The Role of Protein Phosphatase-1 in Insulin Action

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ABSTRACT

Insulin is the most-potent physiological anabolic agent known, promoting the synthesis and storage of carbohydrates and lipids and inhibiting their degradation and release into the circulation. This action of the hormone is due in part to the acute regulation of metabolic enzymes through changes in their phosphorylation state. In fat, liver, and muscle, insulin stimulates the dephosphorylation of a number of enzymes involved in glycogen and lipid metabolism via activation of protein phosphatases. Numerous studies have indicated that protein phosphatase-1 (PP1) is the primary phosphatase involved in insulin action. Although PP1 is a cytosolic protein, the phosphatase is compartmentalized in cells by discrete targeting subunits. These proteins confer substrate specificity to PP1 and mediate the specific regulation of intracellular pools of PP1 by a variety of extracellular signals. Four proteins have been described that target the phosphatase to the glycogen particle. G_M and G_L are expressed exclusively in striated muscle and liver, while protein targeting to glycogen (PTG) and R6 are more widely expressed. Despite a common targeting function, these four proteins are not highly conserved, suggesting profound differences in the mechanisms by which they contribute to the hormonal regulation of PP1 activity. Overexpression studies in cell lines or animals have revealed major differences among these proteins regarding basal glycogen levels and hormonal responsiveness. Furthermore, alterations in the expression or function of PP1 glycogen-targeting subunits may contribute to the onset of insulin resistance and type 2 diabetes.

I. Introduction

Insulin is the principal anabolic hormone that regulates glucose and lipid metabolism. However, the precise molecular mechanisms by which this important factor exerts its effects on target cells are still unclear. Although insulin stimulates protein phosphorylation by the activation of several well-characterized kinase cascades, many of the metabolic effects of this hormone are mediated through protein dephosphorylation. Most of these dephosphorylation reactions are thought to be catalyzed by protein phosphatase-1 (PP1), which has been implicated in the regulation of several rate-limiting enzymes in both glucose and lipid metabolism.

The activation of PP1 by insulin is complex and paradoxical. A number of pharmacological studies have revealed that the insulin-dependent dephosphorylation of metabolic enzymes such as glycogen synthase, hormone-sensitive lipase,

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and acetyl CoA-carboxylase is catalyzed by PP1 (Haystead *et al.*, 1989; Hess *et al.*, 1991). However, PP1 is involved in the regulation of a wide variety of cellular processes that are not influenced by insulin, such as cell division, vesicle fusion, and ion channel function (Berndt, 1999; Kwon *et al.*, 1997; Peters *et al.*, 1999; Westphal *et al.*, 1999). Moreover, in a single cell type, insulin can stimulate the phosphorylation of some proteins, while simultaneously reducing the phosphorylation of others (Saltiel, 1996). To further complicate matters, insulin produces the dephosphorylation of only a small subset of proteins at discrete locations, whereas PP1 is ubiquitously expressed and found in virtually all cellular compartments. These findings suggest that mechanisms must exist for the targeted regulation of PP1 in insulin-responsive cells. Furthermore, these mechanisms must selectively permit activation of the enzyme only at these sites.

One clue in understanding the compartmentalized regulation of PP1 activity has emerged from the interaction of the protein with "targeting" subunits. A number of studies have shown that PP1 is localized to the nucleus, plasma membrane, neuronal dendrites, myosin filaments, and glycogen particles through binding to a growing family of PP1-targeting proteins (Alessi *et al.*, 1992; Allen *et al.*, 1998; Hsieh-Wilson *et al.*, 1999; Hubbard and Cohen, 1993; Schillace and Scott, 1999; Terry-Lorenzo *et al.*, 2000). These interactions allow for the compartmentalized activation of the phosphatase by a variety of extracellular signals, resulting in the specific dephosphorylation of discrete pools of proteins within the cell. This chapter will focus on the role of PP1 glycogen-targeting subunits in the regulation of glycogen metabolism by insulin.

II. Regulation of Glycogen-metabolizing Enzymes

A. GLYCOGEN SYNTHASE IS REGULATED BY ALLOSTERIC AND COVALENT MODIFICATION

Glycogen is an important form of energy storage in mammals. As such, its synthesis and degradation are subject to multiple levels of hormonal and allosteric regulation via control of the enzymes glycogen synthase (GS) and phosphorylase. Although glucose and its metabolites can modulate enzymatic activity and localization, GS and phosphorylase are principally regulated by phosphorylation. Glycogen synthase, the rate-limiting enzyme in glycogen synthesis, is phosphorylated on up to nine regulatory serine residues by several kinases, resulting in progressive inactivation (Lawrence and Roach, 1997). These kinases — which include cAMP-dependent kinase (PKA), calmodulin-dependent kinases, glycogen synthase kinase-3 (GSK-3), and adenosine monophosphate (AMP) kinase — respond to a variety of catabolic stimuli to reduce glycogen synthesis. In contrast, phosphorylase, which catalyzes glycogen breakdown, is activated by phosphorylation of a single serine by phosphorylase kinase. The opposing activities of GS and phos-

phorylase arise from a summation of allosteric regulators and covalent modifications that allow glycogen levels to fluctuate in parallel to the energy needs of the cell.

In peripheral tissues, insulin modulates glycogen accumulation through a coordinate increase in glucose transport and regulation of glycogen-metabolizing enzymes (Azpiazu et al., 2000; Brady et al., 1999b) (Figure 1). Insulin increases glycogen synthesis via the activation of GS by promoting the net dephosphorylation of the protein. Insulin both attenuates the activity of kinases such as PKA or GSK-3 (Cross et al., 1995; Welsh and Proud, 1993) and activates serine/threonine phosphatases, particularly PP1 (Brady et al., 1997a; Dent et al., 1990; Ragolia and Begum, 1998; Wang et al., 1995). Additionally, insulin stimulates the PP1-mediated dephosphorylation of both phosphorylase and phosphorylase kinase.
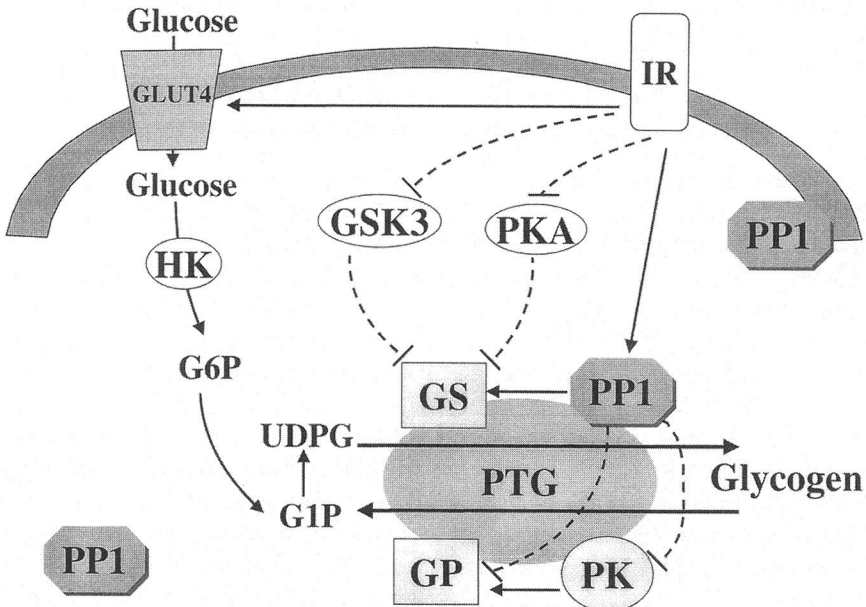

FIG. 1. Insulin regulates glycogen metabolism by promoting protein dephosphorylation. Insulin binds to its receptor and stimulates glycogen synthesis by increasing glucose transport via GLUT4 and modulating enzymatic activities. Insulin induces the dephosphorylation and activation of glycogen synthase (GS) through activation of PP1 and inactivation of upstream kinases (e.g., PKA, GSK-3). Additionally, insulin inhibits glycogenolysis by promoting the PP1-mediated dephosphorylation and inactivation of phosphorylase and phosphorylase kinase. Thus, modulation of glycogen-metabolizing enzymatic activities by insulin requires both phosphatase activation and kinase inactivation. Stimulatory pathways are denoted with solid lines; inhibitory pathways are denoted with dashed lines. Abbreviations: IR = insulin receptor; GSK3 = glycogen synthase kinase-3; PKA = cAMP-dependent kinase; PP1 = protein phosphatase-1; GS = glycogen synthase; GP = glycogen phosphorylase; PK = phosphorylase kinase; HK = hexokinase; G6P = glucose-6-phosphate; G1P = glucose-1-phosphate; UDPG = UDP-glucose.

resulting in enzymatic inactivation and a decrease in glycogenolysis (Figure 1). Thus, the insulin-mediated dephosphorylation of GS and phosphorylase involves both phosphatase activation and kinase inactivation. Insulin-stimulated glucose transport also plays a critical role in regulating GS, since removal of extracellular glucose markedly reduces the activation of GS by insulin (Brady *et al.*, 1999a; Lawrence and James, 1984; Lawrence and Larner, 1978). Increased intracellular levels of glucose-6-phosphate (G6P) and uridine diphosphate (UDP)-glucose induce the translocation of cytosolic GS to glycogen-containing fractions in primary hepatocytes and 3T3-L1 adipocytes (Brady *et al.*, 1999a; Fernandez-Novell *et al.*, 1992a,1992b). Additionally, G6P binding to GS allosterically activates glycogen synthase and increases its susceptibility to dephosphorylation (Lawrence and Roach, 1997; Villar-Palasi and Guinovart, 1997). Therefore, insulin modulates GS activity through covalent modification, translocation, and allosteric regulation.

B. STIMULATION OF GLYCOGEN SYNTHASE ACTIVITY BY INSULIN INVOLVES KINASE INHIBITION AND PHOSPHATASE ACTIVATION

The molecular mechanisms by which insulin regulates glycogen metabolism are complex and may vary among cell types. Recently, a kinase cascade has been described that may mediate the activation of GS through inhibition of GSK-3 (Cohen *et al.*, 1997). Stimulation of phosphatidylinositol 3-kinase (PI 3-K) by extracellular signals results in an increase in membrane phosphatidyl inositol (3,4,5)-trisphosphate (PIP$_3$) levels and the sequential translocation, phosphorylation, and activation of Akt-kinase by another membrane-bound, PIP$_3$-stimulated kinase, PDK. Akt subsequently phosphorylates and inactivates GSK-3 kinase, decreasing the rate of phosphorylation of GS, resulting in enzymatic activation. Insulin inhibits GSK-3 activity in a variety of cell types (Cross *et al.*, 1995,1997; Welsh and Proud, 1993), concomitant with dephosphorylation of GS. Overexpression of wild-type and mutant GSK-3 constructs diminished basal and insulin-stimulated GS activity (Eldar-Finkelman *et al.*, 1996; Summers *et al.*, 1999). Use of pharmacological agents has suggested a role for GSK-3 inactivation in insulin metabolic signaling, since the PI 3-K inhibitor wortmannin blocks both GSK-3 inactivation and GS activation (Hurel *et al.*, 1996; Moule *et al.*, 1995; Shepherd *et al.*, 1995). Additionally, the GSK-3 inhibitor lithium stimulates GS activity in cell lines and primary muscle (Furnsinn *et al.*, 1997; Orena *et al.*, 2000; Summers *et al.*, 1999).

Despite this evidence, the precise role for GSK-3 inactivation in the regulation of GS activity is unclear. Inhibition of GSK-3 is not sufficient to mediate GS activation, since GSK-3 does not phosphorylate several of the residues on GS that are dephosphorylated by insulin (Lawrence and Roach, 1997; Skurat *et al.*, 1994). Furthermore, wortmannin inhibits the activation of PP1 (Brady *et al.*, 1998; De

Luca *et al.*, 1999) and GLUT4 translocation by insulin (reviewed in Czech and Corvera, 1999), both of which contribute to GS activation. Additionally, GSK-3 inactivation by other factors does not result in GS activation (Brady *et al.*, 1998; Moule *et al.*, 1997). Thus, stimulation of GS activity by insulin requires increased glucose transport, kinase inactivation, and phosphatase activation, although the relative contribution of each signaling component is unclear. Furthermore, insulin promotes the dephosphorylation of phosphorylase (Zhang *et al.*, 1989) and inhibition of glycogenolysis. Conversely, glycogenolytic agents may act, in part, by promoting the dissociation of PP1 from the glycogen particle (Hiraga and Cohen, 1986; Hubbard and Cohen, 1993), thus further enhancing the phosphorylation of GS and phosphorylase. Together, these findings indicate that PP1 plays a central role in the hormonal control of glycogen metabolism.

III. Structure/Function Analysis of PP1 Glycogen-targeting Subunits

As mentioned earlier, insulin does not globally activate PP1 but rather specifically targets discrete pools of phosphatase activity. Insulin has been reported to activate a cytosolic pool of PP1 (Villa-Moruzzi, 1989) via a mitogen-activated protein kinase (MAPK)-dependent pathway (Wang *et al.*, 1995). However, since inhibition of MAPK had no effect on metabolic signaling by insulin (Lazar *et al.*, 1995), the significance of these results is unclear. Insulin primarily increases PP1 activity localized at the glycogen particle. Thus, glycogen-targeting subunits were initially proposed to mediate the compartmentalized activation of PP1 by insulin (Hubbard and Cohen, 1993). However, recent data indicate that these targeting proteins actually serve as "molecular scaffolds," bringing together the enzyme directly with its substrates in a macromolecular complex. In the process, they exert profound effects on PP1 activity in a substrate-specific manner.

A. PP1 IS LOCALIZED TO GLYCOGEN BY A FAMILY OF TARGETING SUBUNITS

Four different proteins have been reported to target PP1 to the glycogen particle. $G_M/R3$ and $G_L/R4$ are primarily expressed in striated muscle and liver, respectively, while PTG/R5 and R6 are more ubiquitously distributed (Armstrong *et al.*, 1997; Doherty *et al.*, 1996; Printen *et al.*, 1997). G_M is a 124-kDa protein that is significantly larger than the other three approximately 35-kDa family members. The nonhomologous portion of G_M mediates localization at the sarcoplasmic reticulum (Hubbard and Cohen, 1993; Tang *et al.*, 1991). Despite a proposed common function, no two targeting subunits share more than 50 percent sequence homology, which is largely confined to the PP1- and putative glycogen-binding regions (Figure 2A). Additionally, northern blot analysis revealed that three subunits are expressed in both liver and skeletal muscle (Armstrong *et al.*,

1997; Doherty *et al.*, 1995; Printen *et al.*, 1997; Tang *et al.*, 1991). These observations suggest that each targeting subunit may possess unique regulatory properties in regard to PP1 activity and hormonal responsiveness.

Sequence analysis predicted that PP1 binds to the N-terminal region of the glycogen-targeting subunits through a consensus V̲X̲F̲ binding motif that is conserved in other PP1 binding proteins (Zhao and Lee, 1997) (Figure 2B). This supposition was confirmed for G_M, G_L, and PTG through peptide competition, deletion analysis, and crystallization studies (Armstrong *et al.*, 1998; Egloff *et al.*, 1997; Fong *et al.*, in press; Johnson *et al.*, 1996). Furthermore, mutagenesis of the invariant V̲ and F̲ residues in PTG completely abrogates PP1 binding (Fong *et al.*, in press). Although the importance of these conserved residues explains the mutually exclusive binding of PP1 to glycogen-targeting subunits, substitution of the single amino acid in the X̲ position on G_M with the corresponding residue from PTG also disrupted PP1 binding (Liu *et al.*, 2000). This result indicates that the immediate consensus motif is not necessarily interchangeable between targeting proteins. Additionally, a series of point mutations in PP1 differentially affected interaction with the glycogen-targeting subunits in a two-hybrid array (N.M. Fong, M.J. Brady, and A.R. Saltiel, unpublished observation). These results suggest that multiple contact points mediate the interaction of PP1 with each glycogen-targeting subunit.

B. DIFFERENTIATAL MODULATION OF PP1 ACTIVITY BY BINDING TO GLYCOGEN-TARGETING SUBUNITS

The glycogen-targeting proteins confer substrate specificity to PP1 by localizing the phosphatase to glycogen particles. The conserved region on the targeting subunits that binds to glycogen has been loosely defined (Figure 2A) through deletion analysis, mutagenesis, and sequence comparison (Armstrong *et al.*, 1997; Doherty *et al.*, 1996; Fong *et al.*, in press; Wu *et al.*, 1998). However, these subunits also have differential effects on phosphatase activity against glycogen-localized substrates. PTG stimulates the PP1-catalyzed dephosphorylation of phosphorylase *in vitro* (Brady *et al.*, 1997b), while G_M increases PP1 activity against GS, phosphorylase, and phosphorylase kinase (Hubbard and Cohen, 1989b). In contrast, the binding of PP1 to G_L inhibits phosphatase activity against phosphorylase, while increasing the dephosphorylation of GS (Doherty *et al.*, 1995). These effects on phosphatase activity can be reversed by interfering with PP1 interaction with PTG or G_L, either through mutagenesis or addition of a peptide corresponding to the PP1 binding site on G_M (Fong *et al.*, in press; Johnson *et al.*, 1996). Thus, the unique PP1-binding pockets of the various glycogen-targeting proteins may differentially modulate PP1 activity against the same glycogen-metabolizing enzymes.

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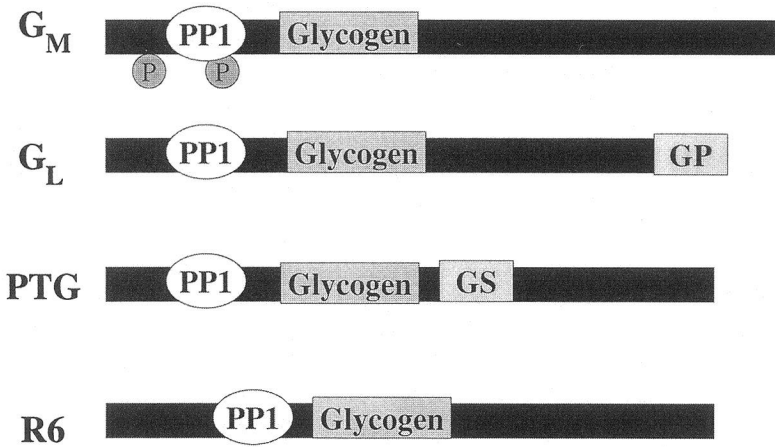
B

PP1-GTS	AA	Sequence
GM	62-76	GGRRVSFADNFGFNL
GL	58-72	VKKRVSFADNQGLAL
PTG	58-72	AKKRVVFADSKGLSL
R6	99-113	QKLRVRFADALGLEL

FIG. 2. (A) Schematic diagram of the functional domains of PP1 glycogen-targeting subunits. Abbreviations: GS = glycogen synthase; GP = glycogen phosphorylase. The PP1-, glycogen-, and GS-binding domains of the glycogen-targeted subunits are indicated by ovals and squares. G_M is a 124-kDa protein, while the other three targeting subunits are around 35 kDa. Only the homologous N-terminal portion of G_M is shown. The Ser-48 and Ser-67 residues on G_M that may be phosphorylated in response to hormones are indicated by shaded Ps. PP1 binds to the N-terminal region of all four targeting subunits. Glycogen phosphorylase binds to the extreme C-terminus of G_L, while mutation of two residues in the GS-binding region of PTG completely abrogates interaction with the enzyme. (B) Alignment of the PP1-binding domains from glycogen-targeting subunits. The VXF motif in the PP1-binding domains is highly conserved with other PP1-binding proteins. However, substitution of Ser-67 on G_M with Val-63 from PTG completely abrogated PP1 binding, indicating that other residues must be involved in the binding of PP1 by glycogen-targeting subunits. Abbreviations: GTS = glycogen-targeting subunit; AA = amino acid residue; invariant residues are shaded gray.

Glycogen-targeting subunits also form specific complexes with PP1 sub-strates that regulate glycogen synthesis. PTG directly binds to GS, phosphorylase, and phosphorylase kinase (Printen et al., 1997). Deletion analysis revealed that the glycogen regulatory enzymes bind to a single domain located in the C-terminal region of PTG (Fong et al., in press). Mutagenesis of two acidic residues in this region completely blocked the binding of these proteins to PTG, while having no effect on PP1 binding. Conversely, mutagenesis of the two highly conserved residues in the PP1-binding domain of PTG abrogated PP1 interaction without affecting complex formation with the glycogen-metabolizing enzymes (Fong et al., submitted). Overexpression of wild-type PTG in Chinese hamster ovary cells overexpressing insulin receptor (CHO-IR) cells induces a redistribution of PP1 and GS from the cytosol to glycogen particles and a commensurate marked increase in glycogen synthesis rate (Printen et al., 1997). Interestingly, disruption of either PP1 or GS binding to PTG mutants completely blocked glycogen accu-mulation (Fong et al., in press). These results demonstrate that targeting PP1 to glycogen alone is not sufficient to increase glycogen storage and implicate PP1 substrate binding as a critical function of PTG.

G_L does not bind to GS but does bind to phosphorylase with an approximately 1000-fold higher affinity than PTG (Armstrong et al., 1998). The extreme C-ter-minus of G_L contains a phosphorylase-binding site that is not conserved in the other glycogen-targeting subunits (Armstrong et al., 1998). PP1 binding to G_L potently inhibits phosphatase activity against phosphorylase. Furthermore, the G_L-PP1 complex is subject to allosteric inhibition by phosphorylase, which de-creases PP1 activity against GS in vitro (Doherty et al., 1995). Removal of the last 17 amino acids from G_L completely abrogated phosphorylase binding (Arm-strong et al., 1998). However, the effects of this deletion on PP1 activity or glycogen metabolism were not reported. Interestingly, recent data suggest that G_L preferentially regulates glycogenolysis, while PTG primarily mediates insulin-stimulated glycogen synthesis (Gasa et al., 2000). Thus, multiple glycogen-tar-geting 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.

IV. Tissue-specific Regulation of Glycogen-targeted PP1

Insulin-stimulated glycogen synthesis depends on increased glucose uptake, which coordinately regulates glycogen-metabolizing enzymes. In skeletal muscle and adipose tissue, insulin-stimulated glucose uptake is mediated by the translo-cation of the glucose transporter GLUT4 to the cell surface. In contrast, hepatic glucose transport is primarily mediated by GLUT2, which enables the rate of glucose uptake to change in parallel with plasma glucose levels, independently of insulin. PP1-targeting subunits also display tissue-specific expression patterns. In

skeletal muscle, PP1 is primarily localized to glycogen through binding of G_M (Tang *et al.*, 1991), although PTG and R6 are expressed at lower levels (Armstrong *et al.*, 1997; Printen *et al.*, 1997). In adipose tissue, PTG is the principal PP1 glycogen-targeting subunit (Printen *et al.*, 1997). However, in liver, PTG and G_L are expressed at equivalent levels (O'Doherty *et al.*, 2000), which may facilitate the utilization of hepatic glycogen for both glucose storage and hepatic glucose output. Thus, the tissue-specific hormonal regulation of glycogen metabolism partially arises from differential expression of facilitative glucose transporters in combination with PP1 glycogen-targeting subunits.

A. HORMONAL REGULATION OF GLYCOGEN-TARGETED PP1 IN STRIATED MUSCLE MAY INVOLVE PHOSPHORYLATION OF G_M

In rabbit skeletal muscle, G_M was reported to be phosphorylated on Ser-48 and Ser-67 in response to both insulin and adenylyl cyclase activators (Dent *et al.*, 1990; Hubbard and Cohen, 1993). Phosphorylation of Ser-48 in response to insulin increased PP1 activity against phosphorylase kinase and GS but not phosphorylase (Dent *et al.*, 1990). This is a further indication that glycogen-targeting subunits may preferentially regulate PP1 activity against specific glycogen-localized substrates. The proposed involvement of the MAPK cascade in the insulin-stimulated phosphorylation of G_M (Dent *et al.*, 1990) has been disproven (Azpiazu *et al.*, 1996; Lazar *et al.*, 1995). The putative insulin-activated kinase that phosphorylates Ser-48 has not been identified. However, mutagenesis of Ser-48 to alanine has no effect on GS activation by insulin, indicating that other regulatory mechanisms are involved (Liu and Brautigan, 2000). Additionally, conflicting reports exist regarding whether the insulin-stimulated phosphorylation of G_M occurs in primary tissue and cell lines (Begum, 1995; Dent *et al.*, 1990; Liu and Brautigan, 2000; Walker *et al.*, 2000).

G_M is also phosphorylated on Ser-48 and Ser-67 in response to elevation of intracellular cAMP levels (Hubbard and Cohen, 1989a; Walker *et al.*, 2000). Ser-67 is located in the middle of the PP1-binding region. Phosphorylation of this site results in dissociation of the phosphatase from G_M and glycogen (Hubbard and Cohen, 1989a; Liu *et al.*, 2000) and a corresponding reduction in PP1 activity against glycogen-bound substrates (Hubbard and Cohen, 1989b) (Figure 3). Thus, disruption of PP1 binding to G_M would further enhance the stimulation of glycogenolysis induced by cAMP elevation. Substitution of Ser-67 with a threonine residue blocks PKA-mediated phosphorylation as well as the release of PP1 from G_M (Liu *et al.*, 2000). Thus, while the hormonal regulation of glycogen metabolism in skeletal muscle may involve the phosphorylation of G_M, the putative regulatory phosphorylation sites are not conserved in the other glycogen-targeting
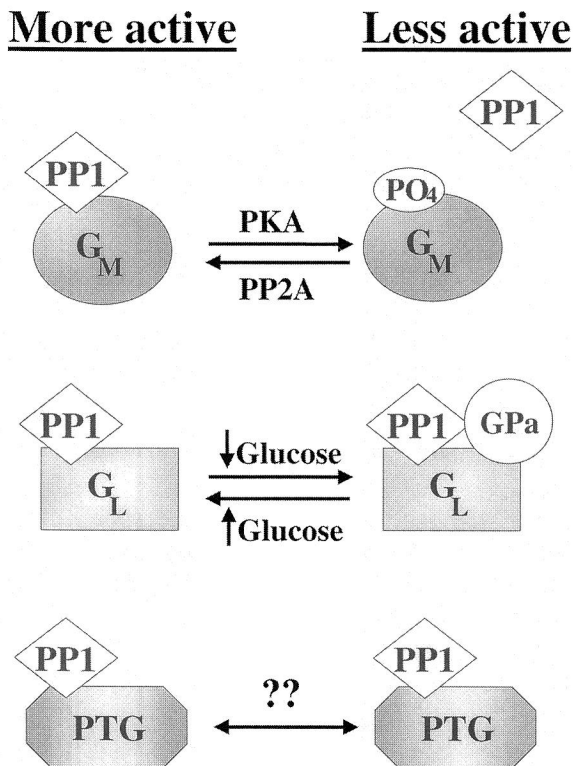
More active   Less active

FIG. 3. Regulation of glycogen-targeted PP1 activity. The PKA-mediated phosphorylation of Ser-67 on G_M disrupts PP1 binding to the targeting subunit. The disassociation of PP1 from glycogen further enhances glycogen breakdown by glycogenolytic agents. In liver, reduction of intracellular glucose levels results in binding of activated phosphorylase to G_L and allosteric inhibition of PP1. Conversely, increased intracellular glucose levels removes phosphorylase from G_L, causing PP1 activation. The mechanisms of hormonal regulation of the PP1-PTG complex are unknown. Abbreviations: PKA = cAMP-dependent protein kinase; PP2A = protein phosphatase 2A; GPa, glycogen phosphorylase a.

subunits, indicating that additional mechanisms must be utilized by insulin to activate glycogen-targeted PP1.

B. HORMONAL REGULATION OF
HEPATIC GLYCOGEN METABOLISM

Glycogen metabolism in liver is responsive to changes in both extracellular glucose and hormone levels (Villar-Palasi and Guinovart, 1997). Since GLUT2 acts as a glucose sensor, hepatic glucose levels change in parallel to plasma

glucose concentrations. During hyperglycemic conditions, glucose is stored as glycogen, while, conversely, hypoglycemia induces glycogenolysis, which contributes to the elevation of hepatic glucose output. The regulation of the G_L-PP1 complex by phosphorylase binding may mediate these effects. During hyperglycemic conditions, glucose binds to phosphorylase, relieving the allosteric inhibition of glycogen-targeted PP1 (Figure 3). Additionally, elevation of intracellular G6P levels increases PP1 activity against GS. Conversely, during hypoglycemic conditions, when hepatic glycogen stores are mobilized to increase hepatic glucose output, phosphorylase allosterically inhibits the G_L-PP1 complex, reducing GS activity (Figure 3). Insulin and glycogenolytic agents can further regulate these enzymes by modulating kinase activities, thus establishing a delicate balance between hepatic glycogen synthesis and the energy needs of peripheral tissues. However, since G_L and PTG are expressed at equal levels in the liver (O'Doherty *et al.*, 2000), multiple, undefined, second messenger cascades most likely are also involved in the hormonal regulation of hepatic glycogen metabolism.

C. INSULIN ACTIVATES THE PTG-PP1 COMPLEX

The mechanisms of hormonal regulation of PP1 bound to PTG are unclear. Insulin stimulates both PP1 and GS activity in 3T3-L1 adipocytes (Brady *et al.*, 1997a), which contain high levels of PTG (Printen *et al.*, 1997). However, treatment of adipocytes with either insulin or adenylyl cyclase activators does not cause phosphorylation of PTG or any detectable movement of PP1 between cellular fractions (Brady *et al.*, 1997b). The potential regulation of the PTG-PP1 complex by DARPP-32 in 3T3-L1 adipocytes has been suggested (Brady *et al.*, 1997a). Following phosphorylation of a single site by PKA, DARPP-32 specifically and potently inhibits PP1 (Hemmings *et al.*, 1984). Increased phosphorylation of DARPP-32 might mediate inhibition of PP1 by cAMP elevation, while disruption of the PTG-PP1-DARPP-32 complex by insulin would allow for the specific activation of glycogen-targeted PP1. However, recent data indicate that PTG and DARPP-32 share overlapping binding sites on PP1, precluding trimeric formation (Zhao and Lee, 1997). Additionally, mouse models lacking PP1 inhibitor proteins displayed no perturbations in the stimulation of GS by insulin (Scrimgeour *et al.*, 1999). However, novel PP1-inhibitor proteins recently were described that differentially inhibited PP1 bound to myosin- and glycogen-targeting subunits (Eto *et al.*, 1999). The potential interaction of the PTG-PP1 complex with other regulatory proteins is currently under investigation.

V. Modulation of PP1 Glycogen-targeting Subunit Levels Markedly Alters Glycogen Metabolism *in Vivo*

A. CELLULAR OVEREXPRESSION OF GLYCOGEN-TARGETING SUBUNITS

PTG overexpression in CHO-IR cells results in a dramatic increase in glycogen accumulation. Although there was no change in cellular GS or PP1 protein levels, PTG overexpression resulted in a redistribution of both enzymes to glycogen particles and a corresponding marked increase in GS activity and glycogen synthesis (Printen *et al.*, 1997). Similarly, basal and insulin-stimulated GS activity was elevated in muscle cell lines overexpressing G_M (Liu and Brautigan, 2000; Ragolia and Begum, 1997). These results indicate a critical role for PP1 glycogen-targeting subunits in the control of key regulatory enzymes involved in glycogen metabolism.

Recently, Newgard and colleagues have expanded these initial findings through use of adenoviral-mediated gene transfer into primary cell cultures and animals. Transfection of primary hepatocytes with PTG resulted in a dramatic increase in basal cellular glycogen levels, even when the extracellular medium was supplemented only with amino acids (Berman *et al.*, 1998). Furthermore, glycogen stores in the cultures infected with PTG were refractory to breakdown by agents that raise intracellular cAMP levels. These results suggested that PTG overexpression locks the cell into a glycogenic mode by pulling carbon chains into glycogen through various metabolic pathways, desensitizing the cell to glycogenolytic stimuli (Berman *et al.*, 1998). Similar findings were reported when rats were inoculated with the PTG adenoviral construct, which results in preferential protein expression in liver (O'Doherty *et al.*, 2000). Hepatic glycogen stores were markedly elevated and did not substantially decrease during fasting conditions. Interestingly, despite increased glucose deposition into glycogen, the transgenic animals displayed no alterations in plasma glucose, insulin, free fatty acid, or triglyceride levels (O'Doherty *et al.*, 2000). Recently, the same group has reported that transfection of primary hepatocytes with G_L or PTG results in similarly large increases in glycogen levels, while G_M overexpression has more-modest effects (Gasa *et al.*, 2000). Cells containing G_L were no longer responsive to insulin-stimulated glycogen synthesis, while PTG-overexpressing cells were refractory to glycogenolytic stimuli. These results suggest that PTG may play a greater role in mediating insulin-stimulated glycogen synthesis, while G_L may preferentially transduce cAMP elevation into glycogen breakdown.

B. PP1-GLYCOGEN-TARGETING SUBUNIT
KNOCKOUT MOUSE MODELS

Two transgenic mouse strains have been made that lack either G_M or PTG. The G_M knockout mice exhibited no apparent developmental defects but showed reduced GS activity and enhanced phosphorylase activity. The mice produced a 90 percent reduction in skeletal muscle glycogen levels (Depaoli-Roach *et al.*, 1999). Despite the dramatic disruption of peripheral glucose storage, fasting blood glucose and rate of clearance of an injected glucose challenge were normal. In contrast, PTG knockout mice are not viable, although the cause and precise timing of the embryonic lethality is not yet known (M.J. Brady, S.M. Crosson, P.A. Hansen, J.E. Pessin, and A.R. Saltiel, unpublished observation). Due to the redundancy of expression, disruption of an individual PP1 glycogen-targeting subunit may not be sufficient to produce fasting hyperglycemia. Interbreeding of the glycogen-targeting knockout lines and crossbreeding with other diabetic rodent models may be needed before perturbations in glucose homeostasis are realized.

C. POTENTIAL ROLE FOR ALTERATIONS IN PP1
GLYCOGEN-TARGETING SUBUNIT FUNCTION
IN TYPE 2 DIABETES

Insulin-stimulated glucose transport and storage is inhibited in all experimental models of non-insulin dependent or type 2 diabetes. Additionally, reduced glucose clearance by peripheral tissues is one of the first defects detected in insulin-resistant patients, which may be the primary lesion leading to the development of diabetes. Shulman and colleagues (Cline *et al.*, 1999) recently have pinpointed reduced glucose transport as the primary defect causing insulin resistance. However, the activation of GS and PP1 by insulin is also diminished (Freymond *et al.*, 1988). Overexpression of PP1 glycogen-targeting subunits has been reported to markedly alter glycogen synthesis rates without affecting protein levels of key regulatory enzymes (Berman *et al.*, 1998; Gasa *et al.*, 2000; Printen *et al.*, 1997; Ragolia and Begum, 1997). Thus, defects in the expression or function of PP1 glycogen-targeting subunits might impact on the metabolic actions of insulin *in vivo*, resulting in insulin resistance. However, a detailed, comprehensive characterization of PP1 glycogen-targeting subunit expression in normal, obese, and diabetic patients has not been reported.

Genetic linkage analyses have suggested possible alterations in G_M function and/or expression in the onset of insulin resistance and type 2 diabetes. A widespread amino acid mutation in G_M was associated with altered rates of glucose metabolism in Danish subjects *in vivo* (Hansen *et al.*, 1995) and increased sensitivity to glycogenolytic agents *in vitro* (Ragolia *et al.*, 1999). However, despite the widespread penetrance of this allele in the Japanese population, no linkage could be established between the mutation and insulin resistance (Shen *et al.*,

1998). A second mutation in the 3′ noncoding region of G_M has been reported to result in mRNA instability (Xia et al., 1998,1999) and reduced G_M protein levels and may be linked to development of insulin resistance in Native American populations (Hegele et al., 1998; Xia et al., 1998). Clearly, more analysis will be required to address the potential link between aberrant PP1-glycogen-targeting subunit function and the development of insulin resistance and type 2 diabetes.

VI. Summary

Significant progress has been made in identifying many of the signaling components involved in insulin action and the development of insulin resistance (Cline et al., 1999; Virkamaki et al., 1999). However, relatively little is known concerning the precise molecular mechanisms by which insulin regulates glycogen metabolism. Insulin modulates the activities of the two rate-limiting enzymes of glycogen synthesis — GS and phosphorylase — through allosteric activation, intracellular translocation, and covalent modification. Increased glucose uptake and metabolism mediate the allosteric regulation and localization of GS and phosphorylase. Insulin also promotes the dephosphorylation of both enzymes, resulting in activation of GS and inactivation of phosphorylase. Although insulin inhibits upstream kinases for both enzymes, the activation of PP1 plays a crucial role in the control of glycogen levels. PP1 is present in nearly every cellular compartment through the action of targeting subunits. However, insulin promotes the dephosphorylation of a limited number of proteins that are mainly involved in glycogen metabolism. These results suggest that mechanisms must exist whereby PP1 is targeted to the glycogen particle and insulin can specifically activate this pool of phosphatase, while not affecting PP1 activity in other cellular compartments.

Recently, four proteins have been described that bind to PP1 and glycogen, thus targeting the phosphatase to the glycogen particle. These proteins are not highly homologous but display overlapping tissue expression, suggesting distinct functions. The mechanisms by which insulin activates PP1 activity bound to each targeting subunit are still poorly understood. The continued construction of chimeric targeting subunits, through point mutagenesis and swapping of putative domains, will aid in the elucidation of tissue-specific mechanisms for the regulation of glycogen synthesis. Additionally, these studies should help to elucidate why so many relatively nonconserved proteins are required to target PP1 to the glycogen particle. Finally, comparison of this family of glycogen-targeting proteins to other PP1-targeting subunits will establish the importance of intracellular localization in the specific regulation of protein dephosphorylation by a variety of hormones and extracellular signals in different cell types.

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