Overexpression of Protein Targeting to Glycogen (PTG) in Rat Hepatocytes Causes Profound Activation of Glycogen Synthesis Independent of Normal Hormone- and Substrate-mediated Regulatory Mechanisms*

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Protein targeting to glycogen (PTG), also known as PPP1R5, is a widely expressed member of a growing family of proteins that target protein phosphatase-1 (PP-1) to glycogen particles. Because PTG also binds to glycogen synthase and phosphorylase kinase, it has been suggested that it serves as a “scaffold” for efficient activation of glycogen synthesis. However, very little is known about the metabolic effects of PTG. In this study, we have used recombinant adenovirus to overexpress PTG in primary rat hepatocytes, a cell type with high glycogenic capacity. We find that overexpression of PTG potently activates glycogen synthesis in cultured hepatocytes. Surprisingly, the glycogenic effect of PTG is observed even in the complete absence of carbohydrates or insulin in the culture medium. Furthermore, glycogenolytic agents such as forskolin or glucagon are largely ineffective at activating glycogen degradation in PTG overexpressing hepatocytes, even though large increases in cAMP levels are demonstrated. These metabolic effects of PTG overexpression are accompanied by a 3.6-fold increase in glycogen synthase activation state and a 40% decrease in glycogen phosphorylase activity. Our results are consistent with a model in which PTG overexpression “locks” the hepatocyte in a glycogenic mode, presumably via its ability to promote interaction of enzymes of glycogen metabolism with PP-1.

Glycogen storage in liver is a process that has central importance in control of glucose homeostasis (1). In recent years, evidence has begun to accumulate in support of the notion that regulation of glycogen metabolism involves movement of relevant enzymes and regulatory factors within cells and the assembly and disassembly of complexes of proteins that mediate glycogen synthesis and degradation (2–14). This has focused attention on a growing gene family known collectively as glycogen targeting subunits can be used to enhance glucose storage in liver cells, possibly serving as an approach for lowing blood glucose levels in non-insulin-dependent diabetes, a disease in which hepatic glycogen synthesis is impaired (17). We find that PTG overexpression profoundly activates glycogen synthesis, even in the absence of glucose, and that normal hormone- and substrate-mediated regulatory mechanisms are largely overridden by this maneuver.

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1 The abbreviations used are: PP-1, protein phosphatase-1; PTG, protein targeting to glycogen; DMEM, Dulbecco’s modified Eagle’s medium; G6P, glucose 6-phosphate.

MATERIALS AND METHODS

Generation of Recombinant Adenoviruses—A cDNA clone containing the entire protein coding sequence of mouse protein targeted to glycogen (PTG) was prepared by polymerase chain reaction amplification of 3T3-L1 adipocyte cDNA, using oligonucleotide primers derived from the published mouse PTG cDNA sequence (5′-CCGATTCGGNACGAGATTGCTAGATCC-3′ (sense), encompassing nucleotides (−) 3–26, and 5′-GCGAAGTTGCTGGAATTCTCAGTAG-3′ (antisense), encom-
passing nucleotides 1116–1090; Ref. 6). A 1.1-kilobase EcoRI fragment was verified as authentic by sequence analysis and used to prepare a recombinant adenovirus (AdCMV-PTG) as described previously (18). In addition, AdCMV-βGAL, a virus containing the bacterial β-galactosidase gene (19), was used as a control in the metabolic studies described below.

Hepatocyte Isolation and Treatment with Recombinant Adenoviruses—Monolayer hepatocyte cultures were prepared from overnight fasted male Wistar rats by incubation of freshly isolated cells for 1 h at 37 °C in “attachment medium,” consisting of DMEM (Life Technologies, Inc.) containing 5% fetal bovine serum, 100 uM dexamethasone, insulin, and penicillin/streptomycin. Cells were washed twice with phosphate-buffered saline and then resuspended in “culture medium,” consisting of DMEM containing 0.2% bovine serum albumin, and unless otherwise specified, 10 μM dexamethasone, 1 μM insulin, no glucose, 2 mM pyruvate, and 2% penicillin/streptomycin. Cells were washed twice with phosphate-buffered saline and, unless otherwise specified, experiments were performed following 36 h of further incubation in culture medium. These incubations included a replenishment of fresh culture medium 24 h after adenovirus treatment.

RNA Blot Hybridization Analysis—Total RNA was isolated from rat hepatocytes by guanidine isothiocyanate–phenol extraction (TriZOL, Life Technologies, Inc.), electrophoresed in 2% agarose, 7.5% formaldehyde gels, transferred to nylon membranes (ICN), and hybridized overnight at 65 °C with a fluorescein-N4-dATP labeled probe (NEN Life Science Products) prepared by random priming from a 1.1-kilobase EcoRI fragment of the PTG cDNA in 250 mM NaHPO4, pH 7.2, 1 mM EDTA, 0.5% blocking reagent (NEN Life Science Products), 5% SDS. Hybridized fluorescein-labeled probe was detected using ECL chemiluminescence reagents (Amersham).

Enzyme Activity Assays—Hepatocytes were grown in 6-cm plates, flash frozen in liquid nitrogen for 10 min, and collected with a plastic scraper into 200 μl of ice-cold homogenization buffer (10 mM Tris, pH 7.0, 150 mM KF, 15 mM EDTA, 10 mM mercaptoethanol, 10 μM leupeptin, 1 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride). Glycogen synthase activity was measured as described (10). In one-half of the samples, glucose 6-phosphate (G6P) was added to a final concentration of 10 μM, and the glycogen synthase activity ratio was defined as the activity in the absence compared with the presence of G6P (10). Glycogen phosphorylase activity was measured as described (21).

Metabolic Measurements—Glycogen levels in hepatocytes cultured in 6-cm dishes were measured by the method of Chan and Exton (22), with minor modifications as described (23). Intracellular concentrations of glucose 6-phosphate were measured as described (24). cAMP levels in media samples were measured with an enzyme immunoassay (Titer-Zyme, PerSeptive Biosystems).

RESULTS AND DISCUSSION

Overexpression of PTG in Hepatocytes Activates Glycogen Synthesis in a Glucose- and Insulin-independent Manner—In a previous study, Saltiel and colleagues (6) demonstrated that transient transfection of CHO-IR cells (a CHO cell line stably overexpressing the insulin receptor) with PTG resulted in a 4.5-fold increase in incorporation of radiolabeled glucose into glycogen relative to control cells. As stated by the authors, this increase may have been underestimated due to the approximate 10–20% transfection efficiency predicted for the transient method used. We have shown previously that recombinant adenovirus allows delivery of genes to various primary cell types and cell lines with near 100% efficiency, and as such is an ideal method for studies on metabolic control strength of specific enzymes (9–11, 18, 25). This has allowed us, in the current study, to obviate the metabolic impact of efficient PTG overexpression in primary rat hepatocytes.

Hepatocytes from fasted rats were treated with AdCMV-PTG or AdCMV-βGAL and collected at intervals 3–48 h after viral treatment for measurement of glycogen levels (Fig. 1). Initial experiments were conducted in DMEM medium lacking glucose but containing 2 mM pyruvate. As shown in Fig. 1A, hepatocytes treated with AdCMV-βGAL maintained a low constant level of glycogen throughout the time course. To our surprise, a sharp increase in glycogen content was evident in AdCMV-PTG-treated cells, even in the complete absence of glucose, beginning between 15 and 24 h after viral treatment. By 48 h, glycogen levels in AdCMV-PTG-treated cells were 10.9-fold higher than in controls. Fig. 1B shows that the time course of glycogen accumulation in response to PTG overexpression in primary hepatocytes, Panel A, hepatocytes were isolated from fasted rats and treated with AdCMV-PTG (filled squares) or AdCMV-βGAL (open squares) for 1 h. Cells were then cultured in DMEM lacking glucose but containing 2 mM pyruvate and harvested at the indicated times post-viral treatment for measurement of glycogen levels. Data represent the mean of two independent experiments. Panel B, cells treated identically and in parallel with those in A were collected for preparation of total RNA. Blot hybridization analysis was then carried out, using a PTG cDNA as probe, as described under “Materials and Methods.” The blot shown is representative of two independent experiments.

The experiments in Fig. 1 were conducted in DMEM medium lacking glucose but containing 2 mM pyruvate and 1 nM insulin. We therefore carried out experiments to learn more about the substrate and hormonal requirements for PTG activity. AdCMV-βGAL-treated control hepatocytes incubated with no carbohydrates, 2 mM pyruvate, or 2 mM glucose contained very low levels of glycogen after 36 h of incubation (6.5–9.8 μg of glycogen/mg of protein), while inclusion of 20 mM glucose raised the...
levels to 34.6 μg of glycogen/mg of protein. However, glycogen content in control cells incubated at 20 mM glucose was still only 22% and 9.5% of that achieved in AdCMV-PTG-treated cells incubated in the absence of carbohydrates (158 μg of glycogen/mg of protein) or at 20 mM glucose (364 μg of glycogen/mg of protein), respectively (Fig. 2A). For comparison, glycogen content in freshly isolated liver from 18-h fasted rats was found to be 2.8 ± 0.5 μg of glycogen/mg of protein, while that in ad libitum fed animals was 291 ± 44 μg/mg (n = 5 for both measurements). DMEM contains 4 mM glutamine and a total amino acid concentration of 10.7 mM, and gluconeogenic conversion of these amino acids to glycogen is the presumed mechanism by which glycogen synthesis proceeds in PTG overexpressing hepatocytes in the absence of media carbohydrates. Consistent with this idea, AdCMV-PTG-treated cells incubated in Hepes-buffered saline supplemented only with 8 mM glutamine, 0.2% bovine serum albumin, 1 mM insulin, 10 mM dextromethasone, and 2% penicillin/streptomycin accumulated 317 ± 16 μg of glycogen/mg of protein over 36 h (n = 3), equivalent to the amounts found in cells incubated in DMEM supplemented with carbohydrates. Note that in AdCMV-PTG-treated cells, glycogen accumulation was approximately doubled by inclusion of 20 mM glucose relative to cells incubated in 2 mM glucose, 2 mM pyruvate, or in the absence of carbohydrates. However, in experiments carried out for a longer time period (48 h), glycogen accumulation in PTG overexpressing cells incubated at low glucose or in its absence became equal to that in cells incubated at 20 mM glucose (data not shown).

The experiments in Fig. 2A were conducted in the presence of 1 mM insulin. To determine whether the hormone is required for the glycogenic effect of PTG, we performed 36-h incubation experiments in DMEM supplemented with 1 mM glucose, with the addition of 0, 1, 10, or 100 nM insulin. As shown in Fig. 2B, insulin is clearly not required for activation of glycogen synthesis by PTG, since there was abundant glycogen in AdCMV-PTG-treated hepatocytes in the absence of insulin relative to AdCMV-βGAL-treated control cells. Insulin instead modestly enhances PTG-mediated glycogenesis (65%, 103%, and 122% enhancements in glycogen accumulation at 1, 10, and 100 nM insulin, respectively, relative to cells incubated in the absence of the hormone). In sum, neither glucose nor insulin is required to observe a large glycogenic effect of PTG overexpression in rat hepatocytes, but either agent is able to exert a modest potentiating effect when applied at high concentrations.

PTG Overexpression Largely Prevents cAMP-mediated Glycogenolysis—We next tested whether the remarkable capacity of PTG overexpression to promote glycogenesis was reflected in an altered capacity to activate glycogenolysis. We cultured AdCMV-PTG-treated hepatocytes in DMEM containing 1 mM glucose, 2 mM pyruvate, and 1 nM insulin for 36 h, then replaced this with DMEM containing no insulin, 1 mM glucose, and 2 mM pyruvate in the presence or absence of varying concentrations of forskolin, glucagon, or 8-bromo-cyclic AMP for 4 h. As shown in Fig. 3, treatment of cells with 1, 10, or 100 μM forskolin caused 3-, 20-, and 67-fold increases in cyclic AMP levels, respectively, relative to cells cultured in the absence of the drug. Despite these large changes in cAMP levels, the higher concentrations of forskolin caused only a 14 and 22% decrease in glycogen content, respectively. Addition of glucagon in a range of concentrations from 10 nM–1 μM had a smaller effect on cAMP levels than forskolin (maximal increase in levels of 10-fold at 1 μM), but caused a similarly small decrease in glycogen content (Fig. 3). Only treatment of cells with very high levels of 8-bromo-cyclic AMP (2.5 mM) or incubation of cells in Hepes-buffered saline in the complete absence of nutrients was able to cause decreases in glycogen content of around 50% over the 4-h experimental period (Fig. 3). Thus, overexpression of PTG in hepatocytes largely blocks activation of glycogenolysis by agents that act via generation of cAMP.

Overexpression of PTG Causes Activation of Glycogen Synthase in the Absence of an Increase in Glucose 6-Phosphate Levels—Previous studies have shown that activation of glycogen synthesis in liver is accompanied by a rise in glucose 6-phosphate (G6P) levels, leading to the suggestion that G6P participates in
regulation of glycogen synthesis by its capacity to promote activation of glycogen synthase (27). As shown in Fig. 4, total glycogen synthase activity (measured with G6P added to the assay mixture) was increased by approximately 25% in AdCMV-PTG-treated hepatocytes compared with AdCMV-βGAL-treated controls. More importantly, however, “activated” glycogen synthase (measured in the absence of added G6P) was increased by nearly 5-fold in the PTG overexpressing cells. Calculation of the glycogen synthase activity ratio (defined as the activity measured in the absence of G6P relative to activity in the presence of G6P) from these data revealed an increase from 0.18 to 0.64 in response to AdCMV-PTG treatment, a 3.6-fold enhancement. These changes in glycogen synthase activity were accompanied by a 40% decrease in glycogen phosphorylase activity in AdCMV-PTG-treated hepatocytes compared with AdCMV-βGAL-treated control cells (Fig. 4). G6P concentrations were not different in the AdCMV-βGAL- and AdCMV-PTG-treated hepatocytes used for the enzyme assays (79.9 ± 15 μM versus 84.2 ± 13 μM, respectively), indicating that the large change in glycogen synthase activation state could not be attributed to a PTG-induced increase in G6P levels. It remains possible, however, that G6P levels increase in concert with the increase in glycogen deposition in AdCMV-PTG-treated hepatocytes observed at 20 compared with 2 mM glucose.

Several new properties of PTG are revealed by the foregoing experiments. First, PTG overexpression profoundly stimulates glycogen synthesis in the hepatocyte, showing that its effects are not specific to CHO cells, and consistent with the idea that the metabolic impact of PTG in the prior transient transfection studies (6) was underestimated. Second, the ability of PTG overexpression to activate glycogen synthesis is independent of the carbon source. Thus, similar amounts of glycogen are synthesized in PTG overexpressing cells incubated in media containing only amino acids as in media containing pyruvate or pyruvate plus 2 mM glucose. Glucose has previously been ascribed an important regulatory role in control of hepatic glycogen metabolism, in part due to the capacity of the sugar to bind directly to glycogen phosphorylase, resulting in inhibition of enzyme activity by both allosteric and dephosphorylation mechanisms (26). Overexpression of PTG appears to override this form of regulation, since glycogen synthesis clearly does not require glucose in AdCMV-PTG-treated cells.

These findings suggest that overexpression of PTG “locks” the cell in a glycogenic mode, probably by targeting of PP-1 to the glycogen particle, where it maintains glycogen synthase in a dephosphorylated and active state and glycogen phosphorylase and phosphorylase kinase in dephosphorylated and inactivated states. Previous studies showing direct binding of PTG to glycogen synthase and phosphorylase kinase provide further support for the proposal (6). We speculate that the capacity of PTG overexpression to lock the hepatocyte in a glycogenic mode may be a specific effect of this member of the family of glycogen

**Fig. 3.** Limited effect of glycogenolytic agents in PTG overexpressing hepatocytes. **Panel A,** hepatocytes were isolated from fasted rats and treated with AdCMV-PTG for 1 h, cultured in DMEM containing 1 mM glucose, 2 mM pyruvate, and 1 nM insulin for 36 h. After removal of the culture medium and washing, cells were then incubated for an additional 4 h in DMEM containing 1 mM glucose, 2 mM pyruvate, and no insulin, with addition of the agents noted along the x-axis of the panel. At the conclusion of this protocol, cells were collected for measurement of glycogen content. Data represent the mean ± S.E. for four independent experiments. **Panel B,** media from a subset of the cell groups described in A were collected for assay of cAMP. Data represent the mean ± S.E. for four independent measurements.

**Fig. 4.** Alterations in glycogen synthase and glycogen phosphorylase activities induced by PTG overexpression in hepatocytes. Hepatocytes isolated from fasted rats were treated with AdCMV-βGAL or AdCMV-PTG for 1 h, incubated in DMEM lacking glucose and containing 2 mM pyruvate and 1 nM insulin for 36 h, and harvested for assay of glycogen synthase (A) and glycogen phosphorylase (B) enzyme activities as described under “Materials and Methods.” Glycogen synthase activity was assayed in the absence (open bars) or presence (black bars) of 10 mM glucose 6-phosphate. For both panels, data represent the mean ± S.E. for three independent experiments.
targeting subunits, based on consideration of the properties of the various isoforms. G\textsubscript{M}, for example, is phosphorylated on serine 46 in response to insulin, and this appears to enhance the capacity of the G\textsubscript{M}-PP-1 complex to dephosphorylate and activate glycogen synthase, while \beta-adrenergic agonist-mediated phosphorylation of serine 65 results in dissociation and inactivation of the G\textsubscript{M}-PP-1 complex (15). G\textsubscript{I} lacks serine 46, and while it contains a serine at position 65, it appears not to be regulated by a phosphorylation/dephosphorylation mechanism, but is instead allosterically regulated by binding to phosphorylase \textit{a} (4). Neither the hormonal nor allosteric mechanisms appears to be operative in PTG (5, 6). Based on this one might predict that glycogen metabolism will be more sensitive to hormonal or nutritional regulation in G\textsubscript{M} or G\textsubscript{I} overexpressing cells compared with PTG overexpressing cells. However, it is also possible that yet undiscovered regulators of PTG are overwhelmed in the face of the large overexpression achieved with the adenovirus/CMV promoter combination, and that PTG may not truly "lock" the cell in a glycogenic mode if expressed at more modest levels.

Our studies also provide fresh support for the concept that formation and disassembly of complexes of metabolic enzymes at discrete subcellular sites is central to the regulation of glycogen metabolism. The fact that several enzymes of glycogen metabolism are directly associated with the glycogen particle has long been appreciated (2, 3). More recently, it has been shown that glucokinase (hexokinase IV) and glycogen synthase are sequestered in the nucleus under basal conditions and that both enzymes are translocated to the cytosol upon exposure to nutrients (7, 8, 11, 13, 14, 28). Furthermore, hepatic overexpression of hexokinase I, an enzyme that is activated by binding to porin in mitochondrial membranes (29), has no effect on activation of glycogen synthase or glycogen deposition, while overexpression of glucokinase impacts both parameters, suggesting that the subcellular localization of these enzymes determines their impact on glycogenesis (9, 10). Finally, electron microscopy studies provide evidence that glycogen synthesis occurs in a spatially organized fashion, with initial appearance of glycogen synthase and glycogen particles near the plasma membrane, followed by further synthesis in a gradient toward the central aspect of the cell (12). The data in the current study provide new support for the idea that members of the glycogen targeting subunit gene family play a vital role in the compartmentalization and regulation of glycogen synthesis.

In closing, one can consider the potential therapeutic relevance of up-regulated expression of PTG or its family members for treatment of diabetes. A fundamental metabolic defect in individuals with non-insulin-dependent diabetes is a failure to suppress hepatic glucose output in the fed state, and this is reflected in lower levels of hepatic glycogen in such individuals (17). While it is clear that PTG overexpression strongly enhances glycogen synthesis in liver cells, the fact that this protein appears to override normal control mechanisms for glycogen metabolism raises concerns about the therapeutic relevance of such a maneuver. Expression of other family members, such as G\textsubscript{M} or G\textsubscript{I}, in contrast, may allow continued substrate and hormone-mediated regulation of glycogen turnover. Since recombinant adenovirus is clearly an effective means for delivery of genes to liver of intact animals (30, 31), these ideas should be testable in future experiments.

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