Perspectives in Diabetes

Organizing Glucose Disposal

Emerging Roles of the Glycogen Targeting Subunits of Protein Phosphatase-1

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Glucose is stored in mammalian tissues in the form of glycogen. Glycogen levels are markedly reduced in liver or muscle cells of patients with insulin-resistant or insulin-deficient forms of diabetes, suggesting that impaired glycogen synthesis may contribute to development of hyperglycemia. Recently, interest in this area has been further stimulated by new insights into the spatial organization of metabolic enzymes within cells and the importance of such organization in regulation of glycogen metabolism. It is now clear that a four-member family of glycogen targeting subunits of protein phosphatase-1 (PP1) plays a major role in coordinating these events. These proteins target PP1 to the glycogen particle and also bind differentially to glycogen synthase, glycogen phosphorylase, and phosphorylase kinase, thereby serving as molecular scaffolds. Moreover, the various glycogen-targeting subunits have distinct tissue expression patterns and can influence regulation of glycogen metabolism in response to glycogenic and glycogenolytic signals. The purpose of this article is to summarize new insights into the structure, function, regulation, and metabolic effects of the glycogen-targeting subunits of PP1 and to evaluate the possibility that these molecules could serve as therapeutic targets for lowering of blood glucose in diabetes. *Diabetes* 49:1967–1977, 2000

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DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; G_M/R_{GL}, glycogen-targeting subunit in muscle; GSK3, glycogen synthase kinase 3; NMR, nuclear magnetic resonance; OGTT, oral glucose tolerance test; PKA, cAMP-dependent kinase; PP1, protein phosphatase-1; PTG, protein targeting to glycogen; STZ, streptozotocin; UDP, uridine 5′-diphosphate.

The primary storage form of glucose in mammals is glycogen. After ingestion of a meal containing carbohydrate, conversion of glucose into glycogen in muscle, liver, and fat is a key event in the maintenance of glucose homeostasis. Conversely, in the fed-to-fasted transition, glycogen stores represent a major source of energy and serve to protect against hypoglycemia. Importantly, liver glycogen stores are reduced in concert with an increase in net hepatic glucose production in all forms of human diabetes studied to date (1–3), and muscle glycogen deposition is also impaired in type 2 diabetes (4). These metabolic perturbations clearly contribute to development of hyperglycemia and may be a site for therapeutic intervention.

Glycogen synthesis and degradation are tightly regulated by hormones and metabolic signals, primarily via modulation of glycogen synthase and glycogen phosphorylase enzymatic activities. The foremost mechanism by which these enzymes are regulated in response to changing physiological conditions is phosphorylation and dephosphorylation, although their activities and/or subcellular distributions can also be modulated by allosteric effectors such as glucose-6-phosphate, glucose, uridine 5′-diphosphate (UDP)-glucose, and AMP (5–9). In the catabolic state (e.g., fasting or stress), glycogen synthase, the rate-limiting enzyme in glycogen synthesis, is phosphorylated on six regulatory serine residues by several kinases, resulting in its progressive inactivation (7). These kinases, which include cAMP-dependent kinase (PKA), calmodulin-dependent kinases, glycogen synthase kinase 3 (GSK3), protein kinase C, and others, respond to a variety of catabolic stimuli. Catabolic signals also promote PKA-mediated phosphorylation of phosphorylase kinase, which in turn phosphorylates glycogen phosphorylase on serine 14 (5), resulting in activation of the enzyme and stimulation of glycogenolysis. Under anabolic conditions, insulin stimulates activation of glycogen synthase and glycogen synthesis by promoting the net dephosphorylation of the enzyme. Insulin both attenuates the activation of kinases such as PKA and GSK3 (10,11) and activates serine/threonine phosphatases, particularly protein phosphatase-1 (PP1) (12–15). The insulin-dependent dephosphorylation of glycogen synthase is catalyzed by PP1 (12,16). PP1 also catalyzes the dephosphorylation and inactivation of glycogen phosphorylase, further contributing to stimulation of glycogen deposition.

The relative contributions of kinase inhibition and phosphatase activation to control of glycogen metabolism are incompletely understood and may vary between tissue and cell type. However, in recent years, PP1 and its associated proteins have clearly been shown to play an important role.

There is growing evidence that glucose disposal in general, and glycogen metabolism in particular, are spatially organized pathways within cells. Both processes begin with the transport of glucose across the cell membrane, enabled by members of the family of facilitated glucose transporters. In muscle, glucose transport is primarily regulated by the GLUT4 glucose transporter, which is stored in intracellular vesicles in the absence of insulin and translocated to the cell surface in response to the hormone. Once transported, glucose is immediately phosphorylated by one of the members of the hexokinase gene family. In liver, the primary glucose-phosphorylating enzyme is hexokinase IV, or glucokinase. In the fasted state, cytosolic glucokinase enzyme activity is low because of sequestration of the enzyme in the nucleus via its binding to an inhibitory glucokinase regulatory protein (17–19). In the postprandial state, glucose stimulates the translocation of glucokinase from the nucleus to the cytosol. In muscle, glucose is phosphorylated by hexokinase II, which is most active when bound to mitochondrial porins, allowing direct access to ATP as it exits the organelle (20). In liver, the balance between glucose disposal and glucose production is largely controlled by the relative rates of glucose uptake and phosphorylation on the one hand and hydrolysis of glucose-6-phosphate on the other. Glucose-6-phosphate hydrolysis is catalyzed by the glucose-6-phosphatase enzyme complex. The complex is comprised of a catalytic subunit sequestered within the endoplasmic reticulum, a glucose-6-phosphate translocase known as T1 that delivers glucose-6-phosphate to the catalytic subunit, and putative endoplasmic reticulum glucose and inorganic phosphate transporters (T2, T3) that move the reaction products back into the cytosol (21,22). The discrete localization of glucose phosphorylating enzymes in muscle and liver and of the glucose-6-phosphatase complex in liver (muscle lacks glucose-6-phosphatase activity) clearly has potential for influencing flux of glucose-6-phosphate into the pathways of glycolysis, glycogen synthesis, and the pentose shunt pathway and for modulating net hepatic glucose disposal and production.

The enzymes of glycogen metabolism also exhibit spatial organization. Thus, in liver cells, glycogen synthase is translocated from an intracellular site to the cell membrane in response to glucose and insulin, resulting in synthesis of glycogen in a gradient from the membrane surface toward the interior of the cell (23,24). Under basal conditions in muscle cells, glycogen synthase appears to be sequestered in the nucleus and is translocated out to the cytosol when glucose and insulin are provided (25). A role for glucose-6-phosphate in translocation of glycogen synthase has been suggested, based in part on findings that 2-deoxyglucose, a glucose analog that accumulates as 2-deoxyglucose-6-phosphate, stimulates movement of the enzyme in liver cells (6,8). However, 2-deoxyglucose is without effect on glycogen synthase localization in 3T3-L1 adipocytes, whereas the translocation event can be restored in extracts of such cells by addition of UDP-glucose but not glucose-6-phosphate (9). The precise mechanisms by which glucose-6-phosphate in liver and UDP-glucose in adipocytes mediate glycogen synthase translocation remain to be established.

Controlled movement of glycogen synthase within cells is complemented by targeting of PP1 to the glycogen particle. Such targeting is remarkable, considering the fact that PP1 is involved in the regulation of a wide variety of cellular processes other than glycogen metabolism, such as cell division, vesicle fusion, and ion channel function, which occur in virtually all cellular compartments (26–29). Significant understanding of the compartmentalized regulation of PP1 activity has been gained from the recent emergence of a family of PP1 targeting subunits. This family's many members can be divided into subclasses that mediate localization of PP1 to the nucleus, plasma membrane, neuronal dendrites, myosin filaments, or glycogen particles via subclass-specific binding to each of these subcellular targets (30–35). These interactions allow for compartmentalized activation of the phosphatase by a variety of extracellular signals, resulting in the specific dephosphorylation of discrete pools of proteins within the cell.

It is now appreciated that most mammalian cells contain at least one of four known glycogen-targeting subunits of PP1. Understanding of the structure, protein-protein interactions, regulatory properties, and metabolic effects of these proteins has blossomed in the last 3–4 years. In this article, we will summarize important advances in knowledge about these proteins, examine their role in the spatial organization of glucose disposal and glycogen metabolism, and discuss their overall impact on control of fuel homeostasis. We will also highlight important unanswered questions about the function of these molecules. Finally, we will address the issue of whether manipulation of the expression or activity of glycogen-targeting subunits is worthy of consideration as a potential therapeutic target for lowering blood glucose levels in diabetes.

STRUCTURE/FUNCTION ANALYSES OF GLYCOGEN-TARGETING SUBUNITS OF PP1

Insulin does not globally activate PP1, but rather specifically stimulates phosphatase activity associated with the glycogen particle. Thus, glycogen-targeting subunits were initially proposed to mediate the compartmentalized activation of PP1 by insulin (12,33). However, recent data indicate that these targeting proteins actually serve as "molecular scaffolds," juxtaposing the enzyme with its substrates in a macromolecular complex and, in the process, exerting profound effects on PP1 activity and glycogen metabolism.

The concept of targeting subunits of PP1 originated from studies of glycogen metabolism by Cohen and coworkers (36). These investigators purified a heterodimeric complex associated with rabbit skeletal muscle glycogen particles consisting of a 37-kDa catalytic subunit (serine/threonine phosphatase) and a 103-kDa regulatory component (36). The 37-kDa catalytic subunit is common to all targeted PP1 complexes in mammalian cells. The regulatory protein isolated from rabbit skeletal muscle was subsequently shown to be a proteolytic fragment (37), and its actual size as determined from a full-length cDNA clone is now known to be 124-kDa (38). Biochemical studies revealed that this protein contains binding sites for glycogen and the 37-kDa catalytic subunit. It was also shown to contain two serines that are phosphorylated in response to different hormonal signals, designated sites 1 and 2 (12). Phosphorylation of site 2 occurs in response to glycogenolytic agents such as epinephrine and causes dissociation of the regulatory targeting subunit and the

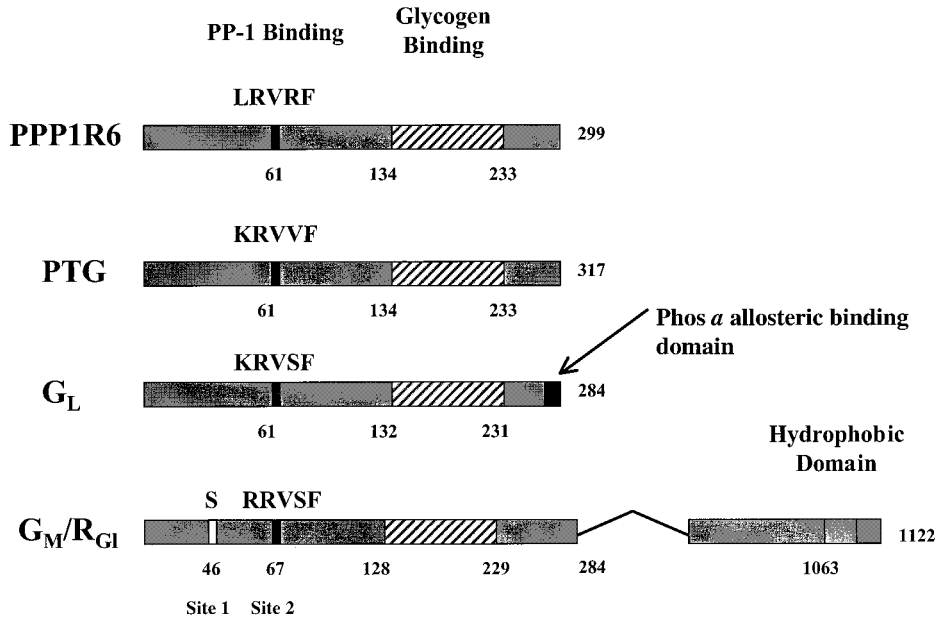

FIG. 1. Schematic alignment of the mammalian glycogen-targeting subunits of PP1. The four known members of the family are shown. G_M/R_{Gl} is expressed primarily in striated muscle, G_L is expressed primarily in liver, and PTG and PPP1R6 are expressed in many tissues. G_M/R_{Gl} is approximately three times larger than the other forms, and its COOH-terminal extension has no homology with the other family members. The COOH-terminal domain of G_M/R_{Gl} contains a patch of hydrophobic amino acids that are thought to mediate its binding to sarcoplasmic reticulum. G_M/R_{Gl} also contains two serines (designated site 1 and site 2) that are phosphorylated in response to glycogenolytic agents. None of the other forms contain serine at site 1, and they contain varying sequences at site 2, as shown for each form. A conserved glycogen binding motif is also indicated. Finally, G_L is the only targeting subunit that exhibits allosteric regulation by phosphorylase a, via binding of the enzyme to the COOH-terminal tail of the G_L molecule. Numbers under each targeting subunit refer to the amino acid sequence of each protein.

catalytic subunit, resulting in reduced PP1 activity (39). The regulatory role of site 1 has been the subject of some controversy and debate, as summarized below.

The skeletal muscle glycogen–targeting subunit has been known by several names. It was initially referred to as the G subunit, to indicate that it was the entity responsible for targeting PP1 to the glycogen particle (36). It was subsequently renamed R_{Gl}, for glycogen-binding regulatory subunit of PP1 (38). Very recently, as it has become apparent that the skeletal muscle glycogen–targeting subunit is a member of a gene family whose other members have varying tissue expression patterns, the human form of R_{Gl} has been given the name G_M, for muscle glycogen–targeting subunit, or PPP1R3. In the remainder of this article we will refer to this form of glycogen-targeting subunit as G_M/R_{Gl}.

G_M/R_{Gl} was the first glycogen-targeting subunit of PP1 to be cloned (38,40). It was found to be expressed primarily in striated skeletal muscle. More recently, three other forms have been described. G_L is a 35-kDa protein that is preferentially expressed in liver (41). Protein targeting to glycogen (PTG) (42), also known as PPP1R5 (43), and a fourth form, PPP1R6 (44), are similar in size to G_L but differ from G_L and G_M/R_{Gl} in that they are expressed in a wide variety of tissues based on Northern blot analysis (41,42,44).

Figure 1 presents a schematic alignment of these four glycogen-targeting subunits of PP1 that highlights key structural features. An important first point is that despite their common function, no two targeting subunits share >50% sequence homology when human sequences are used for comparison. In contrast, in those cases in which specific forms of glycogen-targeting subunits have been cloned from more than one species (e.g., rabbit and human G_M/R_{Gl}; mouse, human, and rat PTG), the interspecies homologies range from 70 to 85%, suggesting that specific isoforms have conserved functions across species (42,43,45,46). The sequence homology among family members is centered around the PP1- and putative glycogen-binding regions (44,47). An obvious distinguishing feature of G_M/R_{Gl} is that it is approximately three times as large as the other family members. The NH_2-terminal domain of G_M/R_{Gl} is homologous to the other family members and contains its PP1- and glycogen-binding regions. The COOH-terminal two-thirds of G_M/R_{Gl} shares no homology with other family members, but contains a hydrophobic sarcoplasmic reticulum–binding domain (38). All four family members contain the consensus PP1 binding motif (R/K) (V/I) X F (the actual sequence present in each human protein is shown in Fig. 1). G_M/R_{Gl} and G_L, but not PTG or PPP1R6, contain serines at the X position of this motif, but only G_M/R_{Gl} also contains the RR consensus sequence for PKA-mediated phosphorylation immediately upstream (the G_L sequence is KR). This serine (serine 65 in the human G_M/R_{Gl} sequence) is the site 2 phosphorylation site of G_M/R_{Gl}. The site 1 phosphorylation site of G_M/R_{Gl} (RRGS, serine 46 in the human G_M/R_{Gl} sequence) is also unique. Thus, even this cursory inspection of structural features, coupled with the differences in tissue expression patterns, strongly suggests that each targeting subunit may possess unique regulatory properties that could influence regulation of glycogen metabolism in a tissue type–dependent fashion in response to changes in physiological conditions.

The consensus PP1 binding motif (R/K) (V/I) X F is conserved in all PP1 binding proteins (48), including the *GAC1* gene product of yeast, which encodes an 88-kDa protein with clear homology to G_M/R_{Gl} (49). That *GAC1* plays an important

role in regulation of glycogen metabolism in yeast is indicated by studies in which its overexpression was shown to cause hyperaccumulation of glycogen, whereas disruption of the gene reduced glycogen levels (49). The PP1-binding function of this motif has been confirmed for GM/R_{Gl}, G_L, and PTG through peptide competition, deletion analysis, and crystallization studies (50–53). Further, mutagenesis of the invariant V and F residues in PTG completely abrogates PP1 binding (53). Although these conserved residues clearly direct the binding of PP1 to glycogen-targeting subunits, substitution of the single amino acid in the X position of the G_M/R_{Gl} PP1-binding region with the corresponding residue from PTG also disrupts PP1 binding (54), indicating that the immediate consensus motif is not necessarily interchangeable between targeting proteins. Additionally, a series of point mutations in PP1 differentially affected interactions with the glycogen-targeting subunits in a two-hybrid array (N.M. Fong, M.J.B., A.R.S., unpublished observations). These results suggest that multiple contact points are involved in the interaction of PP1 with each glycogen-targeting subunit.

The various glycogen-targeting subunits have differential effects on phosphatase activity against glycogen-localized substrates. Both G_M/R_{Gl} and PTG stimulate the PP1-catalyzed dephosphorylation of phosphorylase a in vitro (55,56). In contrast, the binding of PP1 to G_L inhibits phosphatase activity against phosphorylase a, while increasing the dephosphorylation of glycogen synthase (41). These effects on phosphatase activity can be reversed by disrupting PP1 binding to PTG or G_L, either through mutagenesis or addition of a peptide corresponding to the PP1 binding site (52,53). Thus, the unique PP1-binding pockets of the various glycogen-targeting subunits appear to differentially modulate PP1 activity against the same glycogen-metabolizing enzymes.

Glycogen-targeting subunits also bind directly to enzymes that regulate glycogen synthesis. For example, PTG binds to glycogen synthase, phosphorylase, and phosphorylase kinase (42). Deletion analysis reveals that the glycogen regulatory enzymes bind to a single domain located in the COOH-terminal region of PTG (53). Mutagenesis of two acidic residues in this region completely blocked the binding of these proteins to PTG, but had no effect on PP1 binding. Conversely, mutagenesis of the two highly conserved residues in the PP1-binding domain of PTG abrogated interaction with PP1 without affecting complex formation with the glycogen-metabolizing enzymes. Overexpression of wild-type PTG in CHO-IR cells induced a redistribution of PP1 and glycogen synthase from the cytosol to glycogen particles and also caused a marked increase in glycogen levels. Interestingly, disruption of either PP1 or glycogen synthase binding sites in PTG completely blocks the stimulatory effect of the protein on glycogen accumulation (53). Similar results have recently been reported for G_M/R_{Gl} (57). These results demonstrate that targeting of PP1 to glycogen alone is not sufficient to increase glycogen storage, and they demonstrate the critical role for PP1-substrate binding in the function of glycogen-targeting subunits.

G_L binds to phosphorylase a with an ~1,000-fold higher affinity than PTG (50). The extreme COOH-terminus of G_L contains a phosphorylase a binding site that is not conserved in the other glycogen-targeting subunits (Fig. 1). Binding of phosphorylase a to the G_L-PP1 complex results in allosteric inhibition of PP1 activity, resulting in reduced dephosphorylation and activation of glycogen synthase in vitro (41). Removal of the last 17 amino acids from G_L completely abrogated phosphorylase binding (50), but the effects of this deletion on PP1 activity or glycogen metabolism were not reported. Thus, the glycogen-targeting subunits differentially modulate PP1 substrate specificity through intracellular localization, distinct mechanisms of binding to PP1, and direct interaction with phosphatase substrates.

ROLE OF GLYCOGEN-TARGETING SUBUNITS IN TISSUE-SPECIFIC REGULATION OF GLYCOGEN METABOLISM

In skeletal muscle and adipose tissue, insulin activates glucose uptake by stimulating translocation of the glucose transporter GLUT4 to the cell surface and phosphorylation of glucose by hexokinase II once it enters the cell. In contrast, hepatic glucose transport and phosphorylation are primarily mediated by GLUT2 and glucokinase (hexokinase IV), whose kinetic features enable the rate of glucose uptake and phosphorylation to change in parallel with plasma glucose levels, independent 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/R_{Gl} (38), although PTG and PPP1R6 are also expressed at lower levels (42,44). In adipose tissue, PTG is the principal PP1 glycogen-targeting subunit (42). In liver of fed rats, PTG and G_L are expressed at equivalent levels, although interestingly, G_L undergoes much larger changes in expression in response to fasting and feeding than does PTG (46), suggesting that the two forms may make differential contributions to glycogen metabolism dependent on dietary conditions. Finally, liver and muscle express different isoforms of glycogen phosphorylase and glycogen synthase, which exhibit different regulatory properties (5–7,58). Thus, tissue-specific hormonal regulation of glycogen metabolism is mediated by the expression of tissue-specific isoforms of the proteins and enzymes that participate in the relevant metabolic pathways. Among these, the different forms of glycogen-targeting subunits of PP1 may play a particularly important role by coordinating the formation of a complex of metabolic enzymes involved in glycogen metabolism, as detailed below.

In rabbit skeletal muscle, G_M/R_{Gl} was reported to be phosphorylated on site 1 and site 2 in response to insulin (33). Phosphorylation of site 1 in response to insulin was reported to increase PP1 activity against phosphorylase kinase and glycogen synthase, but not phosphorylase (12). However, the proposed involvement of the mitogen-activated protein kinase cascade in the insulin-stimulated phosphorylation of G_M/R_{Gl} has been disproved (59,60), and the putative insulin-activated kinase that phosphorylates site 1 has not been identified. Furthermore, mutagenesis of site 1 to alanine has no effect on glycogen synthase activation by insulin (61), indicating that other regulatory mechanisms are involved. Additionally, there are conflicting reports as to whether the insulin-stimulated phosphorylation of G_M/R_{Gl} occurs in skeletal muscle (12,62). Thus, further studies will be required to determine whether the effect of insulin in activating glycogen metabolism involves any direct modification of glycogen-targeting subunits.

G_M/R_{Gl} is also phosphorylated on sites 1 and 2 in response to elevation of intracellular cAMP levels (39,62). Site 2 is located in the middle of the PP1-binding region, and phosphorylation of this site results in disassociation of the phos-

phatase from G_M/R_{Gl} and glycogen (39,61). Disruption of PP1 binding to G_M/R_{Gl} impairs dephosphorylation of glycogen synthase, phosphorylase, and phosphorylase kinase, favoring glycogenolysis. Substitution of serine 67 (the site 2 serine in rabbit G_M/R_{Gl}) with a threonine residue blocks PKA-mediated phosphorylation as well as the release of PP1 from G_M/R_{Gl} (61). Although regulation of glycogen metabolism in skeletal muscle in response to glycogenolytic agents appears to involve the phosphorylation of G_M/R_{Gl}, these regulatory phosphorylation sites are not conserved in the other glycogen-targeting subunits, indicating that additional mechanisms must be operative in the many glycogen-metabolizing tissues that do not express G_M/R_{Gl}.

The mechanisms of hormonal regulation of PP1 bound to PTG are unclear. Treatment of adipocytes with either insulin or adenylate cyclase activators does not cause phosphorylation of PTG or any detectable movement of PP1 between cellular fractions (55). The potential regulation of the PTG-PP1 complex by dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) was suggested (55). After phosphorylation of a single site by PKA, DARPP-32 is a specific and potent inhibitor of PP1 (63). Increased phosphorylation of DARPP-32 might mediate its inhibition of PP1, whereas disruption of the PTG–PP1–DARPP-32 complex by insulin would allow for the specific activation of glycogen-targeted PP1. However, recent studies indicate that PTG and DARPP-32 share overlapping binding sites on PP1 (48), apparently precluding formation of trimers. Additionally, transgenic mice in which PP1-inhibitor proteins are knocked out display no perturbations in the stimulation of glycogen synthase by insulin (64). However, novel PP1-inhibitor proteins have recently been described that differentially inhibit PP1 bound to myosin- and glycogen-targeting subunits (65). The potential interaction of the PTG-PP1 complex with other regulatory proteins is currently under investigation.

Glycogen synthesis in the liver can be regulated through modulation of extracellular glucose concentrations, independently of insulin. Elevation of intracellular glucose-6-phosphate levels stimulates glycogen synthase activity through cellular redistribution of the enzyme, allosteric activation, and activation of PP1-mediated dephosphorylation (6). Increased glucose levels also relieve the inhibition of the G_L-PP1 complex, in part by direct binding of glucose to phosphorylase a, rendering the enzyme a better substrate for PP1 (5,58). This effect of glucose results in increased PP1 activity against glycogen synthase. Conversely, as plasma glucose levels fall, phosphorylase a levels increase, and the enzyme reassociates with G_L, inhibiting PP1 activity against glycogen synthase. These mechanisms for regulation of glycogen metabolism by glucose are made possible in liver by its expression of the low-affinity, high-capacity GLUT2 glucose transporter. Interestingly, semiquantitative polymerase chain reaction techniques have recently been used to demonstrate that G_L is also the predominant glycogen-targeting subunit expressed in pancreatic islets (Y. Lee, T. Kakuma, Y.-T. Zhou, P. Jensen, R.H. Unger, C.B.N., unpublished observations), again fitting with expression of GLUT2 in these cells. However, both liver and islets also express PTG (42,46; Y. Lee, T. Kakuma, Y.-T. Zhou, P. Jensen, R.H. Unger, C.B.N., unpublished observations). Expression of this form of glycogen-targeting subunit may enhance the overall capacity for glycogen storage in a constitutive fashion or allow for regulation of glycogen deposition by signals other than glucose and its metabolites. Insights into subtle differences in regulatory properties of glycogen-targeting subunits is beginning to emerge, but more information will be required to fully understand why some tissues express more than one form of glycogen-targeting subunit.

METABOLIC STUDIES, PATHOPHYSIOLOGY, AND THERAPEUTIC IMPLICATIONS

It is clear from the work summarized in the first portion of this review that distinct glycogen-targeting subunits of PP1 have evolved with discrete regulatory properties. What then is the evidence that these proteins have any significant impact on the capacity of mammalian cells to synthesize glycogen under different physiological conditions, or that they have any more global impact on fuel homeostasis? One potentially conclusive means of demonstrating a biological role for a given protein is to reduce its level of expression by homologous recombination, or knock-out, of its encoding gene. In the case of glycogen-targeting subunits, experiments of this nature are ongoing in several laboratories but have yet to appear as full publications.

In one report published in abstract form, homozygous knock-out of the gene encoding G_M/R_{Gl} in mice resulted in substantial reductions in PP1 activity and glycogen synthase activity state and a 90% decrease in muscle glycogen content, but surprisingly, the animals remained normoglycemic (66). More indirect evidence of a critical role of glycogen-targeting subunits in control of normal glycogen metabolism comes from studies of streptozotocin (STZ)-induced diabetic or adrenalectomized fasted mice. In both models, liver glycogen levels were dramatically decreased in concert with reduced glycogen particle–associated PP1 and glycogen synthase activities (67). G_L protein levels were found to be sharply reduced in both models, whereas expression of the 37-kDa catalytic subunit of PP1 was unaltered, suggesting a critical role of the targeting subunit in normal glycogen synthesis. Much more can be learned from future studies of transgenic animals in which glycogen-targeting subunit genes are knocked out, either alone or in various combinations.

Another approach to defining the role of genes in metabolic control is to demonstrate a genetic linkage in human disease. A hallmark of type 2 diabetes is reduced glycogen storage in liver and skeletal muscle (1–4). In Pima Indians, a Native American population with a high incidence of type 2 diabetes, there is evidence that the defect in nonoxidative glucose disposal in muscle is linked to reduced glycogen synthase activity (68). The rapid advances in knowledge about glycogen-targeting subunits have logically led to several recent investigations of the potential role of these proteins in impaired glucose disposal. Thus, a widespread amino acid mutation in G_M/R_{Gl} has been associated with altered rates of glucose metabolism in Danish subjects in vivo (69) and increased sensitivity to glycogenolytic agents in vitro (70). However, despite the widespread penetrance of this allele in the Japanese population, no linkage could be established between the mutation and insulin resistance (71). A second mutation in the 3′ noncoding region of G_M/R_{Gl} has been reported to result in mRNA instability (72–74) and reduced G_M/R_{Gl} protein levels and has been linked to development of insulin resistance in Native American populations (72,75). Other point mutations in human G_M/R_{Gl}, such as Asp905Tyr

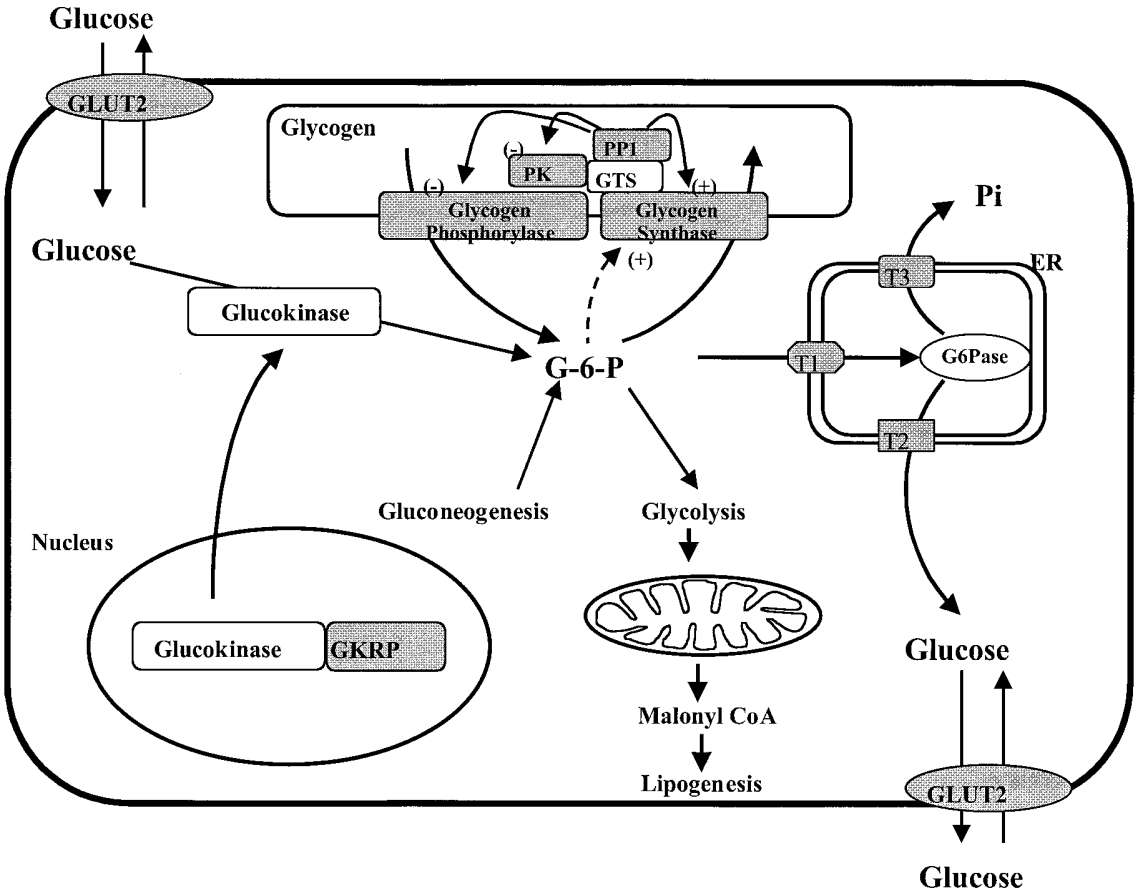

FIG. 2. Spatial organization of glucose and glycogen metabolism in liver cells. The figure shows some of the key regulatory steps controlling hepatic glucose disposal and production and emphasizes how these steps are compartmentalized. Examples of this include translocation of glucokinase from the nucleus to the cytoplasm in response to nutritional stimulation, localization and regulation of enzymes of glycogen metabolism via the glycogen-targeting subunits of PP1, and sequestration of the glucose-6-phosphatase catalytic subunit in the endoplasmic reticulum. The figure shows that glucokinase overexpression can affect lipid homeostasis by increasing malonyl-CoA levels, which diverts fatty acids from oxidative to esterification pathways. In contrast, overexpression of glycogen-targeting subunits is predicted to enhance glucose disposal by activation of glycogenesis, possibly without affecting lipid metabolism. ER, endoplasmic reticulum; G-6-P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GKRP, glucokinase regulatory protein; GTS, glycogen-targeting subunits; PK, phosphorylase kinase; T1, T2, T3, components of the glucose-6-phosphatase enzyme complex serving as translocases for glucose-6-phosphate, glucose, and inorganic phosphate, respectively.

or Arg883Ser, have been described with suggested linkages to reduced glucose disposal in several populations, but no functional defects in these proteins were uncovered when they were overexpressed in L6 myotubes (74,76). Thus, it is clear that more analysis will be required to understand fully the potential link between aberrant PP1 glycogen-targeting subunit function and the development of insulin resistance and type 2 diabetes.

Meanwhile, some important insights into the relative role of glycogen-targeting subunits in control of glucose disposal have been gleaned from overexpression studies in hepatocytes and intact liver. In considering pathways of glucose disposal and storage, several strategies for enhancing net hepatic glucose uptake can be envisioned that might result in improved control of glucose homeostasis in type 2 diabetes (Fig. 2). For example, increasing the expression and/or activity of the principal glucose phosphorylating enzyme of liver, glucokinase, would be predicted to increase hepatic glucose disposal. Alternatively, downregulation or inhibition of one or several of the components of the glucose-6-phosphatase enzyme complex could achieve a similar result. Indeed, in one study, near-normalization of blood glucose levels was achieved in STZ-induced diabetic transgenic mice in which glucokinase was overexpressed in liver under control of the PEPCK (phosphoenolpyruvate carboxykinase) promoter (77). Surprisingly, the high levels of fatty acids, triglycerides, and ketone bodies found in STZ-induced diabetes were also normalized in the transgenic mice. However, it should be noted that these animals are completely insulin deficient, making it difficult to predict the metabolic impact of increased glucokinase expression in the presence of the hormone, as would be the case in type 1 patients receiving insulin therapy or in patients with type 2 diabetes.

To investigate this further, recombinant adenovirus was recently used to overexpress glucokinase in liver of normal rats (78). Overexpression of glucokinase by sixfold in liver resulted in a 40% decrease in blood glucose levels and a 70% decline in circulating insulin levels relative to those in animals treated with a control virus. However, the decrease in glucose levels came at the expense of large increases in circulating triglycerides and free fatty acids. Glucokinase overexpression in liver cells results in both increased glycogen

deposition and increased glycolytic flux (79–81). Thus, the effects of glucokinase overexpression on lipid homeostasis are likely explained, at least in part, by increased carbon flux into lipogenic pathways in liver, probably mediated by increased malonyl-CoA levels and inhibition of fatty acid oxidation. These findings raise concerns about manipulation of glucokinase activity as a viable strategy for treatment of diabetes.

In contrast to the findings with glucokinase, overexpression of glycogen-targeting subunits would be predicted to preferentially stimulate glucose conversion into glycogen, thereby enhancing hepatic glucose disposal without affecting lipid homeostasis (Fig. 2). Consistent with this idea, early studies in CHO-IR cells demonstrated that transient overexpression of PTG improved the capacity of cells to store glycogen (42). This appeared to be a consequence of increased PP1 and glycogen synthase binding to PTG and subsequent translocation of the glycogen synthase–PTG–PP1 complex to the glycogen granule. However, CHO-IR cells have very limited capacity for glycogen storage and are therefore not an optimal system for predicting the metabolic effects of glycogen-targeting subunit overexpression in metabolically active tissues such as liver.

To gain further insights into metabolic effects of targeting subunits, a recombinant adenovirus containing the mouse PTG cDNA (AdCMV-PTG) was used to overexpress the protein in primary rat hepatocytes (82), allowing evaluation of its metabolic impact in a cell type that is known to have a large glycogenic capacity. Overexpression of PTG in hepatocytes isolated from fasted rats resulted in potent activation of glycogen synthesis. Glycogen content was increased four- to fivefold at 2 mmol/l glucose in PTG-overexpressing cells relative to control cells and reached levels of 200 μg/mg protein (equivalent to that in liver of ad lib fed rats). Further studies revealed that the glycogenic effect of PTG was observed even in the complete absence of carbohydrates or insulin in the culture medium (82). These data suggest that PTG overexpression activates the conversion of gluconeogenic precursors into glycogen. PTG overexpression also prevented the normal glycogenolytic action of agents such as forskolin and glucagon (82). These metabolic effects of PTG overexpression were accompanied and at least partially explained by a 3.6-fold increase in glycogen synthase activity state and a 40% decrease in glycogen phosphorylase activity, relative to control cells without overexpression of the targeting subunit. Taken together, these data demonstrate that PTG overexpression in hepatocytes is sufficient to increase glycogen levels, decrease glycogen mobilization, and alter intrahepatic glucose flux, suggesting that an in vivo intervention that increased PTG expression in the liver would be sufficient to alter glycogen and glucose homeostasis.

To test this possibility, the AdCMV-PTG virus was used to cause overexpression of the glycogen-targeting subunit in liver of normal rats (46). In a first set of studies, animals were allowed to feed ad libitum for 90 h and thereafter were either fasted overnight or allowed to continue feeding for 24 h. Liver glycogen was nearly completely depleted in fasted control animals, whereas both fasted and fed PTG-overexpressing animals had glycogen levels that were 70% higher than in ad libitum fed controls. Despite these large changes in glycogen metabolism, plasma glucose, triglycerides, free fatty acids, ketones, and insulin were normally regulated in response to fasting and feeding in both PTG-overexpressing and control groups. Note that it is unclear whether overexpression of PTG in human liver would have the same dramatic effects on fed and fasted glycogen levels because the rate of degradation of the glycogen depot in humans in response to fasting is slower than that in rodents.

In a second set of studies, animals were fed ad libitum for 90 h after viral treatment and then deprived of food for a period of 24 h before administration of an oral bolus of [U-^{13}C]glucose. PTG-overexpressing animals exhibited a modest but significant improvement in clearance of the orally administered glucose from the circulation relative to controls. This was correlated with a sevenfold increase in hepatic glycogen content and a 70% increase in incorporation of [^{13}C]glucose into glycogen, as assessed by ^{13}C nuclear magnetic resonance (NMR). However, incorporation of labeled glucose accounted for only 6% of the glycogen synthesized in PTG-overexpressing animals in response to the glucose bolus.

The following conclusions were drawn from these two sets of experiments (46): *1*) Overexpression of PTG in liver improves whole-body glucose tolerance, but in contrast to glucokinase overexpression, this is achieved without perturbation of lipid homeostasis. *2*) PTG overexpression activates both direct and indirect pathways of glycogen synthesis. *3*) Overexpression of PTG in liver of normal rats prevents the normal activation of glycogenolysis in response to fasting, consistent with the finding of impaired glycogenolysis in response to forskolin and glucagon in PTG-overexpressing hepatocytes.

These data provide both encouraging and cautionary indications in terms of the potential utility of glycogen-targeting subunits for treatment of diabetes. They indicate that increasing the expression of glycogen-targeting subunit proteins in liver might serve to enhance glucose disposal and glycogen storage in diabetic patients, potentially without exacerbation of hyperlipidemia. However, among the isoforms of glycogen-targeting subunits, PTG may not be the optimal candidate for manipulation, because it appears to override normal glycogenolytic signals, potentially leaving the subject at risk for hypoglycemic episodes in response to fasting or exercise. The potentially damaging effects of prolonged elevations in hepatic glycogen stores induced by PTG overexpression must also be considered.

We have recently begun to evaluate the possibility that expression of other, more regulated members of the family of glycogen-targeting subunits, such as G_M/R_{Gl} or G_L, may stimulate glucose disposal while still allowing substrate- and hormone-mediated regulation of glycogen turnover. To this end, recombinant adenoviruses containing the cDNAs encoding G_L (AdCMV-G_L), G_M/R_{Gl} (AdCMV-G_M/R_{Gl}), and PTG (AdCMV-PTG) were used to overexpress these proteins in isolated hepatocytes, leading to several key observations (83).

First, G_L had the highest glycogenic potency among the three forms studied. Similar to results described earlier for PTG overexpression, G_L overexpression caused significant accumulation of glycogen in the absence of glucose or at low concentrations, but also allowed potent activation of glycogen accumulation as glucose concentrations were raised. Glucose increased glycogen synthesis more effectively in cells overexpressing G_L than in cells overexpressing

PTG. These effects became apparent at glucose concentrations ≥3 mmol/l and maximal at 20 mmol/l glucose, where G_L-overexpressing cells accumulated 50% more glycogen that PTG-overexpressing cells. In contrast to G_L and PTG, overexpression of G_M/R_{Gl} caused no significant increases in glycogen synthesis at low glucose, whereas high glucose induced a maximal increase of glycogen content of threefold relative to that in control cells, only 7% of the amount in G_L-overexpressing cells (83).

Second, glycogen synthase activity ratio was much higher in G_L-overexpressing cells than in PTG- or G_M/R_{Gl}-overexpressing cells. Thus, at moderate levels of G_L overexpression, glycogen synthase activity was increased by insulin treatment, but at higher levels of G_L expression, insulin was no longer required to achieve maximal synthase activity. In contrast, cells with high levels of PTG overexpression retained dose-dependent regulation of glycogen synthesis and glycogen synthase enzyme activity by insulin, and they never attained the degree of glycogen synthase activation observed in G_L-overexpressing cells (83).

Third, G_L- and G_M/R_{Gl}-overexpressing cells exhibited a strong glycogenolytic response to forskolin, whereas PTG-overexpressing cells were poorly responsive to these agents, consistent with earlier findings. This difference may be explained in part by a lesser forskolin-induced increase in glycogen phosphorylase activity in PTG-overexpressing cells than in G_L- or G_M/R_{Gl}-overexpressing cells (83).

Thus, both G_L- and G_M/R_{Gl}-overexpression enhance glycogen deposition in a glucose-sensitive manner in hepatocytes, while allowing the cells to retain responsiveness to glycogenolytic agents. However, recent preliminary studies from our laboratories serve to illustrate that care must be taken in extrapolating results from cultured cells to whole animals. For example, despite its dramatically enhanced capacity to activate glycogen synthase and glycogen deposition in isolated hepatocytes, overexpression of G_L did not lower glucose levels during an oral glucose tolerance test (OGTT) in rats made glucose intolerant by 6 weeks of feeding on a high-fat diet. G_L overexpression in liver of these animals did cause a large increase in glycogen deposition during the OGTT relative to that in control animals. In contrast, overexpression of a truncated G_M/R_{Gl} molecule, which lacks the COOH-terminal domain that is unique to this molecule and that is thought to mediate its association with sarcoplasmic reticulum, largely normalized glucose tolerance during OGTT in animals fed the high-fat diet (R. Gasa, R.-J. Yang, C. Clark, M.J.B., C.B.N., unpublished observations). This truncated G_M/R_{Gl} molecule was approximately twice as effective at promoting glycogen synthesis as full-length G_M/R_{Gl} in hepatocyte studies, although not nearly as effective as G_L (R.-J. Yang, R. Gasa, M.J.B., A. DePaoli-Roach, C.B.N., unpublished observations).

One interpretation of these findings may be that different forms of glycogen-targeting subunits activate direct and indirect pathways of glycogen synthesis to varying degrees. Preferential activation of direct conversion of glucose to glycogen by G_M/R_{Gl} could explain its superior effects on glucose disposal despite its lesser capacity, relative to G_L, to cause glycogen deposition. Further studies involving ^{13}C and ^2H_2O NMR to measure the relative contributions of direct and indirect pathways of glycogen synthesis (46,84) will be required to prove or disprove these ideas.

OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Glycogen-targeting subunits of PP1 have clearly emerged as important proteins in control of glycogen metabolism and glucose disposal. Although much knowledge has been gained about these molecules in the past decade, much more remains to be learned. Some key issues that remain to be investigated are as follows:

What is the exact role played by glycogen-targeting subunits in regulation of glycogen metabolism by hormones, particularly insulin? As summarized earlier, glycogenolytic agents have clear regulatory effects on G_M/R_{Gl} (via phosphorylation) and G_L (via binding of phosphorylase a). However, there is as yet no evidence for modulation of PTG localization or function by insulin or glycogenolytic agents, and the role of insulin in regulation of G_M/R_{Gl} and G_L remains obscure. Further insight into these issues may be gained by continued development of site-directed mutants of the individual targeting subunits and, possibly, by construction of chimeric proteins. Other important insights will come from studies of animals in which individual targeting subunit genes are knocked out.

Why do some tissues contain more than one glycogen-targeting subunit of PP1? In particular, why does the liver contain one glycogen-targeting subunit whose expression is highly regulated by fasting and feeding (G_L) and one whose expression is largely constitutive (PTG). Further, in animals in which GM/R_{Gl} has been knocked out (66), how is glycogen metabolism regulated in skeletal muscle by PTG and PPPR16? Again, answers to these questions may be forthcoming as transgenic lines with single or combined knockouts of glycogen-targeting subunits emerge.

Does the existence of multiple glycogen-targeting subunit isoforms explain differential regulation of glycogen metabolism in tissues in response to physiological perturbations? It is known, for example, that the glycogenolytic response during exercise is greater in muscle than in liver. In contrast, glycogenolysis is greater in liver than in muscle during starvation or fasting. Furthermore, glycogen synthesis is strongly activated in muscle after exercise, particularly in well-trained athletes. The role of glycogen-targeting subunits in mediating these effects will require further investigation.

Do different targeting subunits differentially activate direct and indirect pathways of glycogen metabolism? In studies performed to date in hepatocytes (82,83), we have found that overexpression of either PTG or G_L causes substantial glycogen deposition even in the complete absence of glucose in the culture medium, whereas stimulation of glycogen synthesis by overexpression of GM/R_{Gl} seems to require stimulatory glucose (e.g., ≥5 mmol/l). Further work will be required to determine whether PTG and G_L differ from G_M/R_{Gl} in their capacity to activate glycogen synthesis from gluconeogenic precursors (indirect pathway), and such studies are now underway in our laboratories using ^{13}C and ^2H_2O NMR. Interestingly, in studies of PTG overexpression in liver of normal rats, we found this targeting subunit caused a significant decrease in the levels of glucose-6-phosphatase catalytic subunit mRNA (46), suggesting a means by which gluconeogenically derived glucose might be shunted into glycogen. This finding indicates that careful study of the impact of individual glycogen-targeting subunits on expression and activity of other metabolic enzymes must be investigated.

Finally, we suggest that the effects of glycogen-targeting subunit overexpression on direct versus indirect pathways of glycogen synthesis and on expression of glycolytic or gluconeogenic enzymes may be tissue context–dependent, because cells derived from skeletal muscle or pancreatic islets, for example, lack gluconeogenic enzymes that are expressed in liver. This issue must also be addressed in a systematic fashion.

What role do glycogen-targeting subunits play in organizing interactions between the glycogen particle, glycogen-metabolizing enzymes, and enzymes of glucose disposal and production, such as glucokinase, hexokinase II, and glucose-6-phosphatase? For example, it will be important to understand the time sequence of appearance of proteins on the nascent glycogen particle in the transition from catabolic to anabolic conditions. One possibility is that binding of glycogen-targeting subunits to glycogen is an early event, directing subsequent assembly of enzymes of glycogen metabolism at this site. Alternatively, binding of glycogen-targeting subunits to the glycogen metabolizing enzymes may occur in the cytosol, followed by translocation of the entire complex to the glycogen particle. It will also be of interest to determine whether glycogen-targeting subunits interact in any manner with glucose phosphorylating enzymes or the glucose-6-phosphatase enzyme complex. Of interest in this regard is the finding that overexpression of glucokinase, but not hexokinase I, in hepatocytes stimulates glycogen synthesis and glycogen synthase enzymatic activity (80,81). Overexpressed hexokinase I is bound to mitochondria, whereas glucokinase clearly is not. Whether glycogen-targeting subunits can in any way direct glucokinase to the glycogen particle once it is translocated out of the nucleus in response to glucose remains to be determined.

Is there a form of glycogen-targeting subunit whose overexpression in liver or other tissues will allow enhancement of postprandial glucose disposal without risk of fasting- or exercise-induced perturbations in glucose homeostasis? We are encouraged by our recent finding that hepatic expression of a truncated G_M/R_{GL} molecule normalizes glucose tolerance in rats fed a high-fat diet. However, these animals must be studied in much more detail—and the answers to the basic questions posed above must be gained—before the therapeutic potential of glycogen-targeting subunits can be accurately appraised.

In attempting to answer these fundamental questions about glycogen-targeting subunits of PP1, it seems inevitable that new insights into organization of metabolic pathways and control of fuel homeostasis will emerge.

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