Role of Protein Targeting to Glycogen (PTG) in the Regulation of Protein Phosphatase-1 Activity*

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We have recently cloned from 3T3-L1 adipocytes a novel glycogen-targeting subunit of protein phosphatase-1, termed PTG (Printen, J. A., Brady, M. J., and Saltiel, A. R. (1997) Science 275, 1475–1478). Differentiation of 3T3-L1 fibroblasts into highly insulin-responsive adipocytes resulted in a marked increase in PTG expression. Immobilized glutathione S-transferase (GST)-PTG fusion protein specifically bound either PP1 or phosphorylase a. Addition of soluble GST-PTG to 3T3-L1 lysates increased PP1 activity against 32P-labeled phosphorylase a by decreasing the $K_m$ of PP1 for phosphorylase 5-fold, while having no effect on the $V_{max}$ of the dephosphorylation reaction. Alternatively, PTG did not affect PP1 activity against hormone-sensitive lipase. PTG was not a direct target of intracellular signaling, as insulin or forskolin treatment of cells did not activate a kinase capable of phosphorylating PTG in vitro or in vivo. Finally, PTG decreased the ability of DARPP-32 to inhibit PP1 activity from 3T3-L1 adipocyte lysates. These data cumulatively suggest that PTG increases PP1 activity against specific proteins by several distinct mechanisms.

While much attention has been focused on the activation of protein kinase signaling cascades, many enzymes involved in glucose and lipid metabolism are regulated by dephosphorylation (2). As the main physiological hormone controlling glucose utilization, insulin exerts many of its effects through the mechanisms involved in glycogen metabolism and priming them for the reception of intracellular signals.

PP1 is found in many cellular compartments, including the nucleus, plasma membrane, and glycogen particle. It is thought that the cellular localization of this enzyme is mediated by its association with targeting proteins (4, 5). We have recently identified a novel PP1 glycogen-targeting subunit from 3T3-L1 adipocytes, termed PTG for protein targeting to glycogen (1). PTG is the third member of a family of PP1 glycogen-targeting subunits, which also includes RGL, isolated from muscle (6, 7), and the hepatic GGL protein (8, 9). In contrast to the restricted localization of RGL and GGL, PTG is highly expressed in all insulin-sensitive tissues. In addition to targeting PP1 to the glycogen particle, PTG can also form complexes with PP1 substrate enzymes that regulate glycogen metabolism, namely glycogen synthase, glycogen phosphorylase, and phosphorylase kinase. Overexpression of PTG in the metabolically inactive CHO-IR cell line dramatically increased the levels of basal and insulin-stimulated glycogen synthesis (1). PTG may therefore serve as a scaffolding protein, assembling the proteins involved in glycogen metabolism and priming them for the reception of intracellular signals.

The mechanism by which insulin specifically activates glycogen-targeted PP1 activity remains poorly understood. The proposed phosphorylation and activation of the PP1-RGL complex by pp90RSK (10) has subsequently been challenged (11–15). Further, the two putative PP1 regulatory phosphorylation sites of RGL are not conserved in PTG (1). Therefore, other mechanisms must exist for the regulation and activation of PP1 activity targeted to glycogen by PTG. The results presented here demonstrate that PTG is not a direct target for insulin-activated protein kinases. However, PTG does increase PP1-specific activity against phosphorylase a by three separate mechanisms: by targeting the phosphatase to glycogen, by directly binding and co-localizing PP1 substrates, and by reducing the affinity of PP1 for inhibitor peptides such as DARPP-32.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents, IPTG, glycogen phosphorylase b, and phosphorylase kinase were from Life Technologies, Inc. Okadaic acid was purchased from Calbiochem. [32P]Orthophosphate was obtained from ICN, whereas [$\gamma$-32P]ATP (3000 Ci/mmol) and [$\gamma$-32P]dATP (1250 Ci/mmol) were from NEN Life Science Products. [$\alpha$-32P]dCTP (3000 Ci/mmol) and ECL reagent were obtained from Amersham. Glutathione-Sepharose 4B beads and Ni-NTA agarose beads were purchased from Pharmacia Biotech Inc. and Qiagen, respectively. Polyhistidine-tagged DARPP-32 construct was the kind gift of Drs. J. Biał and A. Nairn (Rockefeller University), while chicken affinity-purified anti-PP1α antibody was generously provided by Dr. J. Lawrence (University of Virginia). GluT-4 antibody was from Dr. G. Lienhard (Dartmouth). Horseradish peroxidase-conjugated rabbit anti-chicken IgG was obtained from Accurate Chemical Corp. (Westbury, NY).

Cell Culture—3T3-L1 fibroblasts and CHO-IR cells were maintained as described previously (14, 16). 3T3-L1 fibroblasts were differentiated as described previously (14, 16). 3T3-L1 fibroblasts were differentiated as described previously (14, 16).

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into adipocytes by a standard protocol (14); adipocytes were routinely used 6–10 days after completion of differentiation. Primary rat adipocytes were isolated from epididymal fat pads and fractionated as described previously (17).

**Blot Analysis**—Total RNA was isolated from 3T3-L1 fibroblasts and fully differentiated adipocytes by acid guanidinium thiocyanate-phenol-chloroform extraction (RNAold; Biotex Laboratories). RNA samples (15 µg) were electrophoresed in 1.2% agarose, 2.2 M formaldehyde, 1 × MOPS, and transferred by capillary diffusion to nylon membranes (Hybond, Amersham). Membranes were pre-hybridized for 1 h in a 1:500 hybridization buffer (10% polyethylene glycol, 1.5 × SSPE, 7% SDS) and then hybridized overnight at 65 °C in the 1:10,000 32P-labeled fragment of PTG, which had been gel-purified and labeled with [γ-32P]dCTP by random priming (>1 × 109 cpm/µg). The blot was washed for 15 min at 65 °C in 2 × SSC, 0.1% SDS, then washed twice at 65 °C in 0.1 × SSC, 0.1% SDS for 15 min each time. Blots were analyzed by autoradiography. RNA loading was determined by ethidium bromide staining and by probing for β-actin.

**Fusion Protein Expression and Purification—PTG** was subcloned into the pGEX-5X-3 expression vector (Pharmacia), and fusion protein was expressed in *Escherichia coli* DH5a. One liter of 2X-YT media plus 100 µg/ml ampicillin was seeded with 10 ml of a saturated overnight culture and allowed to grow at 37 °C for 3.5 h (∆600 = 0.5–0.7). Protein expression was induced with 1 mM IPTG for 3 h at 37 °C. Bacteria pellets were resuspended in 20 ml of 10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 250 µM ampicillin added just before use and were lysed by two passages through a French press (1,000 p.s.i.). 2.5 ml of PBS plus 10% Triton X-100 were added to the lysate which was then gently mixed at 4 °C for 15 min. The supernatant from a 27,000 × g centrifugation spin was applied to 1 ml of glutathione-Sepharose beads equilibrated in PBS. After 30 min of mixing at 4 °C, the beads were washed five times with PBS and analyzed by SDS-PAGE. This protocol was designed to maximize GST-PTG yield at 4 °C, the beads were washed five times with PBS and analyzed by SDS-PAGE. This protocol was designed to maximize GST-PTG yield at 4 °C. Bacterial pellets were resuspended in 20 ml of PBS containing 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol and were lysed by two passes through a French press (2,000 p.s.i.). Samples were also treated with 10 µg/ml of 1,2-␥-glucuronidase and 50 µM 5′-end-labeled substrate in PP1 homogenization buffer (20–30 µl) and were analyzed by measuring measurements, 5 µl of immobilized GST-PTG was resuspended in PP1 homogenization buffer plus 200 mM NaCl and 0.2% BSA. 32P-Labeled phosphoprotein was added to final concentrations of 1, 5, 10, and 15 µM (final volume 30 µl). Samples were incubated at 4 °C with gentle mixing for 30 min and were then washed three times with buffer. Phosphorylase a binding was determined by liquid scintillation counting. Parallel incubations using immobilized GST-PTP1B protein were subtracted as blanks for each phosphorylase concentration. For PP1 binding measurements, immobilized GST-PTG was incubated with increasing amounts of bacterial lysate in PP1 homogenization buffer containing recombinant PP1α. Samples were treated as above, except that binding was analyzed by anti-PP1 immunoblotting and ECL detection. Standard curves of PTG protein were included on the immunoblots. Autoradiograms were analyzed by computer-assisted video densitometry using a Bio Image system (Millipore).

**Other Procedures—Eluted DARPP-32 was thiol-phosphorylated as described elsewhere. Following thiol-phosphorylation, polyhistidine-tagged DARPP-32 was rebound to Ni-NTA agarose beads, washed extensively, and was then eluted and concentrated as above.**

**RESULTS**

**PTG Expression Is Increased upon 3T3-L1 Differentiation—**We have recently identified by two-hybrid screening a novel glycogen-targeting subunit of PP1 from 3T3-L1 adipocytes, termed PTG (1). This protein appears to act as a molecular scaffold for glycogen metabolism and can dramatically increase glycogen synthesis upon overexpression in tissue culture cells. To determine whether PTG expression is correlated with the increase in metabolic activity and insulin responsiveness observed following 3T3-L1 differentiation, PTG expression was examined in fibroblasts and fully differentiated adipocytes by Northern analysis. A single hybridizing mRNA species of 3 kilobases was identified which was dramatically up-regulated following adipogenesis (Fig. 1). These results suggested that the correlation between 3T3-L1 adipocyte differentiation, suggesting a critical role for PTG in the regulation of glycogen synthesis in 3T3-L1 adipocytes.

**PP1 and Phosphorylase a Bind GST-PTG in Vitro—**We have previously demonstrated (1) that PTG can form specific complexes with PP1, phosphorylase a, phosphorylase kinase, and...
PTG in Regulation of PP1 Activity

PTG expression is increased upon 3T3-L1 differentiation. Total RNA from 3T3-L1 fibroblasts and fully differentiated adipocytes was prepared, and Northern blot analysis was performed as described under "Experimental Procedures." PTG and β-actin mRNA bands are indicated.

Fig. 1. PTG expression is increased upon 3T3-L1 differentiation.

Fig. 2. Affinity of PTG for PP1 and phosphorylase a binding. A, PP1 binding to PTG. The affinity of bacterially expressed PP1 for immobilized GST-PTG was measured as described under "Experimental Procedures." Inset, duplicate lanes of a representative anti-PP1 immunoblot quantitated by densitometry scanning. Results are representative of three independent experiments. B, phosphorylase binding to PTG. The affinity of 32P-labeled phosphorylase a for immobilized GST-PTG was measured as described under "Experimental Procedures." Results are representative of two experiments, each performed in duplicate.

Glycogen synthase, the key enzymatic regulators of glycogen synthesis. To better characterize the protein-protein interactions of these enzymes with PTG, the binding affinities of GST-PTG fusion protein for PP1 and phosphorylase a were determined. Immobilized GST-PTG was incubated with varying amounts of recombinant PP1α, the beads were washed and subjected to SDS-PAGE, and binding was quantitated by densitometry scanning of anti-PP1α immunoblot. Binding was saturated at 850 nM PP1, with an EC50 of approximately 335 ± 18 nM (Fig. 2A). The affinity of PTG for glycogen phosphorylase was measured next. Varying amounts of 32P-labeled phosphorylase a were incubated with immobilized GST-PTG, the beads were washed extensively, and binding was determined by liquid scintillation counting. As seen in Fig. 2B, phosphorylase a bound to PTG with an EC50 of 5.45 ± 0.14 μM. In contrast to the results of Doherty et al. (21), these data confirm earlier results demonstrating that PTG can directly associate with phosphorylase a (1).

PTG Increases the Affinity of PP1 for Phosphorylase a—We examined the role of PTG in both the subcellular targeting and the regulation of PP1 specific activity. GST-PTG addition to a 3T3-L1 adipocyte PNS fraction, followed by differential centrifugation, resulted in a dose-dependent, 4–6-fold increase in PP1 activity in the glycogen-enriched fraction (Fig. 3A, GP). PTG addition to 3T3-L1 lysates also reduced the amount of PP1 activity targeted to the plasma membrane fraction (Fig. 3A, PM), indicating that changes in the level of PTG expression can impact on the cellular distribution of PP1 activity (1).

Since PTG forms stable complexes with PP1 substrate proteins, it is possible that PTG can also modulate PP1 specific activity independently of glycogen localization. To test this possibility, varying amounts of soluble GST-PTG were added to 3T3-L1 adipocyte PNS fractions, and then PP1 activity was measured. PTG caused a dose-dependent 2-fold increase in PP1 specific activity against phosphorylase a (Fig. 3B), whereas addition of 500 nM GST protein had no effect on PP1 activity (data not shown). Lineweaver-Burk analysis revealed that PTG decreased the Km of PP1 for phosphorylase a 5-fold, while having no effect on the Vmax (Fig. 3B). Similar results were obtained in a glycogen-free, cytosolic fraction (data not shown), indicating that the stimulation of PP1 activity was independent of glycogen targeting. GST-PTG also caused a dose-dependent 3-fold increase in bacterially expressed PP1α activity against phosphorylase a (data not shown). GST-PTG was completely soluble and did not pellet upon ultracentrifugation in the absence of glycogen, possibly explaining differences with the results of Doherty et al. (21).

The effects of PTG on PP1 activity were dependent on the substrate used in the phosphatase assay. HSL is a lipolyzing enzyme, which is dephosphorylated by PP1 in response to insulin stimulation of adipocytes. Although GST-PTG addition to a 3T3-L1 adipocyte PNS fraction increased PP1 specific activity 2-fold against phosphorylase a (Fig. 3D, Phos a), in parallel assays, there was no change in PP1 activity when 32P-labeled hormone-sensitive lipase was used as substrate (Fig. 3D, HSL). Thus PTG regulates PP1 specific activity by both targeting the phosphatase to glycogen and also by selectively binding and co-localizing certain substrates with PP1.

PTG Is Not a Target of Intracellular Signaling—Because PTG is likely to play a critical role in the regulation of glycogen synthesis by insulin, we examined whether PTG might be phosphorylated in response to hormone treatment. Although the putative phosphorylation sites previously suggested for RGL (10, 22) are not conserved in PTG (1), the possible phosphorylation of other residues on PTG was examined. CHO-IR cells transiently transfected with FLAG-PTG were labeled with [32P]orthophosphate and exposed to either 100 nM insulin or 10 μg/ml forskolin for 5 min. PTG was immunoprecipitated with anti-FLAG antibodies and subjected to SDS-PAGE followed by autoradiography. PTG exhibited a low basal state of phosphorylation, which was not changed by either treatment (Fig. 4A).

In replicate cultures, insulin or forskolin exposure increased MAP kinase or protein kinase A activity 3- and 5-fold, respec-
To determine whether 3T3-L1 adipocytes contained a protein kinase capable of phosphorylating PTG, in vitro phosphorylation assays were performed using PTG as substrate. Cells were exposed to a variety of agents, and cellular lysates were prepared and incubated with GST-PTG and \( \gamma\)-\(^{32}\)P-ATP. Samples were then analyzed by SDS-PAGE and autoradiography. As seen in Fig. 4C, GST-PTG was not phosphorylated in vitro by basal extracts (lane 1 versus 2). Further stimulation of 3T3-L1 adipocytes with either insulin or forskolin did not lead to the activation of a PTG kinase (Fig. 4C, lanes 3 and 4). EGFR or TPA treatment of cells also did not result in any measurable phosphorylation of GST-PTG in vitro (Fig. 4C, lanes 5 and 6).

Finally, PTG was also not phosphorylated in vitro by purified MAP kinase or protein kinase A catalytic subunit (data not shown), further indicating that PTG is not a physiological substrate for insulin- or cAMP-activated kinases.

**Insulin Does Not Change the Subcellular Distribution of PP1**—Translocation of PP1 to and from the glycogen particle in response to the phosphorylation of R\(\text{GL}_1\) has been suggested to underlie hormonal regulation of PP1 activity (10, 22). Although PTG is not phosphorylated in response to external stimuli, possible changes in the cellular distribution of PP1 following insulin treatment were examined. Primary rat adipocytes were isolated and exposed to 10 nM insulin for 30 min, and cellular fractions were prepared by differential centrifugation. Insulin induced a translocation of GluT-4 protein from the low density microsomal fraction to the plasma membrane fraction (Fig. 5A). However, insulin stimulation did not modulate PP1\(\gamma\) protein levels in any fraction, including the low density microsomal fraction, which contains the glycogen pellet (Fig. 5B); identical results were obtained using an anti-PP1\(\gamma\) antibody (data not shown). Further insulin or forskolin treatment of 3T3-L1 adipocytes also did not cause a detectable translocation of PP1 between cellular fractions (data not shown). Taken together, these data indicate that the regulation of PP1 activity by insulin, or agents that elevate intracellular cAMP levels, occurs independently of PP1 translocation.

**PTG Decreases the Inhibition of PP1 by DARPP-32**—PP1 is maintained in a low activity state in 3T3-L1 adipocytes by the binding of phosphorylated DARPP-32.\(^3\) Previous studies (23) suggested that insulin may activate PP1 in primary rat adipocytes by inducing the dephosphorylation and disassociation of

\(^3\)M. J. Brady, A. C. Nairn, and A. R. Saltiel, submitted for publication.
The role of PTG in modulating the regulation of PP1 activity by DARPP-32 was investigated. Thiophosphorylated DARPP-32 specifically inhibited PP1 activity in 3T3-L1 lysates (Fig. 6), with a $K_i$ of 3 nM, consistent with previous results (20). Addition of purified GST-PTG to the lysates caused a rightward shift in the inhibition curve of DARPP-32 ($K_i$ 30 nM, Fig. 6), indicating that PTG lowers the binding affinity of DARPP-32 for PP1. These data suggest that a decrease in the cellular phospho-DARPP-32 concentration in response to insulin might result in the preferential activation of PP1 bound to PTG.

**DISCUSSION**

The regulation by insulin of enzymes involved in glycogen synthesis is primarily mediated by the activation of PP1 (2). The activities of glycogen synthase, phosphorylase $a$, and phosphorylase kinase are modulated by insulin via a mechanism involving their net dephosphorylation, resulting in an increase in glucose storage as glycogen. The paradoxical dephosphorylation of only a limited number of proteins by insulin, despite the ubiquitous presence of PP1 in nearly all cellular compartments, has yet to be explained. Mechanisms must exist for the establishment of discrete pools of PP1 which are preferentially activated by insulin.

PP1 is maintained in discrete subcellular compartments by association with specific targeting subunits. Three proteins have been identified which bind both glycogen and PP1, thus localizing PP1 at the glycogen particle. $R_{GL}$ was first purified from skeletal muscle (6, 7), and $G_L$ was subsequently purified from liver (8, 9). We have recently identified a third PP1 targeting subunit from 3T3-L1 adipocytes, termed PTG, for protein targeting to glycogen (1). By co-localizing PP1 with its substrates at the glycogen particle, PTG acts as a scaffolding protein, assembling metabolic enzymes for the localized reception of intracellular signals.

Studies in different cell lines indicate that the level of PTG expression correlates with cellular metabolic activity. CHO-IR cells contain no endogenous PTG protein and exhibit a low basal rate of glycogen synthesis. Overexpression of PTG in these cells resulted in a 7–10-fold increase in glycogen synthesis (1). PTG binds not only to PP1 and glycogen, but also to the primary enzymatic regulators of glycogen synthesis, namely glycogen synthase, phosphorylase kinase, and phosphorylase $a$ (1). By co-localizing PP1 with its substrates at the glycogen particle, PTG acts as a scaffolding protein, assembling metabolic enzymes for the localized reception of intracellular signals.

PTG appeared to be capable of regulating PP1 specific activity in vitro by several mechanisms. Firstly, a GST-PTG fusion fusion
protein bound to PP1 with high affinity. Moreover, addition of GST-PTG to 3T3-L1 lysates resulted in a concentration-dependent translocation of PP1 to the glycogen-enriched pellet (Fig. 3A). The level of cellular expression of PTG would therefore presumably dictate the localization of PP1 at the glycogen particle (1). Secondly, GST-PTG also bound directly to phosphorylase a (Fig. 2B). Addition of GST-PTG to 3T3-L1 lysates, with no subsequent fractionation, caused a 2-fold increase in PP1 specific activity against phosphorylase a in vitro. Since PTG addition decreased the $K_m$ for phosphorylase a 5-fold, without affecting the $V_{max}$ of the reaction, this increase in phosphatase activity resulted from the formation of a trimolecular complex between PTG, PP1, and its substrate phosphorylase a. This effect of PTG on PP1 activity was restricted to specific proteins. Although HSL is a physiological substrate for PP1 in adipocytes and in vitro, PTG did not affect PP1 activity against this enzyme (Fig. 4D). Thus, PTG regulates PP1 activity against glycogen metabolic enzymes, both by targeting PP1 to glycogen and also by directly binding to and co-localizing specific PP1 substrate proteins.

The precise mechanism by which insulin activates glycogen-targeted PP1 activity remains unclear. Dent et al. (10) reported that the phosphorylation state of two protein kinase A consensus sites on the RGL glycogen targeting subunit regulated PP1 activity in vitro. However, this model has subsequently been disputed (11–15). PTG does not share the putative regulatory phosphorylation sites of RGL (1), and PTG was not phosphorylated in response to either insulin or forskolin treatment of CHO-IR cells (Fig. 4A). Additionally, neither agent could activate a kinase from 3T3-L1 adipocytes capable of phosphorylating exogenous PTG in vitro (Fig. 4C). Finally, insulin treatment had no effect on PP1 binding to PTG in CHO-IR cells (1), and insulin did not increase PP1 localization at the glycogen particle in either primary rat adipocytes (Fig. 5B) or 3T3-L1 adipocytes.3 Taken together, these results indicate that phosphorylation of PTG and/or changes in the affinity of PTG for PP1 do not mediate the hormonal regulation of PP1 activity targeted to glycogen.

In 3T3-L1 adipocytes, PP1 is maintained in a low basal activity state by DARPP-32 binding. DARPP-32 expression is dramatically induced upon differentiation of 3T3-L1 fibroblasts into adipocytes and correlates with a decrease in PP1 basal activity and increase in stimulation by insulin.3 Furthermore, DARPP-32 is expressed in pig brown fat (24), bovine adipose tissue (25), and in primary rat adipocytes, where it has been reported to be dephosphorylated in response to insulin (23). PP1 bound to PTG was resistant to inhibition by DARPP-32 (Fig. 6), in agreement with results with RGL (26). Since PTG reduces the affinity of DARPP-32 for PP1, glycogen-targeted PP1 activity may be more sensitive to possible insulin-induced dephosphorylation of inhibitor peptides in vivo. PTG may therefore not only increase PP1 specific activity against glycogen-targeted enzymes, but also may partially underlie the specific activation of glycogen-targeted PP1 by insulin. Additional work is needed to fully test this proposed model.

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PTG in Regulation of PP1 Activity

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