The activation of protein phosphatase-1 (PP1) by insulin plays a critical role in the regulation of glycogen metabolism. PP1 is a glycogen-targeting protein, which also binds the PP1 substrates glycogen synthase, glycogen phosphorylase, and phosphorylase kinase (Printen, J. A., Brady, M. J., and Saltiel, A. R. (1997) *Science* 275, 1475–1478). Through a combination of deletion analysis and site-directed mutagenesis, the regions on PTG responsible for binding PP1 and its substrates have been delineated. Mutagenesis of Val-62 and Phe-64 in the highly conserved (K/R)V sequence of PTG completely blocked the stimulation of PP1 activity in vitro against phosphorylase, indicating that both binding sites may be important in PTG action. Transient overexpression of wild-type PTG in Chinese hamster ovary cells overexpressing the insulin receptor caused a 50-fold increase in glycogen levels. Expression of PTG mutants that do not bind PP1 had no effect on glycogen accumulation, indicating that PP1 targeting is essential for PTG function. Likewise, expression of the PTG mutants that do not bind PP1 substrates did not increase glycogen levels, indicating that PP1 targeting glycogen is not sufficient for the metabolic effects of PTG. These results cumulatively demonstrate that PTG serves as a molecular scaffold, allowing PP1 to recognize its substrates at the glycogen particle.

Protein phosphatase-1 (PP1) is one of the four major serine/threonine protein phosphatase families expressed in eukaryotic cells (2). The enzyme regulates a variety of cellular functions, including cell cycle progression, RNA splicing, vesicle fusion, ion channel function, and muscle contraction (3–8). PP1 also plays a key role in the hormonal regulation of glycogen metabolism, catalyzing the dephosphorylation of glycogen synthase, glycogen phosphorylase, and phosphorylase kinase (9). These dephosphorylation reactions promote the net synthesis of glycogen by activating glycogen synthase and inhibiting phosphorylase. A number of pharmacological (10) and biochemical (11–13) studies have suggested that insulin stimulates glycogen synthesis, at least in part, through the activation of PP1.

PP1 is ubiquitously expressed and resides in most cellular compartments. However, the hormonal activation of the enzyme is restricted to discrete sites, such as the glycogen particle, suggesting that mechanisms must exist to ensure the localized regulation of the enzyme. For example, although PP1 is found in a number of cellular locations in fat, liver, and muscle cells, insulin produces the dephosphorylation of only a small fraction of phosphoproteins. The compartmentalization of PP1 is mediated by a family of binding or targeting subunits (14, 15). Protein targeting to glycogen (PTG) is one of four targeting proteins that bind to PP1 and glycogen, targeting the phosphatase to the glycogen particle. Unlike the restricted expression of other PP1 glycogen-targeting subunits, PTG is expressed in all insulin-sensitive tissues (1). Overexpression of PTG results in dramatic increases in glycogen accumulation in cell lines and tissues (1, 16, 17), indicating an important role for this protein in the regulation of glycogen metabolism.

In addition to PP1 and glycogen, PTG also directly binds to three PP1- and insulin-regulated enzymes involved in glycogen metabolism as follows: glycogen synthase, phosphorylase, and phosphorylase kinase (1). However, the physiological significance of these interactions was unclear, since each of these enzymes can also bind directly to glycogen (18). To determine whether PTG solely functions to target PP1 to glycogen, or to additionally direct the binding of substrates with the phosphatase, we have delineated the binding domains of PTG by deletion analysis and site-directed mutagenesis. Interestingly, disruption of either PP1 or substrate binding to PTG completely abrogated the ability of PTG to increase glycogen synthesis. Thus, PTG acts as a molecular scaffold, assembling PP1 with its substrates at the glycogen particle and priming them for activation by insulin.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—pGEX-KG plasmid was a generous gift of Dr Kun-Liang Guan (University of Michigan). Cell expression vectors and anti-V-5 antibodies were purchased from Invitrogen (Carlsbad, CA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). Cell culture reagents were obtained from Life Technologies, Inc, with the exception of sera for culture of 3T3-L1 cells, which was supplied by Summit Biotechnology (Ft. Collins, CO). Insulin, differentiation reagents, amylase, and phosphorylase b were from Sigma. ECL*
reagents and glutathione-Sepharose 4B beads were purchased from Amersham Pharmacia Biotech. Affinity purified chicken anti-PP1 and anti-glycogen synthase antibodies were a generous gift from Dr. J. Lawrence, Jr. (University of Virginia). Polyclonal anti-phosphorylase kinase antibody was prepared by Research Genetics, against the recombinant phosphorylase kinase catalytic subunit (kindly provided by P. Bourbonais, Parke-Davis). The antibody was affinity purified as described (13), using a phosphorylase kinase catalytic subunit-affinity column. Horseradish peroxidase-conjugated rabbit anti-chicken antibody was from Accurate Chemical Corp (Westbury, NY), and horseradish peroxidase-conjugated goat anti-rabbit antibody was supplied by Bio-Rad.

**Cloning and Site-directed Mutagenesis**—Full-length, point mutant, and truncated PTG-GST constructs were generated by PCR using the full-length 1.1-kilobase pair BamHI-SalI FLAG-PTG fragment in Bluescript KS as template. Constructs were cloned into the BamHI site of pGEX-KG (19) for GST fusion protein expression or pCDNA3.1/V5/His (Invitrogen) for mammalian cell expression. The resultant constructs were confirmed by sequencing. The V62A/F64A double point mutation was made using the mutagenesis approach in abiotic cells, and truncated PTG-GST constructs were generated by PCR using the mutagenesis approach described previously (20). For some experiments, the fusion proteins were eluted by incubation of the glutathione-Sepharose beads with 3 volumes of 50 mM Hepes (pH 8.0), 20 mM reduced glutathione on ice for 5 min. Beads were pelleted by centrifugation, and the supernatant was removed. After two more batch elutions, the eluates were combined and concentrated with an Amicon-30. Protein concentrations of the GST-PTG constructs were estimated by Bradford and Coomassie Blue staining of polyacrylamide gels.

**Cell Transfection and Glycogen Measurement**—CHO-IR cells were transfected by electroporation as described previously (21). Briefly, cells were trypanotized, washed with phosphate-buffered saline, and electroporated with 120 μg of plasmid DNA using BioCell (Becton Dickinson). Cells were replated on Biocoat tissue culture dishes (Becton Dickinson). Media were changed after 12–24 h, and cells were harvested after 40–48 h. The expression of various PTG constructs was verified by anti-V5 immunoblotting.

Measurement of cellular glycogen levels was performed essentially as described (22), with some minor modifications. Briefly, cells were homogenized and lysed with acid-soluble glycogen, and glycogen was determined as follows: (change in absorbance at 340 nm) × (volume × molecular weight of glucose)/6.3 = mg/liter glucose.

**Extract Preparation, Fractionation, and Binding Assays**—After transient transfection with V5-tagged PTG constructs, CHO-IR cells were collected in homogenization buffer (50 mM Hepes (pH 8.0), 150 mM NaCl, 10 mM EDTA, and protease inhibitors) and lysed by sonication (10 s). Lysates were centrifuged at 500 × g for 10 min to separate cytosolic and particulate, glycogen-containing fractions. 3T3-L1 cells were differentiated into adipocytes as described previously (23). For binding assays, cells were collected in homogenization buffer plus 0.5% Triton X-100. Cellular glycogen was digested by addition of 40 μg/ml amylase and incubation of the lysates at 30 °C for 30 min. Samples were then centrifuged at 100,000 × g for 30 min to pellet any residual glycogen. The supernatants were removed, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Binding assays using GST fusion proteins and glycogen-free lysates or 32P-labeled phosphorylase were performed in homogenization buffer lacking glycogen as described (1). For PP1 assays, extracts from 3T3-L1 adipocytes were prepared as described (24) and stored at −80 °C until use.

**RESULTS**

**PP1 and PP1 Substrates Bind to Separate Domains on PTG**—PTG binds to PP1, glycogen synthase, phosphorylase, and phosphorylase kinase (1). To identify the domains on PTG mediating interaction with these proteins, a series of 18 constructs comprising N- and C-terminal deletions was synthesized in vitro and subcloned into a pGEX expression vector (Fig. 1). The GST fusion proteins were expressed in bacteria, and affinity-purified using glutathione-Sepharose beads. The GST-PTG fusion proteins immobilized on Sepharose beads were then incubated with glycogen-free 3T3-L1 adipocyte lysates and then washed extensively. PP1 (PP1) and glycogen synthase (substrate) binding were measured by immunoblotting and visual inspection. Results are summaries of two to four independent experiments. −, no binding; +, to ++, increasing amount of binding; P, PP1 binding domain; S, PP1 substrate binding domain; G, glycogen binding domain, previously reported by Wu et al. (28).

![Fig. 1. Schematic representation of PTG deletion constructs and summary of in vitro binding results.](image)

The indicated deletion constructs were synthesized by PCR, using full-length PTG (residues 1–295) as template. Numbers correspond to amino acid residues. Constructs were subcloned into the pGEX-KG vector, expressed in bacteria as GST fusion proteins, and affinity purified on glutathione-Sepharose beads. The immobilized fusion proteins were incubated with glycogen-free 3T3-L1 adipocyte lysates and then washed extensively. PP1 (PP1) and glycogen synthase (substrate) binding were measured by immunoblotting and visual inspection. Results are summaries of two to four independent experiments. −, no binding; +, to ++, increasing amount of binding; P, PP1 binding domain; S, PP1 substrate binding domain; G, glycogen binding domain, previously reported by Wu et al. (28).
PTG, a series of C-terminal deletion constructs were prepared, and equal amounts of GST fusion protein were used in pull-down assays from glycogen-free 3T3-L1 adipocyte lysates. After extensive washing, proteins bound to the PTG constructs were analyzed by immunoblotting. Binding of glycogen synthase to full-length PTG (residues 1–295) was readily detected (Fig. 3A, lane 2). Deletions of 45–55 amino acids from the C terminus of PTG increased glycogen synthase binding 2-fold as compared with wild-type PTG (Fig. 3A, lanes 2–4). However, deletion of a further 17–23 residues completely abrogated glycogen synthase binding (Fig. 3A, lane 7 and 8). These progressive C-terminal deletions of PTG had no effect on PP1 binding (Fig. 7, data not shown). Phosphorylase kinase exhibited a similar pattern of binding to the GST-PTG constructs (Fig. 3B). Short C-terminal truncations of PTG increased phosphorylase kinase binding, but continued deletions completely disrupted this interaction. Identical results were obtained in phosphorylase binding assays (Fig. 3C), suggesting that all three PP1- and insulin-regulated enzymes involved in glycogen metabolism bind to the same domain of PTG.

Point Mutations of PTG Disrupt PP1 or Glycogen Synthase Binding—A summary of the binding results is shown in Fig. 1. The PP1 binding domain is located in the N-terminal region of PTG, which contains the consensus (K/R)(V/F)PX1-binding motif that is conserved in other PP1-targeting proteins (27). The PP1 substrate binding domain, which mediates interaction with glycogen synthase, phosphorylase, and phosphorylase kinase, is located in the C-terminal region. The glycogen binding domain has been previously reported to span a rather large, ill-defined region between the other two domains (28). However, deletion analysis can potentially cause secondary effects elsewhere in the molecule, due to alterations in protein folding. Therefore, to confirm these initial results, two full-length PTG constructs were made, with point mutations in either the putative PP1 or PP1 substrate binding domain (Fig. 1). The highly conserved Val-62 and Phe-64 residues in the consensus PP1-binding motif were substituted with alanine. Mutagenesis of the analogous hydrophobic residues in the PP1 nuclear targeting subunit NIPP1 completely ablated PP1 binding (5). The proposed PP1 substrate binding domain on PTG contains a stretch of amino acids, WDNNE, that is predicted by RasMol molecular modeling to protrude from the surface of the molecule. This motif is homologous to the granular starch binding of the Aspergillus niger (1Kul) (29) and is conserved in the other PP1 glycogen-targeting proteins. The two acidic residues in this region, Asp-225 and Glu-228, were therefore also mutated to alanine.

Wild-type PTG and the two full-length, double point mutant PTG constructs were expressed and immobilized as GST fusion proteins and incubated in duplicate with glycogen-free 3T3-L1 adipocyte lysates. Twice as much protein was used in the pulldown assays with the point mutant constructs as compared with wild-type PTG. Glycogen synthase and PP1 binding was then examined by immunoblotting. Wild-type PTG readily bound to both PP1 and glycogen synthase from the lysate (Fig. 4A, lanes 2 and 3). Mutagenesis of two residues in the consensus PP1-binding motif (V62A/F64A) completely blocked PP1 binding without affecting glycogen synthase binding (Fig. 4A, lanes 4 and 5). Conversely, mutagenesis of the two acidic C-terminal residues to alanine (D225A/E228A) blocked glycogen synthase binding without affecting interaction with PP1 (Fig. 4A, lanes 6 and 7). These results confirm the finding that PP1 and glycogen synthase bind independently to PTG, via separate N- and C-terminal domains. Identical results were obtained in phosphorylase binding assays (Fig. 4B). Disruption of the PP1 binding domain on PTG (V62A/F64A) had no effect on interaction with phosphorylase (Fig. 4B, lanes 5 and 6 versus 3). However, phosphorylase did not bind to the D225A/E228A PTG construct (Fig. 4B, lanes 7 and 8), indicating that both glycogen synthase and phosphorylase binding are blocked by the mutagenesis of these two residues.

Inhibition of PP1 or PP1 Substrate Binding Disrupts PTG Function in Vitro and in Vivo—The effects of interfering with either PP1 or PP1 substrate binding to PTG were investigated. The indicated GST-PTG constructs (Fig. 5) were affinity-purified from bacterial lysates, and then eluted off the glutathione-Sepharose beads. Equal amounts of protein were added to 3T3-L1 adipocyte lysates, and PP1 activity was measured in vitro using 32P-labeled phosphorylase a as substrate. As previously reported, addition of wild-type GST-PTG caused a 2–3-fold increase in PP1 activity (Fig. 5, due to a 5-fold reduction in the $K_m$ of the phosphatase for its substrate (20). Ablation of PP1 binding to PTG by mutation of Val-62 and Phe-64 to alanine reduced PP1 activity to control levels (Fig. 5). Additionally, disruption of the PP1 substrate-binding site, either through mutation (D225A/E228A) or deletion (residues 1–217), also blocked the stimulatory effect of PTG on PP1 activity (Fig. 5).
lysates, and PP1 constructs were incubated with glycogen-free 3T3-L1 adipocyte substrate binding. A phosphorylase (Phos) immunoblotting (IB) and are indicated by arrows. B, phosphorylase binding. The indicated PTG constructs were incubated with 32P-labeled phosphorylase (Phos), and binding was detected as in Fig. 3. Lanes 1–295, wild-type PTG; V62A/F64A, full-length PTG, with residues valine 62 and phenylalanine 64 mutated to alanine; D225A/E228A, full-length PTG with residues aspartic acid 225 and glutamic acid 228 mutated to alanine; Lys, starting 3T3-L1 adipocyte lysate before addition of fusion protein beads; Beads, empty glutathione-Sepharose bead control. Results are representative of three or four independent experiments.

5). Addition of 33 residues to the PTG (residues 1–217), C-terminal deletion construct restored both phosphorylase binding to PTG (Fig. 3C) and the PTG-mediated increase in PP1 activity (Fig. 5). These initial in vitro results indicated that binding of both the phosphatase and its substrate to PTG are important for the regulation of PP1 activity.

The effects of disrupting either PP1 or glycogen synthase binding on PTG function were examined in CHO-IR cells. These cells contain low levels of glycogen and no endogenous PTG. Following transient transfection of the various PTG constructs, the cells were harvested, and lysates were prepared. All PTG constructs were expressed at equivalent levels (Fig. 6), and the transfection efficiency was approximately 50% (data not shown). Following transfection, cells were fractionated into a cytosolic and glycogen-containing particulate fraction and analyzed by anti-V5 immunoblotting to detect localization of the PTG constructs. As previously reported (1), wild-type PTG was found exclusively in the glycogen-containing fraction (Fig. 6, lane 6). Mutagenesis of either the PP1- (V62A/F64A) or substrate (D225A/E228A)-binding site had no effect on the binding of PTG to glycogen (Fig. 6, lanes 7 and 8). The latter result confirms previous results indicating that the glycogen binding region of PTG is located in the center of the molecule (28).

In parallel, glycogen levels in the transfected CHO-IR cells were measured (Fig. 7). The top part of Fig. 7 shows results from glycogen synthase and PP1 binding assays using the same constructs expressed as GST fusion proteins. Overexpression of wild-type PTG (residues 1–295) caused a 50-fold increase in glycogen levels as compared with LacZ-transfected CHO-IR cells, adjusting for transfection efficiency (Fig. 7, lanes 1 and 2). PTG overexpression had no effect on PP1 or glycogen synthase protein levels in CHO-IR cells but rather caused a redistribution of PP1 from the cytosol to glycogen (1). The full-length V62A/F64A PTG mutant that bound to glycogen alone did not increase glycogen levels (Fig. 7, lane 3), indicating that PP1 targeting to glycogen is essential for PTG function. However, expression of the full-length D225A/E228A PTG mutant that bound to PP1 but not glycogen synthase also had little effect on glycogen levels (Fig. 7, lane 4). This result indicates that targeting of PP1 to glycogen alone is not sufficient to increase glycogen accumulation. Similarly, a PTG C-terminal truncation construct (residues 1–217) that bound to PP1 but not glycogen synthase also did not increase glycogen accumulation (Fig. 7, lane 5). Addition of 16 amino acid residues (residues 1–233) restored glycogen synthase binding to PTG (Fig. 7, lane 6) and resulted in an increase in glycogen levels com-

FIG. 4. Point mutagenesis of PTG disrupts PP1 or PP1 substrate binding. A, PP1 and glycogen synthase binding. Immobilized PTG constructs were incubated with glycogen-free 3T3-L1 adipocyte lysates, and PP1α and glycogen synthase (GS) binding were detected by immunoblotting (IB) and are indicated by arrows. B, phosphorylase binding. The indicated PTG constructs were incubated with 32P-labeled phosphorylase (Phos), and binding was detected as in Fig. 3. Lanes 1–295, wild-type PTG; V62A/F64A, full-length PTG, with residues valine 62 and phenylalanine 64 mutated to alanine; D225A/E228A, full-length PTG with residues aspartic acid 225 and glutamic acid 228 mutated to alanine; Lys, starting 3T3-L1 adipocyte lysate before addition of fusion protein beads; Beads, empty glutathione-Sepharose bead control. Results are representative of three or four independent experiments.

FIG. 5. PTG stimulation of PP1 activity in vitro requires PP1 and phosphorylase binding. Approximately 100 nM of the indicated soluble PTG constructs were added to 3T3-L1 adipocyte lysates, and PP1 activity was measured in vitro, using 32P-labeled phosphorylase as substrate. Numbers refer to amino acid residues, with full-length PTG corresponding to residues 1–295. PTG point mutants are described in Fig. 4. Results are the mean of three independent experiments, each performed in triplicate.

FIG. 6. Point mutagenesis of PTG does not disrupt glycogen binding. CHO-IR cells were transiently transfected with LacZ (lanes 1 and 5) or the following full-length, V5-tagged PTG constructs: lanes 2 and 6, wild-type; lanes 3 and 7, V62A/F64A; lanes 4 and 8, D225A/E228A. Cell lysates were fractionated into the cytosolic and glycogen-containing particulate fractions by ultracentrifugation. PTG expression was then visualized by anti-V5 immunoblotting (IB). The arrow indicates the V5-PTG bands. PTG point mutants are described in Fig. 4. Result is representative of two independent experiments.
parable to wild-type PTG (Fig. 7, lane 6 versus 2). Furthermore, the PTG deletion construct 1–250, which bound glycogen synthase more avidly than wild-type PTG (Fig. 7, lane 7 versus 2), caused a 125-fold increase in glycogen levels, 2.5-fold higher than wild-type (Fig. 7, lane 7 versus 2). This result indicates that interaction with glycogen synthase may be limiting for the PTG-mediated increase in glycogen accumulation. Additionally, these results cumulatively demonstrate that both PP1 and glycogen synthase binding to PTG are essential for PTG function.

**DISCUSSION**

Insulin stimulates glycogen synthesis by increasing glucose transport and by coordinately increasing the activity of glycogen synthase and inhibiting phosphorylase via changes in the phosphorylation states of the enzymes. The hormone stimulates the dephosphorylation and activation of glycogen synthase through increased glucose uptake and metabolism, inactivation of glycogen synthase kinases, and activation of glycogen-targeted PP1 (30). The relative contribution of these three signaling events to the activation of glycogen synthase by insulin remains controversial and may vary between cell and tissue types. However, insulin also inhibits glycogenolysis through the PP1-catalyzed dephosphorylation and inactivation of phosphorylase and phosphorylase kinase. Furthermore, overexpression of PP1 glycogen-targeting subunits dramatically increases glycogen synthesis (1, 13, 16, 17). Thus, the regulation of glycogen-targeted PP1 activity plays an indispensable role in the control of glycogen metabolism.

PP1 is localized to glycogen through binding to a family of four glycogen-targeting subunits. G_M/R/P1R3 and G_L/PPP1R4 are primarily expressed in striated muscle and liver, respectively, and PTG/PPP1R5 and PPP1R6 are distributed in muscle, liver, and fat (1, 31–33). G_M is a 124-kDa protein that is significantly larger than the other three approximately 35-kDa family members. Despite a proposed common function, there exists no more than 50% sequence homology between any two targeting subunits. Additionally, Northern blot analysis revealed that three of the subunits are expressed in both liver and skeletal muscle (1, 31–33). These observations suggest that each targeting subunit may possess unique regulatory properties regarding PP1 activity and hormonal responsiveness (13).

In addition to PP1 and glycogen, PTG also binds with the following three insulin- and PP1-regulated enzymes involved in glycogen metabolism: glycogen synthase, phosphorylase, and phosphorylase kinase (1). We sought to define the domains on PTG that mediate phosphatase and substrate binding and to assess their relative contributions to PTG function. The PP1 binding domain was located in the N-terminal region, which contains the consensus (K/R)VX F motif present in other PP1-binding proteins (27). Mutagenesis of the PTG residues Val-62 and Phe-64 to alanine completely blocked PP1 binding. Similar results were reported when the corresponding residues were mutated in the PP1-binding protein NIPP1 (5). The (K/R)VX F motif is conserved in the majority of eukaryotic PP1-binding proteins, including all four PP1 glycogen-targeting subunits, and very likely comprises a common site of interaction with the phosphatase (Fig. 8A). The PP1-binding site on G_M was mapped by deletion analysis to a 36-amino acid stretch that contains this conserved motif (26). Furthermore, a peptide from the corresponding region on G_M was successfully co-crystallized with PP1 and bound to a hydrophobic cleft on the phosphatase (34). Additionally, phosphorylation of Ser-67 on G_M, which is in the center of the PP1 interaction motif, disrupts PP1 binding to G_M (35, 36) and promotes dissociation of the phosphatase from glycogen (37, 38). However, substitution of Ser-67 with the corresponding Val-63 from PTG also completely abrogated PP1 binding (36), indicating that the immediate consensus motif is not necessarily interchangeable between targeting proteins. Furthermore, a series of point mutations in PP1 differentially affected interaction with the glycogen-targeting subunits in a two-hybrid array. These results suggest that multiple contact points outside the common (K/R)VX F motif also mediate the interaction of PP1 with each glycogen-targeting subunit.

The extreme C terminus of G_M contains a high affinity binding site for phosphorylase that is not conserved in the other three glycogen-targeting subunits (26). Deletion analysis indicated that phosphorylase, as well as glycogen synthase and phosphorylase kinase, bound to a C-terminal region on PTG, which bore little homology to the phosphorylase-binding site on G_M. This supposition was confirmed, as mutagenesis of the two

**FIG. 7.** PP1 and glycogen synthase binding to PTG are required for stimulation of glycogen accumulation in CHO-IR cells. LacZ control of the indicated PTG constructs was transiently transfected into CHO-IR cells, and cellular glycogen levels were measured 40 h later. All constructs were expressed at equivalent levels, and a transfection efficiency of approximately 50% was achieved. Lanes depicted PP1α or glycogen synthase (GS) binding to the same constructs expressed as GST fusion proteins. Numbers refer to amino acid residues, with full-length PTG corresponding to residues 1–295. PTG point mutants are described in Fig. 4. Results are representative of three to five independent experiments.

**FIG. 8.** Alignment of binding domains from the PP1 glycogen-targeting subunits. The amino acid (AA) sequences from the PP1 binding (A) and PP1-substrate binding (B) regions PTG are shown. The corresponding amino acid sequences from G_M, G_L, and R6 are also displayed. Invariant residues are shaded in gray. GTS, glycogen-targeting subunit.

\(^2\)N. M. Fong, M. J. Brady, and A. R. Saltiel, unpublished observations.
acidic residues Asp-225 and Glu-228 to alanine blocked the binding of all three enzymes to PTG. These residues lie within a WDNXXGNY motif that is relatively well conserved among the various glycogen-targeting subunits (Fig. 8D) and has been proposed to comprise a common glycogen-binding region (26). However, the D225A/E228A PTG mutant still bound to glycogen (Fig. 6), indicating that other residues must also be involved in the binding of PTG to glycogen (28). Initial results indicate that G\textsubscript{L} does not directly bind glycogen synthase,\textsuperscript{3} indicating the WDNXXGNY motif is not sufficient for glycogen synthase binding. Furthermore, several C-terminal PTG constructs, which contained the WDNXXGNY motif, did not bind to glycogen synthase (Fig. 1). These results suggest that either other sites in PTG are also required for interaction with glycogen synthase or that the lack of glycogen synthase binding may be due to misfolding of these truncated proteins. The regions on the three glycogen-regulatory enzymes that bind to PTG are not obvious. Phosphorylase kinase, glycogen synthase, and phosphorylase possess distinct enzymatic properties and thus are not highly homologous. However, all three enzymes bind to glycogen (18) and may use these functionally similar domains to bind to PTG. The mutually exclusive binding of the PP1 substrates on PTG suggests the potential for competitive binding of these enzymes to PTG, which in turn may be hormonally regulated. Mapping of the region of glycogen synthase that binds to PTG and competitive binding assays with the various PP1 substrates are currently under investigation.

Since glycogen synthase, phosphorylase, and phosphorylase kinase directly bind to glycogen, the significance of their interaction with PTG was previously unclear. In the present study, binding assays were performed in the absence of glycogen, demonstrating that PTG forms direct protein-protein complexes with these enzymes, as opposed to glycogen-mediated co-sedimentation (26). Disruption of PP1 binding to PTG completely inhibited the PTG-mediated increase in glycogen levels in CHO-IR cells, confirming the importance of PP1 targeting in PTG function. However, disruption of PP1 substrate binding to PTG, either through C-terminal deletions or point mutagenesis, also completely ablated the ability of PTG to support glycogen synthesis. Conversely, a PTG construct that bound glycogen synthase more avidly than wild-type PTG caused a 2.5-fold larger increase in glycogen levels (Fig. 7). These results clearly demonstrate that targeting of PP1 to glycogen and binding of PP1 substrates are both required for the PTG-mediated increase in glycogen accumulation. However, the binding of substrates to PTG is presumably regulated, allowing for the dephosphorylation of multiple proteins by one PP1-PTG complex. One possibility being investigated is that the phosphorylated substrates possess a higher affinity for PTG, resulting in their dissociation following PP1-mediated dephosphorylation. Additionally, PP1 substrate binding may be influenced by hormonal signaling and/or elevation of intracellular levels of glucose and its metabolites. A family of four targeting subunits localizes PP1 to the glycogen particle. The lack of sequence homology and overlapping tissue distribution suggested that each targeting subunit might confer unique properties onto PP1 activity. Initial work has demonstrated that the G\textsubscript{M}-PP1 binding is uniquely regulated by the phosphorylation of G\textsubscript{M}, whereas the G\textsubscript{L}-PP1 complex is subject to allosteric inhibition by phosphorylation. Additionally, the various glycogen-targeting subunits exert differential effects on PP1 activity against glycogen-bound substrates in vitro (11, 20, 92, 36, 38). Recently, Newgard and colleagues (13) have reported that transfection of primary hepatocytes with G\textsubscript{L} or PTG results in similarly large increases in glycogen levels, whereas G\textsubscript{M} overexpression has more modest effects. Cells containing G\textsubscript{L} were no longer responsive to insulin-stimulated glycogen synthesis, whereas PTG-overexpressing cells were refractory to glycogenolytic stimuli. Thus, multiple glycogen-targeting subunits may be required for the differential modulation of PP1 substrate specificity, through intracellular localization, distinct mechanisms of binding to PP1, and direct interaction with phosphatase substrates. Chimeric analysis of the various glycogen targeting subunits may shed further light on the mechanisms of hormonal regulation and the physiological need for multiple proteins that superficially appear to serve the same function.

Addendum—Recent complimentary results have been reported for G\textsubscript{M/RGL}, the striated muscle-specific PP1-glycogen targeting subunit (39).

REFERENCES
1. Printen, J. A., Brady, M. J., and Saltiel, A. R. (1997) Science 275, 1475-1478
2. Barford, D. (1996) Trends Biochem. Sci. 21, 407-412
3. Kwon, Y. G., Lee, S. Y., Choi, Y., Greengard, P., and Nairn, A. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2168-2173
4. Berndt, N. (1999) Propt. Biotics. 4, D22–D42
5. Trinkle-Mulcahy, L., Ajuy, P., Prescott, A., Claverie-Martin, F., Cohen, S., Lamond, A. I., and Cohen, P. (1999) J. Cell. Sci. 112, 157–168
6. Peters, C., Andrews, P. D., Stark, M. J., Cesaro-Tadic, S., Glatz, A., Podtele-

\textsuperscript{3} B. C. Callaghan and M. J. Brady, unpublished observations.