Protein Targeting to Glycogen Overexpression Results in the Specific Enhancement of Glycogen Storage in 3T3-L1 Adipocytes*

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Protein phosphatase-1 (PP1) plays an important role in the regulation of glycogen synthesis by insulin. Protein targeting to glycogen (PTG) enhances glycogen accumulation by increasing PP1 activity against glycogen-metabolizing enzymes. However, the specificity of PTG's effects on cellular dephosphorylation and glucose metabolism is unclear. Overexpression of PTG in 3T3-L1 adipocytes using a doxycycline-controllable adenoviral construct resulted in a 10–20-fold increase in PTG levels and an 8-fold increase in glycogen levels. Inclusion of 1 μg/ml doxycycline in the media suppressed PTG expression, and fully reversed all PTG-dependent effects. Infection of 3T3-L1 adipocytes with the PTG adenovirus caused a marked dephosphorylation and activation of glycogen synthase. The effects of PTG seemed specific, because basal and insulin-stimulated phosphorylation of a variety of signaling proteins was unaffected. Indeed, glycogen synthase was the predominant protein whose phosphorylation state was decreased in 32P-labeled cells. PTG overexpression did not alter PP1 protein levels but increased PP1 activity 6-fold against phosphorylase in vitro. In contrast, there was no change in PP1 activity measured using myelin basic protein, suggesting that PTG overexpression specifically directed PP1 activity against glycogen-metabolizing enzymes. To investigate the metabolic consequences of altering PTG levels, glucose uptake and storage in 3T3-L1 adipocytes was measured. PTG overexpression did not affect 2-deoxy-glucose transport rates in basal and insulin-stimulated cells but dramatically enhanced glucose incorporation into lipid were unchanged. Cumulatively, these data indicate that PTG overexpression in 3T3-L1 adipocytes results in the development of insulin resistance and type II diabetes (4, 5). However, the molecular mechanisms by which insulin potently regulates energy uptake and storage in vivo are not fully understood.

Glycogen synthase activity is stimulated by insulin in liver, muscle, and adipose tissue via protein dephosphorylation, allosteric activation, and enzymatic translocation. Glycogen synthase is phosphorylated on up to nine residues by a variety of kinases, resulting in its progressive inactivation (6). Insulin increases glycogen synthase activity by stimulating the dephosphorylation of four key residues. Both activation of protein phosphatase-1 (PP1) and inactivation of glycogen synthase kinase-3 (GSK3) have been proposed to mediate this metabolic effect of insulin (7), although the relative contribution of each enzyme remains unclear. Furthermore, maximal stimulation of glycogen synthase activity by insulin also requires increased glucose uptake and metabolism by target cells (8–10). Glucose-6-phosphate is an allosteric activator of glycogen synthase, overriding phosphorylation-dependent inhibition (9, 11, 12), whereas glucose metabolites and glycogen content mediate the redistribution of glycogen synthase (10, 13–15). Thus, insulin potently activates glycogen synthase through the synchronized increase in glucose uptake and protein dephosphorylation (16).

Insulin treatment induces the phosphorylation of a variety of signaling molecules and simultaneously stimulates the PP1-mediated dephosphorylation of a very limited number of proteins, primarily rate-limiting enzymes involved in glucose and lipid metabolism (17). One clue to understanding the discrete regulation of PP1 activity by insulin has emerged from the interaction of the phosphatase with targeting subunits. PP1 is a cytosolic protein yet is found in most cellular compartments via binding to more than 50 PP1-targeting subunits (18). Four proteins have been described which bind to both PP1 and glycogen, thus targeting the phosphatase to the glycogen particle (19). Gγ/RG1/R3 is expressed in striated muscle, Gγ/R4 is primarily expressed in liver (20) but also in skeletal muscle in humans but not rodents (21), and PTG/R5 (22, 23) and R6 (24) are more ubiquitously expressed. Despite a proposed common function, no two targeting subunits share more than 50% sequence homology, which is largely confined to the PP1- and glycogen-binding regions (19). Additionally, adenovirus-mediated overexpression of Gγ/RG1, Gγ, or PTG revealed differences in the regulation of glycogen-metabolizing enzymes and stimulation of glycogen synthesis and breakdown (25, 26). Taken together, these observations suggest that each targeting

Insulin exerts its anabolic actions by promoting the uptake and storage of glucose and lipids in target tissues. Insulin coordinates the stimulation of the translocation of both glucose and fatty acid transporters to the cell surface (1, 2) and the modulation of the activity of key metabolic enzymes (3). In addition, insulin inhibits hepatic glycogenolysis and adipocytic triglyceride breakdown, further lowering excess glucose and free fatty acids levels in the bloodstream. Disruption of the complex interplay between lipid and carbohydrate metabolism results in the development of insulin resistance and type II diabetes (4, 5). However, the molecular mechanisms by which insulin potently regulates energy uptake and storage in vivo are not fully understood.

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‡ The abbreviations used are: PP, protein phosphatase; PTG, protein targeting to glycogen; GSK3, glycogen synthase kinase-3; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; 2-DG, 2-deoxy-glucose; dox, doxycycline.

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subunit may confer differential regulatory properties to basal PP1 activity and/or hormonal responsiveness.

Cellular overexpression of PTG resulted in a significant increase in glycogen accumulation in a variety of cell types (23, 25, 27, 28). These changes were accompanied by the activation of glycogen synthase and/or inactivation of glycogen phosphorylase (25, 28, 29). However, the effects of glycogen-targeting substrates on cellular overexpression of PP1 activity or gross glycogen phosphorylation patterns have not been described. Therefore, we examined the impact of increasing PTG levels in 3T3-L1 adipocytes on PP1 and glycogen synthase activity, insulin signaling, and glucose uptake and storage. Using adenovirus-mediated gene transfer, we found that PTG overexpression resulted in a discrete and potent increase in PP1 activity directed against glycogen synthase and phosphorylase, resulting in the specific enhancement of basal and insulin-stimulated glycogen accumulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents and calf serum were supplied by Mediatech, Inc., and fetal bovine serum (FBS) was obtained from HyClone. The Adeno-X Tet-Off Expression System kit and tetracycline-free FBS were purchased from Clonetech. Cesium chloride and X-gal were obtained from Research Products International; glutaraldehyde, potassium ferrocyanide, and potassium ferricyanide were purchased from Fisher Scientific. Glucose determination kit and all other chemicals were provided by Sigma. UDP-[U-14C]glucose (60 Ci/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO); 2-deoxy-D-[14C]glucose (317 mCi/mmol), [-32P]Pi (4500 Ci/mmol), and [-32P]phosphoric acid were from ICN Pharmaceuticals.

Anti-PTG antibody was generated in rabbits against a peptide comprising amino acids 250–269 of PTG and affinity-purified as described previously (25), using a truncated glutathione S-transferase-PTG construct (amino acids 100–295) immobilized on Affi-gel 10 (Bio-Rad). Commercial sources of antibodies: anti-glycogen synthase, Chemicon; anti-phosphoryinosine, Upstate Cell Signaling Solutions; anti-phospho-p44/p42 MAPK (T262/Y264), anti-phospho-GSK-3 (S21/9), anti-GSK3β, and anti-phospho-Akt (T308), Cell Signaling Technology; anti-PP1, Santa Cruz Biotechnology; anti-GLUT1 and -GLUT4, Alpha Diagnostic International; horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG, Bio-Rad. Enhanced chemiluminescence reagent was supplied by Amer sham Biosciences.

**Cell Culture—**3T3-L1 cells were cultured and differentiated as described previously (10), and infected within 7 days after completion of the differentiation protocol. Human embryonic kidney 293 cells (Clonetech) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.29 mg/ml glucose.

**Adenovirus Preparation—**PTG was subcloned into the tetracycline-responsive Adeno-X DNA vector according to the supplier’s instructions. To obtain high-titer virus stocks, 12 to 24 h before infection, human embryonic kidney 293 cells were plated in 20×T150 flasks at a density of 3×10^4 cells/ml (200 ml/flask). The day of infection, virus was added to fresh medium at a concentration of 10 viral particles per cell. After 50% cell death was achieved (typically 3–5 days), cells and medium were collected in 50 ml tubes and spun at 800×g for 2 min at room temperature. Medium was aspirated and the cell pellet was resuspended in 500 μl of phosphate-buffered saline (PBS). Cells were lysed by five cycles of freezing and thawing in ice/cethanol and thawing at 37 °C. The lysate was spun at 800×g for 2 min at room temperature. 4.4 g cesium chloride was mixed with 6–8 ml of virus-containing supernatant, and PBS was added for a total of 10 ml. The sample was transferred to a 12-ml thin-wall ultracentrifuge tube (Seton Scientific), overlaid with 2 ml of mineral oil, and spun in a SW40 rotor at 32,000 rpm. The band containing the virus (~5 cm from the top of the tube) was removed using an 18-gauge needle. Virus was dialyzed using dialysis cassettes (Pierce) against 4 l of PBS at 4 °C for 2–4 h and then for an additional 12–14 h against fresh PBS. Virus was diluted with an equal volume of storage buffer (10 mM Tris-HCl, pH 8.0, 20% glycerol), quantitated by spectrophotometric analysis, and stored at −80 °C.

**Infection of 3T3-L1 Adipocytes—**Adenovirus encoding the gene of interest and regulatory virus (Adeno-X Tet-off) were diluted to the appropriate concentration in DMEM, 2% FBS, and 0.5 μg/ml poly-t-lysine hydrobromide (30) in sterile tubes and preincubated for 45 min at room temperature. 3T3-L1 adipocytes differentiated in 12-well dishes were washed once with PBS. Unless otherwise noted, 350 μl of preincubated virus mix was added to each well at a concentration of 2.5×10^4 particles of virus per cell and placed in a CO_2 incubator at 37 °C. Mock-infected cells were treated under the same conditions without virus. After 4 h, 0.65 ml/well culture medium was added to all wells (without removing adenovirus-containing medium), in the absence of 0.65 ml/well 2-deoxyglucose (50 μM) and 500 nM okadaic acid for 40–48 h before use. For β-galactosidase controls, after recovery, cells were washed twice with PBS and fixed by addition of 400 μl glutaraldehyde (2.5% in PBS) per well for 5 min. After four washes with PBS, 400 μl of staining solution (0.14% (w/v) potassium ferrocyanide, 0.12% (w/v) potassium ferricyanide, 1 mM MgCl_2, and 2.5 mg/ml X-gal) was added per well, and plates were incubated at 37 °C for 5–15 min. Cells were then washed twice with PBS and analyzed by light microscopy.

**Preparation of Lysates—**After infection, cells were washed twice with Krebs-Ringer buffer supplemented with 25 mM HEPES, pH 7.4, 0.5% bovine serum albumin, and 5 mM glucose, and incubated for 2.5 h in the same medium. Half the cells were treated for 15 min with 100 μM insulin. Cells were washed 3 times on ice with cold PBS and scraped into homogenization buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 10% glycerol, 0.5% Triton X-100, and protease inhibitors added just before use). Whole cell extracts were then analyzed by immunoblotting as described previously (31).

**Measurement of Total Glycogen—**Infected 3T3-L1 adipocytes were washed three times on ice with cold PBS and collected into 200 μl of lysis buffer. 50 μl of the supernatant was removed for the remaining sample was centrifuged at 5000×g for 5 min at 4 °C. 100 μl of the supernatants or glycogen standards (0–200 μg) were spotted onto GF/A filters. Filters were washed in ice-cold 70% ethanol for 15 min followed by two 10-min washes in 70% ethanol at room temperature. After drying overnight, filters were placed in 13×100-mm glass tubes and incubated with 1 ml of amyloglucosidase (0.04% in 0.05% sodium acetate) for 90 min at 37 °C. Glucose present in 100 μl of the reaction was analyzed using a kit (Sigma) according to the manufacturer’s instructions.

**Phosphatase Assays—**32P-labeled phosphorylase was prepared as described previously (31), except that recombinant phosphorylase kinase was used. 32P-labeled myelin basic protein was prepared in the same manner, using 3 mg of substrate and protein kinase A catalytic unit (Promega). Lysates were prepared, and PP1 and PP2A assays were performed as reported (32), except that reactions were run for 4 min at 35 °C. Phosphatase activity inhibited by addition of 3 mM okadaic acid was defined as PP2A, whereas phosphatase activity lost between 3 and 500 mM okadaic acid was defined as PP1. Addition of 500 mM okadaic acid completely inhibited cellular phosphatase activity against both substrates under these conditions.

**32P Labeling of Cells—**After infection and recovery, 3T3-L1 adipocytes plated on 6-well dishes were switched into 1 ml/well of phosphate-free DMEM containing 5% FBS and 250 μM of [32P]phosphoric acid. After 1 h, cells were washed three times on ice with PBS, collected into homogenization buffer lacking Triton X-100, and lysed by 10 strokes in a glass Dounce homogenizer. Samples were then subjected to 1,000×g centrifugation to pellet nuclei, and the resulting supernatants were centrifuged for 1 h at 100,000×g. The pellet was resuspended in homogenization buffer (equal volume to supernatant) using a 1-ml syringe and an 18-gauge needle. Supernatant and resuspended pellet fractions were separated by SDS-PAGE, transferred to nitrocellulose and analyzed by autoradiography. PTG expression and glycogen synthase location were then determined by immunoblotting of the same nitrocellulose membrane.

**Metabolic Assays—**Partitioning of glucose into lipid versus glycogen was determined as described previously (33), with the following modifications. 3T3-L1 cells were differentiated in 12-well dishes. After 15 min incubation with or without 100 nM insulin, 32P-glucose was added to all wells. Cells were incubated at 37 °C for 30 min, washed three times with cold PBS, and collected in 1.1 ml of distilled water. 500 μl of the cell suspension was added to 500 μl of distilled water for extraction with Betafluor (National Diagnostics) and measurement of glucose incorporation into lipid, 500 μl was added to 500 μl of 60% KOH for glycogen synthesis determination, and 50 μl was analyzed by SDS-PAGE and immunoblotting. Glycogen synthase activity was assayed as described previously (31), and 2-deoxy-glucose (2-DG) transport measurements were performed as previously reported (33), with the modifications that glucose-free DMEM plus 0.5% FBS was used as the base medium throughout.
Adenovirus-mediated Overexpression of PTG Increases Glycogen Levels in 3T3-L1 Adipocytes—To determine the effect of modulating PTG levels in 3T3-L1 adipocytes, a tetracycline-controllable (Tet-Off) adenoviral construct encoding PTG was generated. In preliminary experiments, 3T3-L1 adipocytes were infected with adenovirus encoding LacZ and stained for β-galactosidase expression. Results indicated that infection efficiency of >90% was obtained under conditions used (data not shown). Cells were then infected with various amounts of PTG adenovirus, allowed to recover for 48 h, and lysates were prepared. Anti-PTG immunoblotting revealed that PTG levels increased in a dose-dependent manner (Fig. 1, top). A variable amount of a faster migrating PTG breakdown product was also observed (compare Figs. 1A and 2A). However, there was no correlation between the intensity of this closely migrating species and subsequent results obtained. Addition of 1 μg/ml of doxycycline (dox) to the recovery medium repressed PTG overexpression (Fig. 1A, top), providing a control to distinguish between specific effects of PTG overexpression and potential nonspecific viral effects. Total glycogen levels changed in parallel with PTG overexpression, with an 8-fold increase at 2.5 × 10⁵ viral particles/cell (Fig. 1A, bottom). Consistent with immunoblotting results, addition of 1 μg/ml dox to the recovery medium completely blocked PTG-mediated glycogen accumulation (Fig. 1A, bottom). Further elevations of glycogen accumulation were obtained at viral concentrations up to 2 × 10⁶ (Fig. 1A and data not shown), but nonspecific effects, such as changes in cell morphology and cell detachment, were observed. Thus, a viral titer of 2.5 × 10⁵ particles/cell was used for all subsequent infections. To compare endogenous and exogenous PTG levels, lysates from mock-infected cells and various dilutions of lysates from PTG-overexpressing cells were analyzed by immunoblotting (Fig. 2B). These results indicated that adenoviral-mediated PTG overexpression increased cellular PTG levels by 10–20-fold.

PTG Overexpression Specifically Increases Glycogen Synthase Dephosphorylation and Activity—To investigate the mechanism by which PTG overexpression increased glycogen accumulation, glycogen synthase phosphorylation state and activity were compared in control and PTG-overexpressing cells. After infection, recovery, and a 2.5-h serum starvation, cells were treated in the absence and presence of 100 nM insulin for 15 min. Cell lysates were prepared and analyzed by immunoblotting. Dephosphorylation of glycogen synthase corresponds to downhill electrophoretic mobility shifts on SDS-PAGE. Thus, insulin stimulation of control cells caused an increase in the intensity of the faster-migrating glycogen synthase band on immunoblots (Fig. 2A, lane 2 versus 1). PTG overexpression induced a marked increase in glycogen synthase phosphorylation in the basal state (Fig. 2A, lane 5), whereas insulin treatment resulted in further dephosphorylation of glycogen synthase (Fig. 2A, lane 6). Addition of 1 μg/ml dox to the recovery medium suppressed PTG expression and fully reversed the altered mobility of glycogen synthase, but the insulin-mediated dephosphorylation event was preserved (Fig. 2A, lanes 7 and 8). These data indicated that PTG overexpres-
3T3-L1 adipocytes were stimulated with or without 100 nM insulin after serum starvation, control and PTG-overexpressing cells were treated with or without insulin for 15 min. Cell lysates were prepared and analyzed by immuno blotting. Bands corresponding to PTG (α-PTG), protein phosphatase-1 (α-PP1), GSK3β (α-GSK3β), tyrosine-phosphorylated insulin receptor (α-pIR), phospho-Akt (α-pAkt), phospho-GSK3β (α-pGSK3β), and phospho-mitogen-activated protein kinase (α-pMAPK) are indicated by arrows. Results are representative of three to five independent experiments.

To confirm that PTG overexpression resulted in glycogen synthase activation, enzymatic activity was measured in vitro. After serum starvation, control and PTG-overexpressing 3T3-L1 adipocytes were stimulated with or without 100 nM insulin for 15 min, and cell lysates were prepared. In control cells, the basal activity ratio was low, and insulin stimulation doubled the glycogen synthase activity ratio to 0.12 (Fig. 2B). In cells overexpressing PTG, total glycogen synthase activity was unchanged (data not shown). However, the basal glycogen synthase activity ratio was elevated to that measured in insulin-stimulated control cells (Fig. 2B). Furthermore, insulin treatment increased the glycogen synthase activity ratio to 0.25, double the insulin-stimulated activity in control cells. The insulin-induced dephosphorylation and activation of glycogen synthase in PTG-overexpressing cells may reflect inactivation of GSK3 (see below) and/or further PP1-mediated glycogen synthase activation, resulting from increased glucose uptake and metabolism (12, 34). Additionally, the enhanced basal activation state of glycogen synthase probably mediated the robust increase of glycogen levels in cells overexpressing PTG (Fig. 1).

Both GSK3 phosphorylation and inactivation and PP1 activation have been proposed to mediate insulin stimulation of glycogen synthase activity (7). To identify the mechanism by which PTG overexpression increased glycogen synthase activity, several insulin-sensitive proteins were compared in control and PTG-overexpressing cells. After infection, recovery and serum starvation, cells were treated with or without insulin for 15 min. Cell lysates were prepared and analyzed by immunoblotting. Consistent with previous studies (23, 29), PTG overexpression had no effect on PP1 levels (Fig. 3, PP1). In addition, GSK3β expression was also unchanged (Fig. 3, GSK3β), whereas GSK3α is expressed at very low levels in these cells (data not shown). These results indicate that enhanced glycogen synthase dephosphorylation occurred independently of activation increased both basal and insulin-stimulated glycogen synthase dephosphorylation in 3T3-L1 adipocytes.

To better characterize the breadth of PTG-induced protein dephosphorylation, protein phosphorylation patterns were compared in control and PTG-overexpressing cells. After infection and recovery, cells were incubated with [32P]orthophosphate for 1 h. Cells were then lysed with a Dounce homogenizer, and cytotoxic and particulate fractions were obtained by ultracentrifugation. The samples were separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography. Results are representative of three independent experiments.

Table 1. PTG overexpression decreases the phosphorylation of a limited number of proteins. After infection and recovery, 3T3-L1 adipocytes plated on 6-well dishes were incubated in phosphate-free DMEM containing 5% FBS and with 250 μCi of [32P]orthophosphate/well for 1 h. Cells were lysed with a Dounce homogenizer and the supernatant (Cyto) and pellet (Particulate) fractions were obtained by ultracentrifugation. A, samples were separated by SDS-PAGE and transferred to nitrocellulose, and labeled proteins were detected by a 3-day exposure to film at –80 °C (C, ECL 32P autoradiography). B, glycogen synthase (α-GS) and PTG (α-PTG) localization on the same membrane as A was detected by ECL immunoblotting. Results are representative of three independent experiments.

All enzymatic reactions were performed in complete DMEM containing 5% FBS and with 250 μCi of [32P]orthophosphate/well for 1 h. Cells were lysed with a Dounce homogenizer, and cytotoxic and particulate fractions were obtained by ultracentrifugation. The samples were separated by SDS-PAGE, transferred to nitrocellulose, and initially analyzed by autoradiography. Infection of 3T3-L1 adipocytes with PTG adenovirus had very limited effects on the protein phosphorylation pattern in both fractions. A predominant 84 kDa phosphoprotein in the particulate fraction was dephosphorylated upon PTG overexpression (Fig. 4A, particulate). This protein was subsequently identified as glycogen synthase by immunoblotting the same sheet of nitrocellulose (Fig. 4B, α-GS). However, despite an infection efficiency of >90%, glycogen synthase retained significant 32P labeling in the PTG-overexpressing cells (Fig. 4A). Thus, the PTG-PP1 complex may not recognize all nine phosphorylation sites on glycogen synthase, which may underlie the incomplete activation of glycogen synthase in PTG-overexpressing cells (Fig. 2B). In the particulate fraction, PTG overexpression also resulted in the lesser dephosphorylation of an unidentified protein at 60 kDa and, interestingly, the increased labeling of an unidentified 45 kDa protein. The latter effect may result from decreasing PP1 binding to another targeting subunit upon PTG overexpression. Finally, although the exogenous PTG was exclusively located in the particulate fraction (Fig. 4B, α-PTG), PTG overexpression induced the
dephosphorylation of an unidentified 25-kDa protein in the cytosolic fraction (Fig. 4A, cytosol). As before, all effects of infection with the PTG adenovirus were reversed by inclusion of dox in the recovery media. Cumulatively, these data indicate that PTG overexpression directs PP1 activity against a limited number of proteins in 3T3-L1 adipocytes.

PTG Overexpression Increases PP1 Activity against Specific Substrates—Because PTG overexpression did not alter PP1 or GSK3 expression (Fig. 3), PTG probably increased glycogen synthase dephosphorylation via another mechanism. Previous work showed that PP1 binding to PTG in vitro increased PP1 activity against glycogen phosphorylase (29, 35). To determine whether PTG overexpression exerted similar effects on cellular PP1 activity, lysates from control and adenoviral infected cells were prepared, and phosphatase activity was assayed in vitro. Although PTG overexpression did not change PP1 levels (Figs. 3 and 5, inset), PP1 activity against glycogen phosphorylase was increased 6-fold (Fig. 5). In contrast, PTG overexpression did not alter PP1 activity against myelin basic protein, indicating that PP1 was not globally activated (Fig. 5). Furthermore, PTG had no effect on PP2A activity (Fig. 5), a phosphatase that does not bind to PTG (23). Together with the previous results, these data indicate that PTG overexpression specifically enhanced PP1 activity against glycogen-metabolizing enzymes.

PTG Overexpression Specifically Increases Glucose Flux into Glycogen—PTG overexpression stimulated basal glycogen synthase dephosphorylation and activation, resulting in a marked increase in cellular glycogen levels. To determine the effects of PTG on insulin-regulated glucose metabolism in 3T3-L1 adipocytes, rates of glycogen synthesis and lipogenesis were compared in control and PTG-overexpressing cells. After serum starvation and 15-min insulin stimulation, [14C]glucose was added to all wells. Glucose incorporation into glycogen or lipid was measured in parallel from each well. In control cells, basal rates of glycogen synthesis were low, whereas insulin markedly increased glycogen synthesis rates (Fig. 6A, mock). Similar to the results with glycogen synthase activity, PTG overexpression increased basal glycogen synthesis rates to levels comparable to those of insulin-stimulated control cells (Fig. 6A, PTG). Insulin treatment caused a further increase of 5-fold in glycogen synthase rates (Fig. 6A), reflecting stimulation of glucose transport (see below) and further activation of glycogen synthase (Fig. 2B). Thus, PTG overexpression dramatically enhanced basal and maximal insulin-stimulated glycogen synthesis rates. These results were specific for PTG overexpression, because glycogen synthesis rates were unchanged in β-galactosidase-overexpressing cells (data not shown). Interestingly, in 3T3-L1 adipocytes, most of the insulin-stimulated glucose flux was recovered as glycogen (Fig. 6, A versus B; note the 10-fold difference in scale). Despite the marked effects of PTG overexpression on glycogen synthesis, glucose incorporation into lipid was unaffected in basal and insulin treated cells (Fig. 6B). Thus, the discrete protein dephosphorylation measured upon PTG overexpression was mirrored by the specific enhancement of glucose storage as glycogen.

Insulin-stimulated glycogen synthesis in muscle and fat requires increased glucose uptake and glycogen synthase activation. To determine whether PTG overexpression increased facilitated glucose uptake, 2-DG transport was measured in basal and insulin-stimulated cells. Because this glucose analog is not metabolized after phosphorylation by hexokinases, its transport rate is not influenced by altered glucose metabolism, and thus corresponds to glucose transporter activity at the cell surface. Insulin stimulated 2-DG transport to a similar extent in control and PTG overexpressing cells (Fig. 7). However, infection of cells with adenovirus modestly increased basal uptake. This result was probably a nonspecific effect of adenoviral infection, because suppression of PTG expression with dox...
in the absence of glucose. After 30 min, 20 μM insulin was added to all wells. After 2 min at room temperature, the wells were washed three times with PBS, cells were collected and analyzed by Scintillation counting. Cellular GLUT1 and GLUT4 levels were analyzed by immunoblotting (inset). M, Mock; M + D, Mock + dox; P, PTG; P + D, PTG + dox. PTG levels were analyzed by immunoblotting (bottom). Lanes 1–4, Mock; lanes 5–8, PTG; lanes 1, 2, 5, 6, no dox; lanes 3, 4, 7, 8, +1 μg/ml dox. Glucose transport results are the average of two independent experiments, each performed in triplicate, whereas the immunoblots are representative of three independent determinations.

(Fig. 7, lanes 7 and 8) did not reverse the change. To further characterize this effect, lysates were subjected to anti-GLUT1 and -GLUT4 immunoblotting (Fig. 7, inset). PTG overexpression or adenoviral infection had no effect on GLUT4 levels. However, GLUT1 levels were increased in infected cells independently of dox addition (Fig. 7, inset, P, P + D) and most probably accounted for the increase in basal 2-DG transport. However, basal glycogen synthesis rates were unchanged in infected cells incubated with dox (Fig. 6a), indicating that the increased GLUT1 expression alone did not markedly alter glucose storage in these cells. Cumulatively, these data indicated that PTG overexpression specifically increased glucose flux into glycogen in both basal and insulin-stimulated cells, independently of changes in insulin-stimulated glucose uptake or lipogenesis.

DISCUSSION

Protein dephosphorylation plays a critical role in the regulation of glucose and lipid metabolism by insulin. However, the molecular mechanisms by which insulin simultaneously modulates kinase and phosphatase activities to promote glucose transport and storage remain unclear. Recently, the theme of spatial compartmentalization of signaling molecules has emerged to explain the potent and unique stimulation of glucose transport, metabolism, and storage by insulin (16). PP1 regulates numerous, unrelated cellular processes and is also responsive to a wide variety of extracellular signals. This apparent enzymatic flexibility is mediated by the association of PP1 with a growing family of over 50 targeting subunits (18). We have previously reported that PTG is one of four glycogen-targeting subunits for PP1 (19, 23). PTG binds to both PP1 and glycogen, thus targeting the phosphatase to glycogen particles. In the present study, we have examined the effects of increasing PTG protein levels using adenovirus-mediated gene transfer in 3T3-L1 adipocytes. We used a tetracycline-controllable (Tet-Off) adenovirus construct encoding PTG. Inclusion of the tetracycline analog doxycycline in the recovery medium suppressed exogenous PTG protein levels and reversed all effects of PTG overexpression on PP1 and glycogen synthase activities and enhanced glycogen storage. This condition served as an important control; in preliminary experiments, we found that infection of 3T3-L1 adipocytes with higher titers of adenovirus than used in these experiments exerted nonspecific effects. Even at lower titers, adenoviral infection modestly elevated GLUT1 expression and basal 2-DG transport rates independently of dox addition (Fig. 7). Overexpression of PP1 glycogen-targeting subunits in a wide variety of cell types resulted in the dephosphorylation of glycogen synthase and phosphorylase and a marked elevation in glycogen levels (23, 25, 27, 28). In agreement, adenovirus-mediated overexpression of PTG in 3T3-L1 adipocytes caused a marked dephosphorylation of glycogen synthase and a corresponding increase in basal glycogen synthase activity. PTG overexpression also increased glycogen synthase synthesis by insulin 2-fold, resulting in an increase in maximal glycogen synthesis rates and accumulation in these cells. Additionally, as previously reported in hepatocytes (25), PTG overexpression also blocked forskolin-induced glycogenolysis (data not shown), indicating that inhibition of glycogen mobilization may contribute to the enhancement of glycogen stores.

The pattern of protein dephosphorylation in cells overexpressing PTG seemed highly specific. Despite the marked increase in glycogen synthase dephosphorylation, the basal and insulin-stimulated phosphorylation states of several important signaling proteins were unaffected by PTG overexpression. Furthermore, glycogen synthase was the predominant protein dephosphorylated in response to infection with the PTG adenovirus. The apparent specificity of PTG action on cellular protein dephosphorylation was partially explained by results from in vitro PP1 assays. PTG overexpression specifically increased PP1 activity against phosphorylase 6-fold, without any change in PP1 levels or activity against myelin basic protein. Thus, increasing PTG levels stimulated PP1 activity against a very limited number of substrates, principally involved in glycogen metabolism. Furthermore, these effects and the increased glycogen accumulation occurred without any detectable change in glycogen synthase, PP1, or GSK3β levels, indicating that the PTG-mediated funneling of PP1 activity toward glycogen synthase was the primary molecular mechanism underlying these responses.

PP1 glycogen-targeting subunits act by situating the phosphatase at glycogen particles, co-localizing the phosphatase with specific substrates that also bind to glycogen. Thus, PTG may increase PP1 activity toward glycogen-metabolizing enzymes simply by increasing total PP1 activity targeted to glycogen (data not shown). However, G1, PTG, and Rα/GM also directly bind to glycogen synthase and/or phosphorylase. Glycogen phosphorylase binding to G1 inhibited PP1 activity against glycogen synthase and thus acted as a negative regulator of the PP1-G1 complex (20, 36). In contrast, substrate binding to PTG or Rα/GM has been proposed to mediate efficient substrate recognition by PP1 (23, 29, 37). Point mutagenesis of the glycogen synthase-binding site on either targeting subunit completely blocked the enhancement of PP1 activity in vitro and glycogen synthase activation and glycogen accumulation in cells (29, 37). Thus, PTG and Rα/GM may act as scaffolding proteins, assembling PP1 with specific substrates at glycogen. However, the results in the present study do not distinguish between glycogen targeting and binding of specific substrates as the mechanism for the PTG-mediated enhancement of PP1 activity against glycogen synthase. Further work is needed to clarify this issue.
Activation of Glycogen Synthase by PP1

The importance of glycogen-targeted PP1 as a principal enzymatic regulator of glycogen synthase and phosphorylase has recently been confirmed using knock-out animal models. Two groups have reported that the disruption of the R severely reduced glycogen levels in skeletal muscle by more than 80% (39, 40). These changes were accompanied by a decrease in glycogen synthase activity and an increase in phosphorylase activity, reflecting increased phosphorylation of both proteins. Interestingly, insulin-stimulated glycogen synthase activation was largely preserved, indicating that PP1 bound to other glycogen-targeting subunits and/or another phosphatase activity was compensating for the loss of R. Additionally, despite both animal models being generated in similar mouse strains and using comparable targeting strategies, one line developed age-dependent obesity and insulin resistance (39), whereas the other did not exhibit any metabolic abnormalities (39). The reasons for these discrepancies are not clear. Very recently, homozygous disruption of the PTG gene was reported to be lethal, but the cause remains undetermined (40). Although the heterozygous mice exhibited milder alterations in glycogen metabolism in multiple tissues. Furthermore, these transgenic lines will be useful models to elucidate the reasons why several PP1 glycogen-targeting proteins are expressed with overlapping tissue distribution.

Despite the 8-fold increase in glycogen levels in PTG overexpressing cells at the start of experiments, glycogen synthase activity and glycogen synthesis rates were significantly elevated compared with control cells; importantly, they remained insulin responsive for up to 5 days after infection. We have previously reported that short-term increases in glycogen levels in 3T3-L1 adipocytes did not affect insulin-responsive signaling cascades or glucose transport but did completely block glycogen synthase activation (33). These findings were reminiscent of reports from skeletal muscle, where elevation of glycogen levels exerted profound negative effects on glycogen synthase activation and glucose storage by insulin (15, 41). Insulin and increased glycogen levels caused a redistribution of glycogen synthase protein localization in both cell types (10, 15, 33), potentially uncoupling glycogen synthase from its insulin-sensitive activators. The present study suggested that PTG overexpression in 3T3-L1 adipocytes overcame the negative feedback of glycogen levels on glycogen synthase activity, resulting in the marked increase in glycogen levels compared with uninfected cells. Very similar findings were recently reported from primary human skeletal muscle cells, in which PTG overexpression caused a sustained activation of glycogen synthase activity despite a 20-fold increase in glycogen levels (28). However, how glycogen levels feed back to regulate glycogen synthase activity or how increasing PTG levels override this inhibition remain unclear.

Finally, despite the marked enhancement of glucose flux into glycogen in PTG-overexpressing cells, absolute levels of basal and insulin-stimulated glucose storage as lipid (Fig. 6B) and cellular ATP levels (data not shown) were unaltered. The specificity of PTG effects on glucose utilization and storage were somewhat surprising. A priori, one could expect that the dramatic increase in glycogen accumulation upon PTG overexpression would reduce glucose entrance into the glycolysis/ lipogenesis pathway. Conversely, the increased glucose flux into the cells could also “spill over” into these metabolic pathways. For example, tissue-specific overexpression of GLUT4 in adipose tissue of mice resulted in a 20–40-fold increase in glucose uptake and 2–3-fold stimulation of lipid accumulation, resulting in an obese phenotype (42, 43). However, these cellular and animal results are not necessarily conflicting and may be explained from studies in hepatocytes. Adenosine-mediated overexpression of glucokinase in the livers of intact rats resulted in an increase in hepatic glycogen storage and a decrease in plasma glucose levels (44). However, these changes were accompanied by marked elevations in circulating triglyceride and free fatty acid plasma levels, presumably because of increased hepatic glucose flux into triglyceride synthesis. In contrast, infection of rats with adenosinergic constructs encoding PTG or a truncated R construct augmented hepatic glycogen stores and significantly lowered blood glucose levels without altering plasma lipid profile (27, 45). Thus, “pushing” glucose into hepatocytes by overexpressing glucokinase increased glucose utilization by both glycogenic and lipogenic pathways, and “pulling” glucose into liver upon overexpression of PP1-glycogen targeting subunits resulted in the specific enhancement of glycogen accumulation (26). An analogous situation may occur in adipocytes depending on whether GLUT4 (push) or PTG (pull) is overexpressed, resulting in augmented lipid or glycogen accumulation, respectively. Transgenic overexpression of PTG specifically in adipose tissue is currently being pursued to investigate this possibility.

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Activation of Glycogen Synthase by PP1

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