Acetylation Negatively Regulates Glycogen Phosphorylase by Recruiting Protein Phosphatase 1

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DOI 10.1016/j.cmet.2011.12.005

SUMMARY

Glycogen phosphorylase (GP) catalyzes the rate-limiting step in glycogen catabolism and plays a key role in maintaining cellular and organismal glucose homeostasis. GP is the first protein whose function was discovered to be regulated by reversible protein phosphorylation, which is controlled by phosphorylase kinase (PhK) and protein phosphatase 1 (PP1). Here we report that lysine acetylation negatively regulates GP activity by both inhibiting enzyme activity directly and promoting dephosphorylation. Acetylation of GP Lys470 enhances its interaction with the PP1 substrate-targeting subunit, GL, and PP1, thereby promoting GP dephosphorylation and inactivation. We show that GP acetylation is stimulated by glucose and insulin and inhibited by glucagon. Our results provide molecular insights into the intricate regulation of the classical GP and a functional crosstalk between protein acetylation and phosphorylation.

INTRODUCTION

Glycogen phosphorylase (GP) catalyzes phosphorylolytic cleavage of glycogen to produce glucose-1-phosphate for glucose-dependent tissues when body glucose is scarce. GP activity plays an important role in glucose homeostasis and glycogen metabolism. Defects in glycogen synthesis and breakdown in liver, muscle, and other glucose-dependent tissues often cause glycogen storage diseases (Stegelmeier et al., 1995). For example, McArdle disease is caused by mutations in muscle GP, and patients with this disorder are intolerant of exercise and show early fatigue (Andreu et al., 2007; Tang et al., 2003).

Due to their critical roles in glucose homeostasis, regulations of glycogen synthase (GS) and GP activities have been extensively investigated in hopes of finding therapeutic strategy for type II diabetes.

GP was the first allosteric enzyme to be discovered by Carl and Gerty Cori, who demonstrated that GP existed in two interconvertible forms, referred to subsequently as an active a form or inactive b form, that are regulated by adenosine monophosphate (AMP) (Cori and Cori, 1936). GP is activated by the binding of AMP and IMP, whereas it is inhibited by the binding of ATP and glucose-6-phosphate (Barford et al., 1991; Barford and Johnson, 1989). These allosteric controls provide excellent means of GP activity regulation in response to energy and metabolic status. Moreover, GP activity is tightly regulated by reversible phosphorylation and dephosphorylation. In addition to allosteric regulation, GP is also regulated by posttranslational modifications (PTMs) (Johnson, 1992). In fact, GP was also the first protein discovered to be regulated by reversible protein phosphorylation (Fischer and Krebs, 1955; Sutherland and Wosilait, 1955), which exemplifies a signal transduction pathway by phosphorylation cascades. Under high serum glucose conditions, release of insulin indirectly activates protein phosphatase 1 (PP1), which dephosphorylates Ser-15 and converts the active a form of GP to the unphosphorylated inactive b form, leading to the inhibition of glycogen breakdown (Browner and Fletterick, 1992). Conversely, when glucose concentration is low, glucagon triggers a cascade of signal transduction that activates protein kinase A (PKA) which phosphorylates and activates phosphorylase kinase (PhK), which in turn activates GP by phosphorylating Ser-15 and leads to increased glycogen breakdown and ultimately higher glucose levels.

PP1 regulates glycogen metabolism by inhibiting GP activity and activating GS activity. The hepatic glycogen binding subunit, Gl, is a glycogen-metabolizing scaffold protein that binds to PP1, glycogen, GS, and GP (Armstrong et al., 1998). Gl targets PP1 to glycogen, where it dephosphorylates and inhibits GP, in addition to dephosphorylating and activating GS, thereby increasing glycogen synthesis and reducing glucose output (Alemany and Cohen, 1986). Therefore, the phosphorylation status of GP is critically regulated by its interaction with Gl.
Acetylation Negatively Regulates GP Catalytic Activity

In an attempt to profile liver protein acetylation, we previously enriched acetylated peptides of human liver proteins by affinity purification (Zhao et al., 2010). Among the many liver acetylated proteins identified, two peptides were found to contain acetylated lysine 470 (K470) and lysine 796 (K796) of GP (see Figure S1A available online). To confirm GP acetylation, we expressed Flag-tagged GP in Chang’s liver cells and then treated cells with nicotinamide (NAM) and trichostatin A (TSA), two commonly used deacetylase inhibitors that inhibit all four classes of deacetylases with the transfected mutant GP, and whether the mutant GP could dominantly inhibit wild-type GP. In the transfected cells, the ectopically expressed GP was much higher (about 10-fold) than the endogenous protein GP (Figure S1D), indicating that endogenous GP should have a negligible effect on our assay results. Moreover, we purified WT homodimer, WT-K2R heterodimer, and K2R homodimer and assayed their catalytic activity. The WT-K2R heterodimer exhibited activity that was the average of wild-type and K2R homodimers (Figure S1E), showing that the K2R mutant has no dominant inhibition on wild-type GP. Together, our results suggest that K470 and K796 are two major acetylation sites for its enzymatic inhibition. To further test the effect of acetylation on GP activity and glycogen metabolism, we determined cellular glycogen content in response to NAM and TSA treatment. Consistent with an inhibitory effect of acetylation on GP activity, we found that inhibition of deacetylases by NAM and TSA increased cellular glycogen levels (Figure S1F). Next, we determined glycogen hydrolysis in cells expressing different GP mutants. Expression of wild-type GP promoted a faster glycogen hydrolysis than the GPK2Q mutant (Figure 1E), further supporting a lower enzymatic activity of the acetylation mimetic mutant GPK2Q.

GP Acetylation and Activity Are Regulated by Glucose, Insulin, and Glucagon

Given that glucose concentration is a key factor in GP regulation, we determined GP acetylation under different glucose concentrations. The acetylation level of ectopically expressed GP was low when cells were maintained in glucose-free medium, and increased significantly with elevated glucose concentrations in a dose-dependent manner by as much as 4-fold (Figure 2A), suggesting that acetylation of GP was upregulated by glucose. Moreover, acetylation of endogenous GP in L02 human hepatocytes was increased by glucose (Figure 2B, Figure S2A). NAM and TSA treatment of cells cultured in high-glucose medium could further increase the acetylation level of endogenous GP (Figure 2B), showing that deacetylation of GP occurs even under high glucose.

Next, the effect of glucose on GP activity was determined. As shown in Figure 2C, the activity of wild-type GP was significantly inhibited by increasing concentrations of glucose, while GPK2Q was largely refractory to inhibition by glucose (Figure 2C). These results suggest that glucose inhibits GP activity through K470 and K796 acetylation. To further test the function of acetylation in mediating glucose-induced GP inhibition, we measured the effect of glucose on GP activity after NAM and TSA treatment. We hypothesized that since the inhibition of deacetylases would increase GP acetylation, high glucose may not have a significant effect on GP activity in the presence of deacetylase inhibitors. As expected, glucose increased GP acetylation and decreased GP activity in the absence of deacetylase inhibitors (Figure 2D, lanes 1–4). However, in the presence of deacetylase inhibitors, glucose had little effect on GP activity and did not further increase the elevated GP acetylation (Figure 2D, lanes 5–8). These results further support the notion that acetylation plays a major role in GP inhibition in response to glucose.

Both insulin and glucagon are important signals that regulate GP activity and glycogen metabolism in opposite manners.
We tested whether these two hormones could regulate GP acetylation. In L02 human hepatocytes, the acetylation of endogenous GP was increased with insulin treatment in time- and dose-dependent manners (Figure 2E, Figure S2B). Moreover, acetylation of ectopically expressed GP increased within 30 min of insulin treatment (Figure 1A). K470 and K796 are the major acetylation sites in GP. Ectopically expressed Flag-GP and GP^K2Q^ in Chang’s cells were immunopurified and immunoblotted with pan-anti-acetyllysine antibody (Figure 1B). GP catalytic activity is negatively regulated by acetylation. Both wild-type GP and GP^K2Q^ were expressed in Chang’s liver cells with or without NAM+TSA treatment. Catalytic activity of affinity-purified GP proteins was determined and normalized to protein levels. Activity of wild-type GP under no treatment condition was set as 100% (Figure 1C). The acetylation targets K470 and K796 are important for GP inhibition by the deacetylase inhibitor treatment. GP, GP^K470Q^, GP^K796Q^, and GP^K2Q^ were each expressed in Chang’s liver cells with (hatched bars) or without (solid bars) NAM+TSA treatment as indicated. Specific activity of each purified enzyme was determined. Wild-type GP degrades glycogen faster than K2Q mutant. Wild-type GP and mutant GP^K2Q^ were expressed at similar levels in Chang’s liver cells maintained in regular DMEM medium. The medium was replaced by glucose-free DMEM at time 0, and the glycogen degradation rate was measured. All error bars represent standard deviation (SD). n = 3 for each experimental group. (Lok et al., 1994; Massague and Guinovart, 1977).
Figure 2. Regulation of GP Acetylation by Glucose, Insulin, and Glucagon

(A) Glucose increases GP acetylation. Acetylation levels of GP ectopically expressed in Chang’s cells maintained in different glucose concentrations were probed by anti-acetyllysine antibody.

(B) Glucose and deacetylase inhibitor increase endogenous GP acetylation. Human hepatic L02 cells were treated with various concentrations of glucose and deacetylase inhibitors as indicated. Endogenous GP was immunoprecipitated with a GP antibody. Acetylation levels of GP were determined by western blot.

(C) Glucose decreases GP activity through acetylation. Relative specific activity of GP (solid bars) and GP<sup>Q20</sup> (hatched bars) expressed in Chang’s cells maintained in different glucose concentrations was determined. Specific activity of GP from glucose-free medium was arbitrarily set as 100.

(D) Deacetylase inhibitor treatment blocks the glucose inhibition on GP activity. Relative specific activities for GP expressed in Chang’s cells maintained in different glucose concentrations with (hatched bars) and without NAM+TSA treatment (solid bars) were determined.

(E) Insulin and deacetylase inhibitor increase endogenous GP acetylation. Human hepatic L02 cells were treated with increasing concentrations of insulin and treated with deacetylase inhibitors as indicated. Acetylation of immuno precipitated endogenous GP was detected by western blot.

(F) Insulin decreases GP activity through acetylation. Flag-GP (solid bars) and Flag-GP<sup>Q20</sup> (hatched bars) were expressed in Chang’s cells and treated with increasing concentrations of insulin. GP acetylation and activity were determined.

(G) Glucagon decreases GP acetylation. Acetylation levels of Flag-GP expressed in Chang’s cells treated with 10 nM glucagon for different time periods (as indicated) were determined.

All error bars represent standard deviation (SD). n = 3–4 for each experimental group.
addition (Figure S2C). We then examined the combined effect of both glucose and insulin. GP acetylation in cells cultured in the presence of both glucose and insulin was higher than cells cultured in either glucose or insulin (Figure S2D). Consistent with the stimulatory effect of insulin on GP acetylation, we found that insulin inhibited GP activity in a dose-dependent manner (Figure 2F, lanes 1–5). These effects required K470 and K796, as the GpS515D mutant showed little acetylation and its catalytic activity did not respond to insulin treatment (Figure 2F, lanes 6–10). Contrary to insulin treatment, glucagon decreased GP acetylation in a time-dependent manner (Figure 2G). Together, these results suggest that insulin and glucagon may regulate GP activity by affecting K470 and K796 acetylation.

**Acetylation Decreases GP Phosphorylation**

It is well-characterized that GP is activated by PhK-mediated serine phosphorylation at Ser-15 (Nolan et al., 1964). To investigate the mechanism of acetylation in regulating GP activity, we looked into a possible crosstalk between acetylation and phosphorylation by determining GP serine phosphorylation under different conditions that are known to affect GP acetylation levels. We found that deacetylase inhibitor treatment increased GP acetylation and at the same time decreased Ser-15 phosphorylation by 70% (Figure 3A). Similar results were observed when we utilized a panphosphoserine antibody to detect the GP serine phosphorylation level after NAM and TSA treatment (Figure S3A). The inverse relationship between endogenous GP acetylation and phosphorylation in responding to deacetylase inhibitor was also examined in L02 human hepatocytes and freshly isolated mouse primary hepatocytes. In both hepatocytes, endogenous GP showed a significant increase in acetylation and a concomitant decrease in Ser-15 phosphorylation upon NAM and TSA treatment (Figure 3B). Similar results were also observed in freshly isolated mouse skeletal muscle cells (Figure S3B). These observations demonstrate that acetylation negatively regulates GP phosphorylation.

To provide direct evidence that acetylation regulates GP phosphorylation, we examined GP phosphorylation status when GP was coexpressed with deacetylases. When GP was coexpressed with SIRT1 and SIRT2, two cytosolic deacetylases, an evident decrease in GP acetylation and concomitant increase in GP Ser-15 phosphorylation were observed, with SIRT2 being more potent in deacetylating GP and increasing Ser-15 phosphorylation (Figure 3C). Collaborating with these findings, the catalytic activity of GP was also activated by either SIRT1 or SIRT2 coexpression. This result indicates a causal role of acetylation in modulating GP phosphorylation.

To further elucidate the relationship between GP acetylation and phosphorylation, we determined the effect of deacetylase inhibitors on the activity of GP S515D, which had Ser-15 replaced by an aspartic acid and displayed a lower activity (Buchbinder et al., 1997). Interestingly, the activity of GpS515D mutant was not inhibited by deacetylase inhibitor treatment, whereas the wild-type GP was potently inhibited by a similar treatment (Figure 3D). Of note, glucose still increased the acetylation of GpS515D mutant (Figure 3E). Therefore, Ser-15 phosphorylation is not required for high-glucose-stimulated GP acetylation; rather, acetylation may downregulate Ser-15 phosphorylation and activity of GP.

Because of the inhibitory effect of acetylation on phosphorylation, the above data were unable to show whether acetylation may directly affect GP activity. To address this question, we prepared GP with or without acetylation and with or without phosphorylation. Hyperacetylated GP was obtained by expressing Flag-GP in Chang’s liver cells maintained in high-glucose (25 mM) medium supplemented with NAM and TSA, while hypoaucetylated GP was obtained by glucose-free medium without NAM and TSA. The immunopurified GP proteins were treated with λ-phosphatase or PhK in vitro. After verifying both the acetylation and the Ser-15 phosphorylation of GP by western blotting (right panel, Figure 3F), GP activity was determined. We found that phosphorylation by PhK dramatically increased GP activity regardless of whether GP was hyperacetylated (lane 1 versus lane 2, left panel, Figure 3F) or hypoacetylated (lane 3 versus lane 4). Acetylation did not affect GP activity when GP was hypophosphorylated (lanes 1 versus lane 3). However, when GP was hyperphosphorylated, acetylation exhibited an inhibitory effect on GP in vitro (lane 2 versus lane 4). These results indicate that acetylation inhibits GP only when it is hyperphosphorylated and active.

GP is also regulated by allosteric effect, including activation of inactive b form by AMP. To explore the effect of acetylation on allosteric regulation of GP, we determined the activity of hypo- versus hyperacetylated GP in response to AMP. We found that AMP induced a similar activation to both the hyperacetylated (lane 1 versus lane 1A, 20.5-fold, Figure 3F) and hypoacetylated GP (lane 3 versus lane 3A, 20.4-fold) when GP was hypophosphorylated, indicating that acetylation does not directly affect allosteric activation of GP by AMP. However, when GP was fully activated (hypoacetylated/hyperphosphorylated), AMP could not activate GP further (lanes 4 versus 4A).

To provide more direct evidence to determine the effect of acetylation on allosteric regulation, we treated immunopurified GP with recombinant deacetylase CobB in vitro and measured its allosteric activation by AMP after confirming the decrease of acetylation (right panel, Figure 3G). We found that AMP activated GP equally regardless of the levels of GP’s acetylation (5.54-fold, 5.58-fold, and 5.50-fold, left panel, Figure 3G). We therefore conclude that acetylation does not directly affect allosteric activation of GP by AMP.

**Physiological Stimuli Regulate GP Acetylation-Induced Dephosphorylation**

The notion that acetylation negatively regulates GP-Ser-15 phosphorylation was further pursued by analyzing acetylation and phosphorylation levels in response to physiological stimuli. We found that glucose increased GP acetylation and decreased phosphorylation in a dose-dependent manner (Figure 4A), indicating an inverse relationship between GP acetylation and phosphorylation. Moreover, insulin treatment increased GP acetylation and decreased GP phosphorylation (Figure 4B), whereas glucagon treatment decreased GP acetylation and increased GP phosphorylation (Figure 4C). A time-dependent inverse correlation between acetylation and phosphorylation in GP was observed in response to glucose, insulin, and glucagon (Figure 4D, Figures S4A and S4B), supporting the notion that acetylation may inhibit GP phosphorylation. It is worth noting that glucagon can regulate GP activity through a direct signaling
Figure 3. Acetylation Decreases GP Ser-15 Phosphorylation

(A) Deacetylase inhibitor treatment inhibits GP phosphorylation. Flag-GP was expressed in Chang’s liver cells and treated with or without NAM+TSA. Acetylation and GP-Ser-15 phosphorylation levels of Flag-GP proteins were determined by western blot.

(B) Deacetylase inhibitor treatment increases acetylation and decreases phosphorylation of hepatic GP. Human hepatic L02 cells and mouse primary hepatocytes were treated with or without NAM+TSA as indicated. Acetylation and Ser-15 phosphorylation of GP proteins were determined by western blot (L02, *p = 0.0094, n = 3, and **p = 0.0107, n = 3; primary hepatocytes, *p = 0.0090, n = 3, and **p = 0.0270, n = 3).

(C) Coexpression of deacetylases reduces GP acetylation and increases GP phosphorylation. Flag-GP was expressed alone or coexpressed with Sirt1 and Sirt2 in Chang’s liver cells as indicated. Acetylation and Ser-15 phosphorylation of affinity-purified GP were measured. Relative specific activities of GP were determined by normalizing GP activity against GP protein.

(D) GP Ser-15 is required for deacetylase inhibitor-induced GP inactivation. Wild-type GP and GP^{SER15D} mutant GP were expressed in Chang’s liver cells, with or without NAM+TSA treatment. Activities of affinity-purified GP were determined.
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pathway. Of note, although Ser-15 phosphorylation occurred rapidly and was evident as early as 30 min after glucagon treatment, it increased continuously throughout the 4 hr of experimental duration (Figures 2G and 4D). Thus, it is possible that deacetylation may not be required for the acute GP phosphorylation by glucagon but rather may play a role to maintain the phosphorylated and active GP in the active state (Figure S7).

We next examined the response of Ser-15 phosphorylation in GP<sub>K2Q</sub>, the nonacetylatable form of GP, to different glucose concentrations. Contrary to wild-type GP, which showed increased acetylation and decreased Ser-15 phosphorylation by glucose, Ser-15 phosphorylation of GP<sub>K2Q</sub> was largely unaffected by increasing glucose concentrations (Figure S4C), indicating an essential role of acetylation in regulating Ser-15 phosphorylation. Notably, Ser-15 phosphorylation remained constitutively high in GP<sub>K2Q</sub> regardless of the glucose concentrations, suggesting that the deacetylated GP favors being phosphorylated at Ser-15. This notion was supported by the observation that GP<sub>K2Q</sub>, the acetylation mimetic mutant, not only showed a very weak phosphorylation level but that its phosphorylation levels of either GPK2Q or GP<sub>K2Q</sub> (Figure S4E). Collectively, the above data indicate that acetylation of K470 and K796 is required for GP phosphorylation regulation by glucose and insulin.

To obtain in vivo data to support the inverse relationship between GP acetylation and Ser-15 phosphorylation, mouse experiments were performed. Upon feeding, a state that causes high glucose or high insulin, mouse liver GP acetylation level was high, whereas the level of Ser-15 phosphorylation was low (Figure 4E, Figure S4F). After overnight fasting, hepatic GP acetylation was decreased, while hepatic Ser-15 phosphorylation was inversely increased. Furthermore, when mice were intraperitoneally injected with glucose (1 g/kg), insulin (5 U/kg), or glucagon (0.2 mg/kg), in line with our observations in vitro, glucose and insulin injection increased endogenous GP acetylation and decreased Ser-15 phosphorylation (Figures 4F and 4G, Figures S4G and S4H). In contrast, glucagon injection decreased endogenous GP acetylation and increased Ser-15 phosphorylation in mouse livers (Figure 4H, Figure S4I). Together, these data indicate an inverse correlation of GP acetylation and Ser-15 phosphorylation, and the regulation of these two PTMs under physiological conditions.

Acetylation Increases the GP-PP1 Interaction

After establishing a causal relationship between acetylation and phosphorylation, a key question is how acetylation modulates the interaction between GP and PP1, which is controlled by the PhK and PP1. The interaction between GP and PP1 is important for GP dephosphorylation. As expected, coexpression of PP1<sub>α</sub>, the phosphatase that dephosphorylates P-Ser-15 of GP, largely abolished the inhibitory effect of deacetylase inhibitor on GP activity (Figure S5A). We thus investigated whether acetylation affected the PP1<sub>α</sub>-GP interaction. When PPI<sub>α</sub> and GP were coexpressed in Chang’s cells, the interaction between PP1<sub>α</sub> and GP was readily detectable (Figure 5A). Interestingly, the PP1<sub>α</sub>-GP interaction was increased (by more than 100%) after deacetylase inhibitor treatment (Figure 5A), suggesting that acetylation enhances the recruitment of PP1<sub>α</sub> to GP. Consistent with a role of acetylation in promoting the interaction between PP1<sub>α</sub> and GP, deacetylase inhibitor treatment did not further increase the interaction between PP1<sub>α</sub> and GP<sub>K2Q</sub> (Figure 5B).

To further confirm the function of acetylation in enhancing the PP1<sub>α</sub>-GP interaction, we compared the binding of PP1<sub>α</sub> to GP and the acetylation mimic GP<sub>K2Q</sub> mutant. When PP1<sub>α</sub> and GP proteins were coexpressed in Chang’s cells, the amount of PP1<sub>α</sub> protein communoprecipitated with GP<sub>K2Q</sub> was about 2-fold more than that associated with the wild-type GP protein, suggesting that the acetylation of GP may enhance its interaction with PP1<sub>α</sub>. Consistently, the phosphorylation level of GP<sub>K2Q</sub> was about 60% weaker than that of the wild-type GP (Figure 5C). Moreover, deacetylase inhibitor treatment decreased Ser-15 phosphorylation of GP that was already reduced by PP1<sub>α</sub> coexpression (Figure 5A, Figure S5B). Furthermore, coexpression of SIRT2 impaired the interaction between GP and PP1<sub>α</sub> and at the same time stimulated GP serine phosphorylation (Figure 5D). Taken together, the above data support a model in which acetylation of K470 and K796 in GP favors the interaction with PP1<sub>α</sub>, thereby resulting in GP dephosphorylation and inactivation (Figure 7).

K470 Acetylation Increases GP-G<sub>L</sub> Interaction

The PP1<sub>α</sub>-GP interaction is mediated by G<sub>L</sub>, a substrate-targeting subunit of PP1 (Armstrong et al., 1998). To determine whether the observed PP1<sub>α</sub>-GP interaction resulted from increased PP1<sub>α</sub>-G<sub>L</sub>, Flag-PP1<sub>α</sub> and HA-G<sub>L</sub> were coexpressed in Chang’s cells, and the interaction between PP1<sub>α</sub> and G<sub>L</sub> was determined. We found that the association between PP1<sub>α</sub> and G<sub>L</sub> was not altered by deacetylase inhibitor treatment (Figure S6A), indicating that acetylation does not regulate interaction between PP1<sub>α</sub> and G<sub>L</sub>. On the other hand, the interaction between GP and G<sub>L</sub> was increased upon deacetylase inhibitor treatment (Figure 6A, lanes 3 and 4). G<sub>L</sub> binds to GP through its C-terminal 269–284 residues, and deletion of these five residues in G<sub>L</sub> diminishes its interaction with GP (Kelsall et al., 2007; Pautsch et al., 2008).
Figure 4. GP Acetylation and Phosphorylation Are Inversely Regulated by Physiological Stimuli
(A–C) The effect of glucose, insulin, and glucagon on GP acetylation and phosphorylation. Flag-GP-expressing Chang’s cells were treated with different conditions as indicated. Acetylation and serine phosphorylation of Flag-GP were determined by western blotting.

(D) Time course of glucagon on GP acetylation and phosphorylation. Flag-GP-expressing Chang’s liver cells were treated with glucagon (10 nM) for different times as indicated. Levels of GP acetylation and Ser-15 phosphorylation were determined by western blotting.

(E) Fasting decreases GP acetylation and increases GP phosphorylation in mouse liver. Mice were fasted overnight before sacrifice, and GP proteins were immunoprecipitated from the liver. Acetylation and Ser-15 phosphorylation levels of GP were determined by western blot (#p = 0.0021, n = 3; ##p = 0.0064, n = 3).

(F and G) Both glucose and insulin increase acetylation and decrease phosphorylation of GP in mouse liver. Glucose (1 g/kg) or insulin (5 U/kg) was intraperitoneally injected into overnight-fasted mice. At 30 min postinjection, mice were sacrificed and liver samples were harvested. GP proteins were immunoprecipitated from the liver, and acetylation and Ser-15 phosphorylation levels of GP proteins were determined by western blot (glucose, #p = 0.0191, n = 3, and ##p = 0.0013, n = 3; insulin, #p = 0.0021, n = 3, and ##p = 0.0036, n = 3).

(H) Glucagon decreases acetylation and increases phosphorylation of GP in mouse liver. Glucagon (0.2 mg/kg) was intraperitoneally injected into fed mice, and the mice were sacrificed at 30 min postinjection. GP protein was immunoprecipitated from liver, and acetylation and serine phosphorylation were determined by western blotting (#p = 0.0200, n = 3; ##p = 0.0100, n = 3).

All error bars represent standard deviation (SD). n = 3 for each experimental group.
We thus generated a G_44^{AC5} mutant by deleting the C-terminal five residue deletion of G_44 and confirmed that the deletion weakened the binding of G_44 with GP. Notably, inhibition of deacetylases increased the interaction between G_44^{AC5} and GP to a level similar to that of wild-type GP (Figure 6A). This result suggests that the acetylation enhances the GP-G_44 interaction through a site in G_44 that is independent of the C-terminal five residues in G_44. Consistently, when G_44^{K2R} was coexpressed with G_44^{AC5} in Chang’s cells, virtually no interaction was detected between them in either the presence or the absence of deacetylase inhibitors (Figure S6B). Meanwhile, we characterized another protein, PTG (protein targeting to glycogen), which also functions as a molecular scaffold targeting GP to PP1 (Brady et al., 1997), and investigated whether acetylation regulates GP and PTG interaction. The result demonstrated that GP-PTG binding could also be stimulated by NAM and TSA treatment (Figure S6C).

To obtain direct evidence to support a role of acetylation in promoting GP-G_44 interaction, we performed in vitro deacetylation and binding experiments. Hyperacetylated GP was purified from cells treated with deacetylase inhibitors, and the purified GP was deacetylated in vitro by incubating with the NAD+-dependent bacterial deacetylase CobB. We found that CobB treatment decreased GP acetylation and also reduced the interaction of GP-G_44 in a NAD+-dependent manner (Figure 6B), providing direct biochemical evidence that acetylation of GP enhances its interaction with G_44. We extended these experiments in mouse liver. Plasmids encoding epitope-tagged GP and G_44 were intravenously injected into mouse tail veins to express the two proteins in liver. The interaction of the ectopically expressed GP and G_44 in mouse liver was determined in response to glucose signal. When glucose (1 g/kg) was injected intraperitoneally into fasted mice, we found that the hepatic GP-G_44 interaction was increased significantly (Figure 6C). As expected, glucose injection indeed increased blood glucose concentration (Figure S6D). These results further support the notion that acetylation of GP increases GP-G_44 interaction in vivo.

We next investigated the importance of K470 and K796 acetylation sites in regulating the GP-G_44 interaction. HA-G_44 with Flag-GPK^{K470Q} or GPK^{K796Q} was coexpressed in Chang’s liver cells. As a positive control, deacetylase inhibitors increased the interaction between wild-type GP and HA-G_44 (Figure 6D, lanes 3 and 4). In contrast, a similar treatment did not increase the interaction between Flag-GPK^{K470Q} and G_44, and the mutant GPK^{K796Q} displayed a stronger interaction with G_44 than the wild-type GP (Figure 6D, lanes 6 and 7). On the other hand, the binding between GPK^{K796Q} and G_44 was increased by NAM+TSA treatment, and GPK^{K796Q} showed a basal G_44 interaction similar to the wild-type protein (Figure S6E). These results suggest that the acetylation-enhanced interaction between GP and G_44 is predominantly mediated by K470, but not K796. We performed iTRAQ mass spectrometry analyses to quantify GP K470 acetylation. Our results showed that as much as 50% of GP was acetylated at K470 in this assay and that inhibition of deacetylases resulted in an increase in the ratio of acetylated K470 versus unacetylated K470 from roughly 1:1 to 2:1 (Figure 6E, Figures S6F–S6I), suggesting that a substantial fraction of K470 in GP is acetylated in the cell.
Finally, to investigate whether the GP-G\textsubscript{L} interaction is regulated by physiological stimuli, we determined the interaction between GL with either wild-type or K470Q mutant GP in response to glucose concentration and insulin stimulation. We found that the GP-G\textsubscript{L} interaction was increased by approximately 100% after switching from glucose-free medium to 25 mM glucose medium (Figure 6F). However, a similar glucose switch did not affect the interaction between GPK470Q and GL. Furthermore, insulin stimulated the interaction between the wild-type GP, but not GPK470Q, and GL (Figure 6G). These results support the notion that GP-G\textsubscript{L} interaction is regulated by K470 acetylation in response to nutrient and hormonal signals.

**DISCUSSION**

Following the initial discovery of histone acetylation (Allfrey et al., 1964; Phillips, 1963), extensive studies over the last four decades have identified not only the enzymes that catalyze reversible acetylation, the protein lysine acetyltransferases (KATs, formerly termed histone acetyltransferases, HATs), and deacetylases (commonly known as histone deacetylases, or HDACs) but also many nonhistone substrates. Until relatively recently, nearly all well-characterized acetylation substrates were nuclear proteins, including transcription factors and coregulators (Yang and Seto, 2008). Recent studies, in particular...
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Figure 7. Acetylation Negatively Regulates GP Activity by Promoting the Binding of Phosphatase and the Dephosphorylation of GP

The acetylation of GP is stimulated by glucose and insulin and inhibited by glucagon. The acetylation enhances the binding of G_s subunit to GP and dephosphorylation of GP by PP1.

Crosstalk between different protein PTMs, such as phosphorylation-targeted protein ubiquitylation (Hunter, 2007), plays an important role in coordinating and connecting different cellular processes. As metabolism needs to respond to a variety of intracellular and extracellular conditions such as cell growth signals and nutrient availability, metabolic enzymes, which have now been found to be frequently modified by acetylation, are expected to be subject to such crosstalk regulation between acetylation and other type of PTMs. We have demonstrated recently that acetylation of phosphoenolpyruvate carboxykinase (PEPCK1), a rate-limiting enzyme in gluconeogenesis, promotes its association with UBR5/EDD1 HECT E3 ligase and thus its degradation in the presence of high glucose (Jiang et al., 2011). The current study adds another distinct example—promoting protein dephosphorylation by acetylation (Figure S7). It should be noted that the decrease of GP acetylation induced by glucagon may contribute to the magnitude and duration of GP activation in response to glucagon. Glucagon stimulates GP by activating the GP kinase via the classical phosphorylation cascade and also by dissociating GP phosphatase via acetylation (Figure S7). It should be noted that acetylation appears to have no direct role in GP allosteric activation by AMP, although AMP cannot further activate GP when GP is fully active. Although the precise mechanism by which glucose regulates GP acetylation remains to be elucidated, using acetylation machinery to control GP adds another layer of regulation and thus lends cells further versatility in integrating multifaceted signaling pathways and nutrient conditions to this enzyme that is not only central to the glycogen metabolism, but is also interlocked with multiple energy metabolic pathways.

EXPERIMENTAL PROCEDURES

Mouse Liver Collection and Primary Hepatocytes and Muscle Cell Isolation

Male BALB/c mice (4–6 weeks old, 20–25 g) were divided into two groups, fed and overnight fasted. Fasting started from late afternoon and lasted for 16 hr before experiments. The fasted mice were further subdivided into glucose- and insulin-treated groups, and the fed mice were treated with glucagon. Mice blood glucose levels were measured by Accu-Chek Active Blood Glucose Meter (Roche). At 30 min postinjection, mice were sacrificed and liver samples were harvested. In addition, mouse primary hepatocytes and muscle cells were isolated. Please refer to the Supplemental Experimental Procedures for detailed information. Animal experiments were performed at Fudan Animal Center in accordance with the animal welfare guidelines.

Glycogen Content Measurement

Cells were washed twice and then lysed with supersonic. The cell lysate in 1 ml PBS (pH 4.8) was heated at a boiling point for 10 min to liberate stored glycogen. Glycogen content was measured by a commercial kit (Kamoshi). The kit contains a glycogen oxidase, an aminopyrine reagent, and an ethanol reagent. The oxidase oxidized glycogen to glucose, and the aminopyrine reagent oxidized the glucose to aminopyrine, which was then stabilized by the ethanol reagent.
glycogen and to inactivate enzymes, which may produce extra glucose. After centrifugation for 15 min at 13,000 rpm, 4U amylglucosidase (Sigma) was added into the supernatant. The resulting mixture was incubated for 1 hr at 50°C and followed by boiling for 10 min at 99°C. The cell lysate without amylglucosidase was included as a control. Subsequently, the glycogen content was colorimetrically measured using a glucose assay kit (GAGO20, Sigma). The mixtures were incubated for 30 min at 37°C, and absorbance at 540 nm was measured by using a UV/Visible spectrophotometer reader (Ultraspec 3100 pro, Amersham Biosciences).

iTRAQ Quantification
Flag-GP proteins were expressed in Chang’s liver cells and quantified by iTRAQ following the method modified according to Zhao (Zhao et al., 2010). GP was immunopurified from cells untreated or treated with NAM and TSA, resolved on 10% SDS-PAGE, and stained with Coomassie blue and sliced. The dye of gel slice was removed by soaking with 50 μM NH4HCO3 and 50% acetonitrile, followed by water wash twice and removing water by acetonitrile. The gel was dried and digested in 100 μl 50M NH4HCO3 with trypsin (trypsin:protein at 1:30) at 37°C overnight. The trypsin-treated peptides were extracted by a volume containing 50% acetonitrile and 0.1% trifluoroacetic acid (TFA) and then followed by vacuum dry. Standard control peptides and GP samples were separately labeled with different iTRAQ-labeling reagents (ABI) as indicated in Table S1 and then subjected to LTQ-OrbiTrap MS analysis. Quantification of peptides was calculated by comparing relative intensity of the iTRAQ tags.

Cell Treatment
TSA (0.5 μM) and NAM (5 mM) were added to the culture medium 18 and 6 hr before cell harvest, respectively. Glucose-free medium was prepared with DMEM base (GIBCO, #11966) and supplemented with glucose (Sigma), insulin (Sigma), and glucagon (Sigma) of different concentrations as indicated. Glucose, insulin, and glucagon treatments were carried out by culturing cells in DMEM medium for 24 hr before the desired medium was used to replace DMEM medium.

Glycogen Phosphorylase Activity Assay and CobB Treatment
Flag-tagged proteins were expressed in Chang’s liver cells, eluted by Flag peptides (Gilion Biochemical), and measured using the method of Jones and Wright (Jones and Wright, 1970). The GP activity assay consists of 50 mM sodium glycerol-phosphate (pH 7.1), 10 mM potassium phosphate, 5 mM MgCl2, 0.5 mM NAD+, 1 mM DTT, 1.6 unit phosphoglucomutase, 1.6 unit glucose-6-phosphate dehydrogenase, and 0.2% glycogen in a total volume of 0.3 ml. The reaction was started by adding GP into the volume and assayed at 25°C. The reaction was monitored by measuring the increase of fluorescence (excitation 350 nm, emission 470 nm, HITACHI F-4600 fluorescence spectrophotometer) for 2 hr by shaking. The PhK reaction mixtures were incubated for 30 min at 37°C, and absorbance at 540 nm was measured by using a UV/Visible spectrophotometer reader (Ultraspec 3100 pro, Amersham Biosciences).

In Vitro Binding
Flag-GP purified by Flag beads was subject to in vitro deacetylation by CobB before it was mixed with purified HA-GP. The binding was allowed in 4°C for 4 hr before the beads were washed three times by PBS. Proteins on beads were denatured by SDS loading buffer and detected by western blot.

Statistical Analysis
Statistics were performed with a two-tailed unpaired Student’s t test. All data shown represent the results obtained from triplicated independent experiments with standard deviations (mean ±SD). The values of p < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2011.12.005.

ACKNOWLEDGMENTS
We thank the members of the Fudan MCB laboratory for discussions throughout this study. We appreciate Dr. P.T. Cohen for providing the GP Ser-15 phospho-antibody. This work was supported by the 985 Program, 973 Program (grant numbers 2009CB918401, 2011CB910600, 2012CB910101, and 2012CB910303), NSFC (grant numbers 30971485/C0706, 31030042, 31071192), Shanghai key project (grant numbers 09JC1402300 and 11JC1401100), the Shanghai Leading Academic Discipline Project (project number B110), and National Institutes of Health (NIH) grants to Y.X. and K.-L.G.

Received: January 24, 2011
Revised: July 7, 2011
Accepted: December 9, 2011
Published online: January 3, 2012

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