG overexpression also did not alter lipogenic rates (20) or ATP levels (data not shown).

**DISCUSSION**

Insulin is the major anabolic hormone, promoting glucose uptake and storage of glucose and fatty acid in the target organs, muscle, and adipose tissue. To exert these effects, insulin activates PP1 to mediate the dephosphorylation of key enzymes involved in glucose and lipid metabolism, namely, glycogen synthase, glycogen phosphorylase, and hormone-sensitive lipase (8). For localization of PP1 to glycogen particles, five targeting subunits have been identified. These glycogen-targeting subunits of PP1 bind to PP1 via the consensus sequence XRVXF (16, 43) and to glycogen via a central domain with significant homology to starch metabolizing enzymes (41). The first subunit identified, called RGL or Gs, is expressed in skeletal and cardiac muscle and as a 124-kDa protein is significantly larger than the other four 33- to 38-kDa proteins (36, 38). RGL/GM contains two unique consensus protein kinase A sites, one of which overlaps with the conserved PP1 binding domain, and phosphorylation of this site has been proposed to mediate the glycogenolytic response to intracellular cyclic AMP elevation (22, 23). Gt is primarily expressed in liver (14) and also human muscle (31) and is allosterically regulated by phospho-phosphorylase binding to a C-terminal domain (3, 14). PTG/R5 and R3E have overlapping tissue distribution with RGL/GM and Gt as it is expressed in all insulin-sensitive tissues (15, 34). R6 is widely expressed in tissues (2), and identification of a fifth PP1-glycogen-targeting subunit, R3E, has recently been reported (30).

Several animal models have been generated with altered expression of PP1-glycogen-targeting subunits. Two groups independently generated mouse knockout lines lacking the muscle-specific PP1-targeting subunit RGL/GM (13, 37). Both animal lines exhibited a dramatic loss of PP1 catalytic subunit and phosphorylation of this site has been proposed to mediate the glycogenolytic response to intracellular cyclic AMP elevation (22, 23). Gt is primarily expressed in liver (14) and also human muscle (31) and is allosterically regulated by phospho-phosphorylase binding to a C-terminal domain (3, 14). PTG/R5 and R3E have overlapping tissue distribution with RGL/GM and Gt as it is expressed in all insulin-sensitive tissues (15, 34). R6 is widely expressed in tissues (2), and identification of a fifth PP1-glycogen-targeting subunit, R3E, has recently been reported (30).
In contrast to the RGL/GM transgenic animal lines, there was no change in total PP1 levels in the PTG knockout animals, suggesting that RGL/GM plays a more important role for the stabilization of PP1 protein in vivo. Conversely, several groups have also reported that overexpression of these targeting subunits in a variety of cell lines (17–20, 28), by adenoviral-mediated gene transfer in rat livers (33), and in skeletal muscle of transgenic mice (4) all markedly enhance carbon flux into glycogen. Cumulatively, these results demonstrate a critical role for PP1-glycogen-targeting subunits in the regulation of glycogen metabolism in a variety of tissue types (32).

However, the overlapping tissue distribution of five different gene products that superficially perform the same function, i.e., targeting PP1 to glycogen, suggests that each subunit confers unique regulatory properties to the bound phosphatase. To determine the molecular mechanism by which the PTG-PP1 complex regulates glycogen metabolism, 3T3-L1 adipocytes were infected with adenovirus containing siRNA constructs against PTG. This cell line was chosen for the study since PTG was originally identified from a 3T3-L1 adipocyte two-hybrid library (34), and these cells exhibit robust changes in glucose and glycogen metabolism upon insulin stimulation (6). Infection of 3T3-L1 adipocytes with PTG siRNA dramatically reduced PTG transcript and protein levels. There was a parallel reduction in the amount of PP1 targeted to glycogen, although total cellular levels of this phosphatase were unchanged, suggesting that PTG is not the principal binding partner for PP1 in these cells. Delivery of PTG siRNA had no effect on the cellular expression of several insulin-sensitive regulators of glycogen synthesis, namely GLUT4, GSK3, and glycogen synthase, and several proximal insulin receptor signaling events were likewise unaffected. However, ablation of PTG expression resulted in a >85% reduction in cellular glycogen stores, suggesting a critical role for the PTG-PP1 complex in the physiological regulation of glycogen metabolism in 3T3-L1 adipocytes.

In previous studies, the simultaneous increase in glycogen synthase and phosphorylase dephosphorylation upon PTG overexpression did not address the physiological role of the regulation of glycogenesis versus glycogenolysis in PTG action. However, the data from adenoviral-mediated delivery of PTG siRNA demonstrate that the principal function of the PTG-PP1 complex in 3T3-L1 adipocytes is to dampen glycogen phosphorylase activity and limit glycogen breakdown. Infection of 3T3-L1 adipocytes with PTG siRNA adenovirus resulted in a 60% loss of PP1 activity against glycogen phosphorylase measured in vitro from cell lysates, which corresponded with a marked increase in the cellular phosphorylation of glycogen phosphorylase, corresponding to enzymatic activation. In parallel, there was a marked increase in glycogen degradation rates in PTG-depleted cells. These data extend previous work in liver and primary hepatocytes. Cohen and colleagues assayed in vitro phosphatase activity of PTG-PP1 or Glu-PP1 from rat liver against glycogen synthase or phosphorylase. They found that the glycogen synthase phosphatase/phosphorylase phosphatase activity ratio of PTG-PP1 complex was lower than that of Glu-PP1, supporting the idea that PTG is primarily a glycogen phosphorylase phosphatase (9). Also, several groups have demonstrated a dose-dependent inactivation of glycogen phosphorylase with PTG overexpression in hepatocytes and showed that PTG overexpression counteracted glycogen phosphorylase activation and glycogen mobilization by extracellular stimuli (18, 19). Cumulatively, these data from PTG overexpression and reduction studies suggest that glycogen phosphorylase is the principal enzymatic target of the PTG-PP1 complex.

Studies in skeletal muscle indicate that the cell can adapt its metabolic responses to maintain glycogen levels at a certain set point (21). In rodents or humans, glycogen depletion following exercise induces an increase in insulin sensitivity and responsiveness, resulting in enhanced glucose uptake and glycogen synthase activation. Conversely, glycogen supercompensation following exercise and a high-carbohydrate meal induces a transient insulin resistance in muscle, which persists until glycogen levels are reduced to the physiological set point (25, 26). The molecular mechanisms by which skeletal muscle determines a set point for glycogen stores, “senses” intracellular glycogen levels, and adjusts the hormonal regulation of glucose uptake, metabolism, and storage are uncertain. In 3T3-L1 adipocytes, the reduction of glycogen stores achieved in this study correlated with several responses geared toward increasing glucose storage as glycogen, suggesting that 3T3-L1 adipocytes can also adapt to changes in intracellular glycogen stores (24). The marked drop in cellular glycogen levels following delivery of the PTG siRNA construct resulted in increased GLUT1 expression, enhanced basal and insulin-stimulated glucose uptake, and acute glycogen synthetic rates. Disruption of the PTG-PP1 complex also corresponded to a signification reduction in glycogen phosphorylase levels. The effects on GLUT1 and glycogen phosphorylase appeared to be specific, as there was no corresponding change in GLUT4 or glycogen synthase protein amounts. It is tempting to speculate that the physiological expression of PTG and subsequent targeting of PP1 to glycogen in 3T3-L1 adipocytes establish a basal tone of glucose flux into the cell and storage as glycogen. This physiological glycogen set point is somehow monitored by the cell, resulting in appropriate expression of proteins involved in glucose uptake, metabolism, and storage. Disruption of PTG expression in 3T3-L1 adipocytes resulted in a significant decrease in glycogen levels, which the cell attempted to counteract through several compensatory mechanisms that increased acute insulin metabolic action. Ultimately, however, these changes were unable to overcome the loss of PTG, resulting in inappropriate glycogen phosphorylase phosphorylation and dysregulation of glycogenolysis. Further study will be required to explore and define the molecular links between altered glycogen metabolism and these potential changes in gene transcription and increased insulin responsiveness in 3T3-L1 adipocytes.

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