Phosphorylase regulates the association of glycogen synthase with a proteoglycogen substrate in hepatocytes

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Abstract Changes in the glucosylation state of the glycogen primer, glycogenin, or its association with glycogen synthase are potential sites for regulation of glycogen synthesis. In this study we found no evidence for hormonal control of the glucosylation state of glycogenin in hepatocytes. However, using a modified glycogen synthase assay that separates the product into acid-soluble (glycogen) and acid-insoluble (proteoglycogen) fractions we found that insulin and glucagon increase and decrease, respectively, the association of glycogen synthase with an acid-insoluble substrate. The latter fraction had a higher affinity for UDP-glucose and accounted for between 5 and 21% of total activity depending on hormonal conditions. Phosphorylase overexpression mimicked the effect of glucagon. It is concluded that phosphorylase activation or overexpression causes dissociation of glycogen synthase from proteoglycogen causing inhibition of initiation of glycogen synthesis.

Key words: Glycogen; Glycogenin; Glycogen synthase; Phosphorylase; Glucosamine

1. Introduction

Glycogen synthase has a key role in regulation of glycogen synthesis [1]. Its activity is dependent on the phosphorylation state of multiple sites [2] and on the concentration of glucose 6-P, which is a potent allosteric activator [3]. Glucose 6-P also regulates glycogen synthase by making the enzyme a better substrate for synthase phosphatase and by causing translocation of the protein form a soluble state to a particulate fraction [4,5]. Liver glycogen synthase is also regulated by the phosphorylated form of glycogen phosphorylase (phosphorylase-*a*) which is a potent allosteric inhibitor of glycogen synthase phosphatase [1]. The phosphorylation state of liver phosphorylase is dependent on the concentration of glucose, which makes the enzyme a better substrate for phosphorylase phosphatase [1] and on the concentration of glucose 6-P, which also promotes dephosphorylation [6]. Activation of glycogen synthase by glucose in liver cells is considered to involve two convergent mechanisms: an increase in the cellular content of glucose 6-P (which activates by direct and indirect mechanisms) and dephosphorylation of phosphorylase *a* which reverses the inhibition of glycogen synthase phosphatase [1]. The latter mechanism can be demonstrated using potent allosteric inhibitors of phosphorylase which promote the dephosphorylation of both phosphorylase and synthase [7–9]. Changes in the activity of phosphorylase in liver cells have a greater metabolic impact on the rate of glycogen synthesis than can be explained by either cycling between glycogen synthesis and degradation [10] or by the activation state of glycogen synthase [7]. This raises the question whether phosphorylase regulates glycogen synthesis by additional mechanisms.

The first step in the formation of glycogen is the self-glucosylation of glycogenin, the protein backbone of glycogen, in which glucosyl residues are transferred from UDP-glucose to form an oligosaccharide chain of about eight residues [11,12]. This is then elongated by glycogen synthase to form mature glycogen. Glycogenin was first purified from rabbit skeletal muscle [13] and shown to be present in an equimolar ratio to glycogen synthase [14]. This isoform, designated glycogenin-1 (38 kDa) is expressed in rabbit liver [15,16]. A higher molecular weight isoform (60 kDa, glycogenin-2) is expressed in human liver [17]. Synthesis of glycogen requires the association of glycogen synthase and glycogenin, which depends on the glucosylation state of glycogenin [12]. Glycogenin is regulated by a binding protein that enhances the ability of glycogenin to self-glucosylate [18]. Phosphorylase can catalyse the removal of glucosyl residues from glycogenin [19]. However, whether this has a physiological role in regulating the initiation of glycogen synthesis has not been tested.

The aim of this study was to test the hypothesis that hormones that affect phosphorylase activity in hepatocytes can regulate the initiation of glycogen synthesis through changes in the glucosylation state of glycogenin [19]. Although we could not detect changes in the glucosylation state of glycogenin following hormonal treatment, we found changes in the association of glycogen synthase with a protein-bound substrate. This suggests that phosphorylase like glucose 6-P regulates not only the phosphorylation state of glycogen synthase but also its compartmentation.

2. Materials and methods

2.1. Hepatocyte isolation and culture

Hepatocytes were isolated from male Wistar rats (body weight 180–280 g) fed ad libitum [20]. They were cultured in minimum essential medium (MEM) containing 7% (v/v) newborn calf serum in 6-well plates and after attachment (4 h) the medium was replaced by serum-free MEM containing 10 nM dexamethasone and 5 mM glucose. The muscle isoform of phosphorylase was expressed using recombi-

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nant adenovirus [21] as described previously [7]. Incubations for determination of the effects of hormones on enzyme activity or glycogen synthesis were performed after 18 h culture. For determination of enzyme activity the hepatocyte monolayers were snap-frozen in liquid N_2 and stored at −70°C. For determination of glycogen synthesis hepatocytes were incubated in medium containing 25 mM glucose and [U-^{14}C]glucose (2 μCi/ml) for 3 h. Glycogen was isolated by ethanol precipitation [20] and glycogen synthesis is expressed as nmol of glucose incorporated/3 h per mg protein.

2.2. Enzyme activity determination

Glycogenin was assayed by the method of Ercan and colleagues [22]. Hepatocytes were extracted (1 mg protein/150 μl) by sonication in 100 mM NaF, 50 mM β-glycerophosphate, 5 mM EDTA, 2 mM EGTA, 10 mM dithiothreitol (DTT), pH 7.6 [22]. Glycogenin activity was determined by diluting 1:2 in medium containing (final concentrations): 10 mM $MnCl_2$, 0.12% octylglucoside, 21 mM β-glycerophosphate, 3 mM DTT, pH7.6 without or with 2 M LiBr to inhibit glycogen synthase [23] and UDP[1-^{3}H]glucose at final concentrations of 5 μM UDP-glucose (1500–3000 dpm/pmol) or 80 μM UDP-glucose (650–900 dpm/pmol). For electrophoresis UDP[1-^{14}C]glucose was used (5 μM, 9000 dpm/pmol). The assay was performed at 30°C for 5 min or 15 min and was stopped with an equal volume of ice-cold 20% trichloroacetic acid (TCA) and centrifugation at $13\,000\times g$ (5 min). The pellet was washed twice with 20% TCA, dissolved in formic acid and the radioactivity determined. Activity was expressed as pmol/mg protein based on the specific activity of UDP-glucose which was corrected for endogenous UDP-glucose (assayed as in [24]). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% SDS–PAGE gels with a 4% stacking gel) was used to analyse the glycogenin reaction mixture at the end of the incubation with UDP[1-^{14}C]glucose. The protein was transferred to nitrocellulose membrane and exposed to Fuji BAS 2050 phosphor screen for 2–4 days. For determination of glycogen synthase cells were extracted as in [25] and activity was determined in the homogenates, $13\,000\times g$ (10 min) supernatant and pellet fractions by a modification of [25], with 1 mM UDP-glucose and 10 mM glucose 6-P. The reaction was started by addition of 1 mM UDP[1-^{3}H]glucose (50–200 dpm/pmol) and 0.66% glycogen and was stopped after 10 min by precipitation of protein with TCA as in the glycogenin assay. The soluble fraction was spotted on Whatman 31 ET chromatography as in the conventional assay [25] whereas the TCA-insoluble fraction was treated as in the glycogenin assay. The affinity of glycogen synthase for UDP-glucose was determined as in [2]. Apparent V_{max} values were estimated from Eadie plots and $S_{0.5}$ values were determined from Hill plots [2]. For determination of the activity ratio (−/+ glucose 6-P) assays were performed without or with glucose 6-P [25]. Phosphorylase *a* was assayed spectrometrically [7]. In experiments involving overexpression of muscle phosphorylase assays were performed with 0.2 mM AMP or 5 mM AMP to determine active and total enzyme, respectively [7]. Activities are expressed as mU/mg cell protein. Results are expressed as means ± S.E.M. for the number of hepatocyte preparations indicated. Statistical analysis was by the paired *t*-test.

3. Results

3.1. Effects of Mn^{2+} and glucose 6-P on glycogenin-like activity in hepatocyte extracts

The self-glucosylation of glycogenin is conventionally assayed from the Mn^{2+}-dependent incorporation of labelled UDP[^{14}C/^{3}H]glucose into a TCA-insoluble fraction at 5 μM or 80 μM UDP-glucose [17,22,23]. In this study the incorporation of label from 5 μM UDP[^{14}C]glucose into a TCA-insoluble fraction was stimulated by Mn^{2+} and also by glucose 6-P. In the presence of Mn^{2+} the labelling coincided with proteins of 30–70 kDa whereas in the presence of glucose 6-P it was associated with high molecular weight fractions that did not enter the SDS gel (Fig. 1A). Mn^{2+}-dependent activity accounted for 60–80% of total activity at 5 μM UDP-glucose (Fig. 1B) and < 30% at 80 μM UDP-glucose (control, 19 ± 4; 10 mM Mn^{2+}, 27 ± 4, $n = 4$, pmol/5 min per mg). Insulin or glucagon pre-treatment of the cells had no significant effect on the Mn^{2+}-dependent activity (Fig. 1B). However, the Mn^{2+}-independent activity was increased by insulin in both the absence and presence of glucose 6-P and decreased by glucagon in the presence of glucose 6-P (Fig. 1B). In the combined presence of Mn^{2+} and glucose 6-P the stimulation was not additive (control, 1.9 ± 0.3, glucose 6-P, 3.5 ± 0.4; Mn^{2+}, 6.2 ± 0.3; glucose 6-P+Mn^{2+} 5.8 ± 0.6 pmol/5 min per mg), suggesting that Mn^{2+} may inhibit synthase activity or that glucose 6-P may inhibit glycogenin-like activity. The stimulation of label incorporation by glucose 6-P suggests that it represents glycogen synthase. This was confirmed using LiBr (an inhibitor of synthase but not glycogenin [23]), which inhibited activity by 91 ± 3% ($n = 11$) in the absence of Mn^{2+} but caused little inhibition (7 ± 6%) in the presence of Mn^{2+}. The Mn^{2+}-dependent LiBr-insensitive activity was inhibited by 56 ± 10% with 10 mM CDP, an inhibitor of glycogenin [26]. Time course studies showed that label incorporation reached a plateau within 15 min (Fig. 1C). This was not due to substrate depletion during the assay, as confirmed by lack of further incorporation after re-addition of substrate at the end of the incubation (results not shown). There was no effect of pre-treatment with insulin for 4 h on net glucosylation (Fig. 1C). When the effects of hormones or inhibitors were tested for different incubation times, and glucosylation was assayed at 5 min or 15 min there was no effect of: 100 nM glucagon (5–240 min); 10 nM insulin (1–8 h); 25 mM glucose

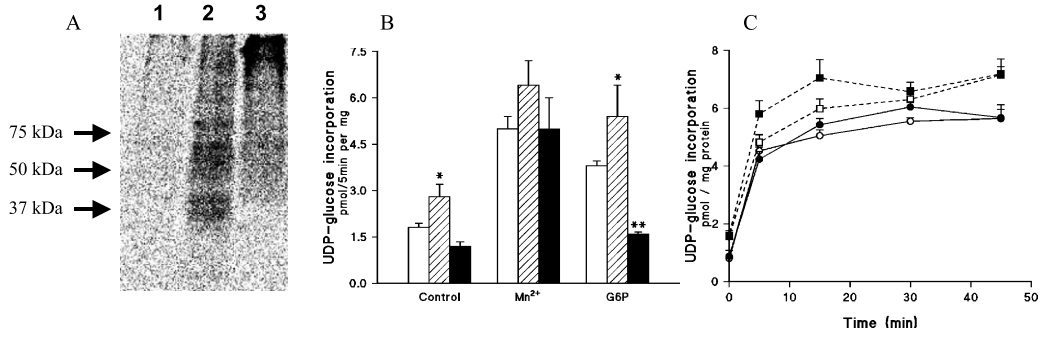

Fig. 1. Mn^{2+}-dependent glycogenin-like activity. A: Radiogram of hepatocyte extracts incubated with UDP[^{14}C]glucose without (1) or with 10 mM Mn^{2+} (2) or 10 mM glucose 6-P (3) and separated by SDS–gel electrophoresis. B: Hepatocytes were incubated for 1 h without (open bars) or with 10 nM insulin (hatched bar) or 100 nM glucagon (solid bar) and glycogenin activity was assayed with 5 μM UDP-glucose as described in Section 2: without (control) or with 10 mM Mn^{2+} or 10 mM glucose 6-P. C: Time course of incorporation of UDP[1-^{3}H]glucose in the presence of 10 mM Mn^{2+} and 2 M LiBr at either 5 μM (○, ●) or 80 μM (□, ■) UDP-glucose in cells that were pre-incubated for 4 h without (○, □) or with (●, ■) 10 nM insulin. Means ± S.E.M. for $n = 4$–5. *$P < 0.05$; **$P < 0.005$ relative to no hormones.

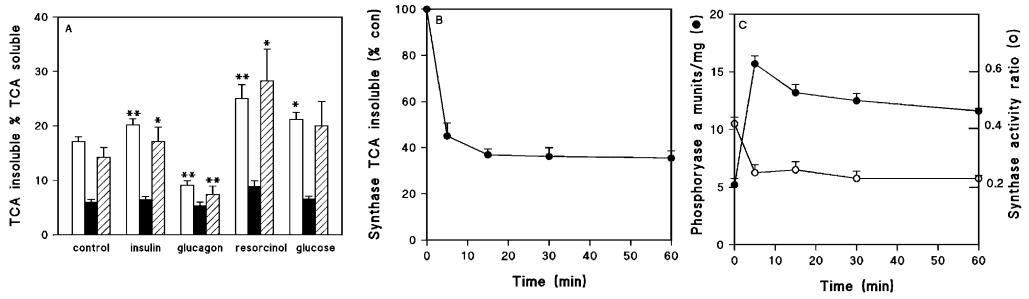

Fig. 2. A: Glycogen synthase activity in hepatocyte homogenates, supernatants and pellets determined by separation of the product into TCA-insoluble and TCA-soluble fractions. Hepatocytes were incubated for 1 h in medium without (control) or with 10 nM insulin, 100 nM glucagon, 150 μM resorcinol or 25 mM glucose. Glycogen synthase was assayed in whole homogenates (open bars), 13 000×*g* supernatant (filled bars) and pellet fractions (hatched bars). On termination of the assay the product was separated into acid-soluble and acid-insoluble fractions and the synthase activity associated with the TCA-insoluble fraction is expressed as % of the TCA-soluble fraction. Means ± S.E.M. $n = 7$–25 $^{*}P < 0.05$, $^{**}P < 0.01$ relative to control. B,C: Effects of incubation of hepatocytes with glucagon on: synthase activity associated with the TCA-insoluble fraction (B) and on phosphorylase *a* and glycogen synthase activity ratio in the TCA-soluble fraction (C). Means ± S.E.M. $n = 5$.

(16 h); or 150 μM resorcinol (60 min), which causes inactivation of phosphorylase [27] (results not shown). The activity of phosphorylase *a* was increased by glucagon and decreased by insulin and resorcinol (control, 8.9 ± 1.2; glucagon 30.5 ± 2.2, $P < 0.01$; insulin, 7.3 ± 1.0, $P < 0.03$; resorcinol, 2.9 ± 0.7, $P < 0.01$; $n = 5$, mU/mg protein). The only treatment that affected glucosylation was 20 mM glucosamine (1 h), which inhibited by 50% (4.6 ± 0.7 to 2.4 ± 0.4, pmol/5 min per mg protein, $n = 6$, $P < 0.003$). This inhibition was reversible after washout of glucosamine (results not shown).

3.2. Glycogen synthase activity associated with TCA-insoluble and TCA-soluble products

To investigate the underlying mechanism for the effects of hormones on the Mn^{2+}-independent glucosylation of a TCA-insoluble substrate, glycogen synthase was assayed by a modification of the conventional assay for glycogen synthase [25] using 1 mM UDP-glucose and 10 mM glucose 6-P, and on termination of the assay the labelled product was separated into TCA-soluble and TCA-insoluble fractions. For this assay the specific activity of UDP[1-^{3}H]-glucose was 10-fold lower than in the glycogenin assay and there was no interference from glycogenin activity, as confirmed by total inhibition by LiBr, which inhibits glycogen synthase but not glycogenin [23]. The label incorporated from UDP[1-^{3}H]glucose into the TCA-insoluble fraction was expressed as a percentage of labelling of TCA-soluble glycogen. Since glycogen synthase translocates from the supernatant to the pellet fraction following incubation with glucose [5] assays were performed on whole homogenates and on supernatant and pellet fractions. In assays on the whole homogenates (Fig. 2, open bars) the radiolabelled product from the synthase assay that was recovered in the TCA-insoluble fraction was significantly increased by insulin, 25 mM glucose and resorcinol and decreased by glucagon. When total glycogen synthase assays was assayed on the supernatant and pellet fractions, we confirmed a similar translocation of enzyme from the supernatant to the pellet fraction after incubation with 25 mM glucose (control, 67 ± 3; glucose, 58 ± 3, $P < 0.05$, $n = 5$, supernatant % total) as reported previously [5]. However, insulin, glucagon or resorcinol did not significantly affect translocation (not shown). Fig. 2A shows the activity in the TCA-insoluble fraction relative to the TCA-soluble fraction for the supernatant (solid bars) and 13 000×*g* pellet fractions (hatched bars). The effects of insulin, glucagon and resorcinol on the glycogen synthase activity associated with the TCA-insoluble product were observed in the pellet (hatched bars) but not in the supernatant fractions (solid bars, Fig. 2A). The action of glucagon on the TCA-insoluble fraction was rapid (complete within 5–15 min Fig. 2B) and similar to the activation of phosphorylase and inactivation of synthase in the TCA-soluble fraction, which were complete within 5 min (Fig. 2C).

3.3. Synthase activity associated with acid-insoluble glycogen has a higher affinity for UDP-glucose

The affinity of synthase for UDP-glucose was determined in whole homogenates and the assay product was separated into the TCA-insoluble and TCA-soluble fractions (Table 1). For all treatments the apparent $S_{0.5}$ for UDP-glucose was lower in the TCA-insoluble fraction ($P < 0.01$) confirming that this labelling is not an artefact due to non-specific entrapment of glycogen in a protein pellet. The apparent V_{max} of the TCA-insoluble fraction was increased by insulin and resorcinol and decreased by glucagon (Table 1), confirming that glucagon

Table 1
The affinity of glycogen synthase for UDP-glucose determined after separation of the product into TCA-insoluble and TCA-soluble fractions

Addition:	Control	Insulin	Glucagon	Resorcinol
TCA-insoluble: $S_{0.5}$ (μM)	316 ± 12	376 ± 36	687 ± 69*	234 ± 9**
TCA-insoluble: V_{max} (mU/mg)	0.14 ± 0.02	0.19 ± 0.03*	0.06 ± 0.01**	0.24 ± 0.04*
TCA-soluble: $S_{0.5}$ (μM)	1551 ± 188	1448 ± 130	2711 ± 211	744 ± 105
TCA-soluble: V_{max} (mU/mg)	1.01 ± 0.12	1.09 ± 0.10	1.04 ± 0.20	0.90 ± 0.09

Hepatocyte monolayers were incubated for 1 h without or with 10 nM insulin, 100 nM glucagon or 150 μM resorcinol. Glycogen synthase activity was determined in homogenates in the presence of 10 mM glucose 6-P and varying concentrations of UDP-glucose and the product was separated into TCA-insoluble and TCA-soluble fractions. $S_{0.5}$ for UDP-glucose were determined from Hill plots. Values are means ± S.E.M. $n = 4$–6. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to control.

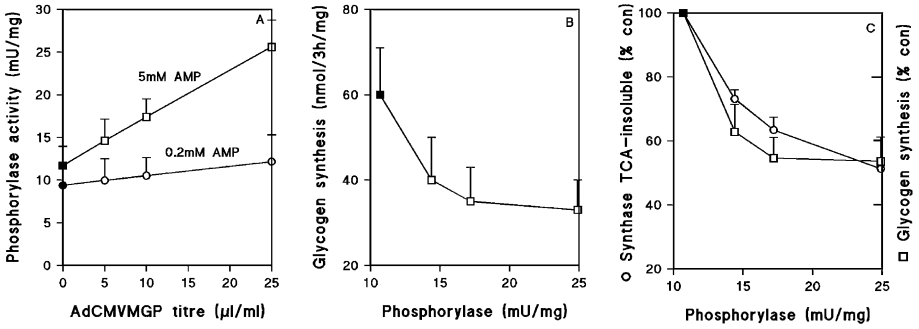

Fig. 3. Expression of muscle phosphorylase in hepatocytes inhibits glycogen synthesis and dissociates glycogen synthase from the TCA-insoluble fraction. Hepatocytes were either untreated (solid symbols) or treated (open symbols) with three titres of recombinant adenovirus AdCMV-MGP for expression of muscle phosphorylase. A: Phosphorylase activity assayed with 0.2 mM AMP (○, ●) or 5 mM AMP (□, ■). B: Glycogen synthesis determined during a 3 h incubation with 25 mM glucose. C: Glycogen synthesis (○) and glycogen synthase activity associated with the TCA-insoluble fraction (□) in cells expressing muscle phosphorylase expressed as % of untreated controls. Means ± S.E.M. $n = 4$.

causes dissociation whereas insulin and resorcinol cause increased association.

3.4. *Phosphorylase overexpression mimics the effect of glucagon*

To determine whether the effect of glucagon on dissociation of synthase from a TCA-insoluble substrate can be explained by the increase in phosphorylase activity we tested the effect of expression of the muscle isoform of phosphorylase using recombinant adenovirus [21]. This isoform unlike the liver isoform is catalytically active in hepatocytes in the dephosphorylated state because of activation by physiological AMP concentrations [7]. Expression of muscle phosphorylase was associated with inhibition of glycogen synthesis (Fig. 3B) and with a decrease in synthase activity on the TCA-insoluble substrate of around 50% (Fig. 3C).

4. Discussion

In this study we found no evidence for hormone-mediated changes in the glucosylation state of glycogenin, as determined from the Mn^{2+}-dependent glucosylation of a TCA-insoluble fraction by the conventional glycogenin assay [22,23]. However, using a modified assay for glycogen synthase, we provide evidence for hormone-dependent changes in the association of glycogen synthase with a proteoglycogen (TCA-insoluble) substrate. The conventional glycogen synthase assay does not distinguish between acid-soluble and acid-insoluble (protein-bound) substrates [25]. By separating the reaction product into TCA-soluble and TCA-insoluble fractions we demonstrate that activity associated with the latter substrate accounts for between 5 and 25% of total activity, depending on the hormonal conditions and has a higher affinity for UDP-glucose than the synthase associated with the acid-soluble substrate.

Previous studies on hepatocytes have shown that high glucose concentration causes translocation of glycogen synthase from the $13\,000 \times g$ supernatant to the pellet fraction by a glucose 6-P-dependent mechanism [5]. This was confirmed in this study. In addition we also show that whereas hormones have negligible effect on translocation of synthase between the $13\,000 \times g$ supernatant and the pellet, they cause translocation of synthase within the $13\,000 \times g$ pellet between a fraction that acts on acid-soluble 'glycogen' and a fraction associated with an acid-insoluble glycogen substrate, which presumably has a higher protein/glycogen ratio. This indicates that there are two distinct translocation mechanisms: a glucose 6-P-dependent mechanism that involves translocation from the soluble to the $13\,000 \times g$ particulate fraction and a hormone-mediated mechanism that regulates binding or dissociation from proteoglycogen within the $13\,000 \times g$ pellet. It is noteworthy that although the glycogen synthase assays in this study contained exogenous glycogen, additional experiments in which the glycogen was omitted showed similar labelling of the acid-insoluble fraction and lower but significant labelling (30–40%) of the acid-soluble fraction in both the supernatant and pellet fractions. This indicates that whereas labelling of the acid-insoluble fraction is entirely due to an endogenous substrate, the labelling of the acid-soluble fraction is in part due to endogenous glycogen and in part to the exogenous glycogen added in the assay.

Recent studies on bovine retina have demonstrated two activities of glycogen synthase associated with acid-soluble glycogen and acid-insoluble proteoglycogen [28]. The latter fraction had a higher affinity for UDP-glucose [28] and contained glycogenin [29]. The synthase associated with the acid-insoluble glycogen may either be a distinct enzyme or alternatively its higher affinity for UDP-glucose may be a consequence of a difference in structure of the polysaccharide moiety [28]. The present findings are analogous to those on bovine retina in that the synthase associated with the acid-insoluble substrate has a higher affinity for UDP-glucose and is confined to the particulate fraction, which also contained the 'glycogenin-like' activity. In addition the present study shows that glucagon causes dissociation of glycogen synthase from the proteoglycogen fraction whereas resorcinol, which causes inactivation of phosphorylase, has the converse effect and phosphorylase overexpression mimics the effect of glucagon. These findings argue in support of a role of phosphorylase activity in regulating the binding of synthase to a proteoglycogen substrate.

Although we found no evidence for hormone-mediated changes in glycogenin activity determined by a Mn^{2+}-dependent assay that has been used previously on liver and other tissues [22,23], which argues against a role for phosphorylase in regulating glycogenin activity, we found 50% inhibition of glycogenin-like activity by pre-treatment of hepatocytes with 20 mM glucosamine. Since glucosamine has been shown to be a minor component of rabbit and pig liver glycogen [30], the inhibition could be due to incorporation of glucosamine into

the glucosyl chain and this may inhibit the further incorporation of glucosyl residues during incubation with UDP-glucose in the conventional assay. An alternative possibility is that glucosamine may inactivate glycogenin by glucosylation of serine or threonine residues after conversion to *N*-acetylglucosamine, as was recently suggested for inactivation of glycogen synthase by glucosamine [31].

A tentative hypothesis to explain the hormone-mediated changes in translocation of glycogen synthase to a TCA-insoluble substrate, is that the proteoglycogen fraction represents nascent glycogen particles and that metabolic conditions associated with increased phosphorylase activity cause dissociation of glycogen synthase and consequent inhibition of glycogen synthesis. The higher affinity for UDP-glucose of the synthase associated with the acid-insoluble proteoglycogen could be explained by either a difference in structure of the polysaccharide substrate as suggested for retina [28] or by the presence glycogen-targeting proteins and synthase phosphatase activity in the proteoglycogen particle, which promote the dephosphorylation of synthase. The glycogen-targeting protein, designated PTG, has a binding site at the C-terminus that can bind glycogen synthase or phosphorylase *a* by a mutually exclusive mechanism [32]. Association of this targeting protein with proteoglycogen may provide a mechanism for competitive binding between phosphorylase *a* and glycogen synthase promoting inhibition or initiation of glycogen synthesis, respectively. It might also explain the lower phosphorylation state (higher affinity for UDP-glucose) of glycogen synthase associated with the proteoglycogen fraction. The changes in association of synthase with the proteoglycogen substrate might also result from changes in the amount of the endogenous acid-insoluble acceptor, because of translocation of enzymes to or from nascent glycogen particles.

It is often assumed that concurrent activity of phosphorylase *a* and glycogen synthase as determined in the conventional assays is indicative of cycling between glycogen synthesis and degradation [33,34]. However, studies using an inhibitor of glycogen phosphorylase provided evidence against cycling in various liver preparations [35]. The present finding that phosphorylase regulates the compartmentation of glycogen synthase provides a potential explanation for the lack of glycogen cycling in metabolic states associated with simultaneous activities of glycogen synthase and phosphorylase, since under conditions of elevated phosphorylase activity there would be inhibition of initiation of glycogen synthesis through dissociation of glycogen synthase from the proteoglycogen particles.

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