Glucose-induced glycogenesis in the liver involves the glucose-6-phosphate-dependent dephosphorylation of glycogen synthase

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Non-metabolized glucose derivatives may cause inactivation of phosphorylase but, unlike glucose, they are unable to elicit activation of glycogen synthase in isolated hepatocytes. We report here that, after the previous inactivation of phosphorylase by one of these glucose derivatives (2-deoxy-2-fluoro- α -glucosyl fluoride), glycogen synthase was progressively activated by addition of increasing concentrations of glucose. Under these conditions, the degree of activation of glycogen synthase was linearly correlated with the intracellular glucose-6-phosphate (Glc-6-P) concentration. Addition of glucosamine, an inhibitor of glucokinase, decreased both parameters in parallel. Further experiments using an inhibitor of either protein kinases (5-iodotubercidin) or protein phosphatases (microcystin) in isolated hepatocytes indicated that Glc-6-P does not affect glycogen-synthase kinase activity but enhances the glycogen-synthase phosphatase reaction. Experiments *in vitro* showed that the

synthase phosphatase activity of glycogen-bound type-1 protein phosphatase was increased by physiological concentrations of Glc-6-P (0.1–0.5 mM), but not by 2.5 mM fructose-6-P, fructose-1-P or glucose-1-P. At physiological ionic strength, the glycogen-associated synthase phosphatase activity was nearly entirely Glc-6-P-dependent, but Glc-6-P did not relieve the strong inhibitory effect of phosphorylase *a*. The large stimulatory effects of 2.5 mM Glc-6-P, with glycogen synthase *b* and phosphorylase *a* as substrates, appeared to be mostly substrate-directed, while the modest effects observed with casein and histone IIA pointed to an additional stimulation of glycogen-bound protein phosphatase-1 by Glc-6-P. We conclude that glucose elicits hepatic synthase phosphatase activity both by removal of the inhibitor, phosphorylase *a*, and by generation of the stimulator, Glc-6-P.

INTRODUCTION

Administration of glucose to intact animals or addition of glucose to isolated hepatocytes induces sequentially the inactivation of phosphorylase and the activation of glycogen synthase [1,2]. The inactivation of phosphorylase appears to be adequately explained by the binding of glucose to phosphorylase *a*, which renders the enzyme a better substrate for phosphorylase phosphatase; the ensuing conversion of phosphorylase *a* to *b* switches off glycogenolysis, but it also relieves glycogen-synthase phosphatase from a potent allosteric inhibitor, phosphorylase *a*. Thus it has previously been proposed that the mere inactivation of phosphorylase would explain the subsequent activation of glycogen synthase [1,2]. However, in glycogen-depleted livers the inhibition of glycogen-synthase phosphatase by phosphorylase *a* is weak or absent [2], and hence the question arises as to how glucose achieves the activation of glycogen synthase under those conditions. An additional mechanism has emerged from experiments with various glucose analogues [3–5], some of which actually bind to phosphorylase *a* with an affinity that is at least one order of magnitude better than that of glucose. In particular, recent work with isolated hepatocytes has shown that the non-metabolized glucose analogue 2-deoxy-2-fluoro- α -glucosyl fluoride (F_2 -Glc) was able to achieve near-complete inactivation of phosphorylase, but activation of glycogen synthase was only observed when a high concentration of glucose was also present [5]. This suggests that a glucose-derived metabolite is required for the activation of glycogen synthase.

The current work provides evidence for a mediatory role of glucose 6-phosphate (Glc-6-P) in the glucose-induced activation of glycogen synthase. Moreover, we show that the synthase

phosphatase activity of the glycogen-bound protein phosphatase-1 (PP-1G) is entirely dependent on the presence of Glc-6-P at physiological ionic strength.

EXPERIMENTAL

Materials

F_2 -Glc was prepared as described by Massillon et al. [5]. 5-Iodotubercidin (ITU) was obtained from RBI (Natick, MA, U.S.A.). Microcystin-LR, histone IIA and the catalytic subunit of protein kinase A from beef heart were purchased from Sigma (St. Louis, MO, U.S.A.). Glucose-6-phosphate dehydrogenase from *Leuconostoc* was obtained from Boehringer. [γ - 32 P]ATP was purchased from Amersham.

Casein was prepared according to the procedure of Mercier et al. [6]. Glycogen synthase *b* and α -particulate glycogen were prepared from dog liver [7]. The catalytic subunit of protein phosphatase-1 (PP-1_C) [8] and phosphorylase *b* [9] were isolated from rabbit skeletal muscle. Phosphorylase *b* was phosphorylated in the presence of [γ - 32 P]ATP by purified phosphorylase kinase [10]. Casein and histone IIA were similarly phosphorylated by the catalytic subunit of protein kinase A [11].

Hepatocytes and liver fractionation

Hepatocytes were isolated from the liver of overnight-fasted male Wistar rats of about 250 g [12]. To ensure full inactivation of glycogen synthase and activation of phosphorylase, 100 nM glucagon was added to the liver perfusion medium (and washed

Abbreviations used: F_2 -Glc, 2-deoxy-2-fluoro- α -glucosyl fluoride; Glc-6-P, glucose 6-phosphate; ITU, 5-iodotubercidin; PP-1_C, catalytic subunit of protein phosphatase-1; PP-1G, glycogen-bound protein phosphatase-1; PP-1S, soluble type-1 protein phosphatase.

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out subsequently during the 3-fold re-suspension of the isolated cells). Unless otherwise indicated, the hepatocytes (10^7 /ml) were incubated at 37 °C in a Krebs–Henseleit medium supplemented with 13.5 mM lactate, 1.5 mM pyruvate and 0.2 mM glycerol. Samples for the assays of glycogen synthase and phosphorylase were immediately diluted with a buffer containing various protein kinase and protein phosphatase inhibitors, and frozen in liquid nitrogen [12].

Subcellular fractions were prepared from rat liver as described [13]. Briefly, overnight-fasted rats were injected intraperitoneally with 0.3 mg of glucagon 30 min before decapitation to ensure a maximal depletion of hepatic glycogen. The livers were homogenized in a buffer containing 50 mM glycylglycine at pH 7.4, 3 mM EGTA, 0.5 mM dithiothreitol, 5% (v/v) glycerol, 5 mM 2-mercaptoethanol, 0.5 mM benzamidine, 0.3 mM PMSF and 0.25 M sucrose. Following centrifugation at 220 000 *g* for 35 min, 2.5 ml of the resulting supernatant was desalted on a Sephadex G25 column (5 × 1 cm). The filtered supernatant was supplemented with α -particulate liver glycogen (5 mg/ml) and a second high-speed centrifugation yielded a post-glycogen supernatant ('cytosolic fraction') and a glycogen pellet with associated enzymes ('glycogen fraction'), which was washed once and re-sedimented. The latter fraction is a specific source of PP-1G [13,14]. The cytosolic fraction contains several protein phosphatases, but its glycogen-synthase phosphatase activity stems exclusively from a 'soluble' type-1 protein phosphatase, termed PP-1S [14].

Assays and statistics

The active *a*-form and the total amount (*a* + *b* forms) of glycogen synthase [7] and phosphorylase [15] were determined at 25 °C as described. Protein phosphatase activities were determined from the rate of activation of purified dog liver glycogen synthase *b* [7], and from the rate of dephosphorylation of phosphorylase *a*, phosphocasein and phosphohistone IIA [11]. In the former case, the samples to be assayed for glycogen synthase *a* were first mixed with 100 μ M ITU and 1 μ M microcystin-LR to prevent further (de)phosphorylation reactions. Subsequently, the samples were incubated for 10 min with glucose-6-P dehydrogenase (2 units/ml), 50 mM Tris/HCl, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA and 15 mM NAD^+ at pH 8.1 to remove Glc-6-P, an allosteric stimulator of glycogen synthase *b*.

Glc-6-P was assayed spectrofluorometrically [16] in neutralized perchloric acid extracts of hepatocytes sedimented for 2 s at 10 000 *g*. Proteins were measured using the method of Bradford [17], with BSA as a standard.

The results are means ($\pm$ S.E.M.) for the indicated number (*n*) of observations. Statistical differences were calculated with Student's paired *t*-test.

RESULTS AND DISCUSSION

Glc-6-P is required for sugar-induced activation of glycogen synthase

In agreement with previous data [5], we found that the addition of 10 mM F_2 -Glc to freshly isolated hepatocytes from fasted rats resulted in near-complete inactivation of phosphorylase, without significant effect on the activity of glycogen synthase (Figure 1). However, while the subsequent addition of glucose did not further affect phosphorylase, it caused activation of glycogen synthase. The extent of synthase activation in response to increasing glucose concentrations (Figure 1B) was almost identical with that seen in experiments without preincubation in the presence of F_2 -Glc (results not shown). This implies that F_2 -Glc

Figure 1 Effects of F_2 -Glc and glucose on the activities of phosphorylase and glycogen synthase in isolated hepatocytes

After a 30 min preincubation of the hepatocytes with 10 mM F_2 -Glc, the indicated concentrations (0, 5, 20, 30 or 60 mM) of glucose were added. Samples were taken at the indicated times for the assays of phosphorylase (0–50 min) (A), Glc-6-P (32–40 min) (A) and glycogen synthase (B). The results represent the means $\pm$ S.E.M. for four hepatocyte preparations.

did not inhibit the activation of glycogen synthase. It also indicates that the notorious low sensitivity of isolated hepatocytes to glucose [12] is not due to the initially high concentration of phosphorylase *a* in freshly isolated hepatocytes.

It has previously been shown that F_2 -Glc is not metabolized and does not affect the transport or the phosphorylation of glucose [5]. In agreement with those findings we observed that, following the inactivation of phosphorylase with F_2 -Glc, increasing glucose concentrations resulted in progressively higher intracellular levels of Glc-6-P (Figure 1A). Analysis of the individual experiments revealed a linear correlation between the level of Glc-6-P and the final activity of glycogen synthase (Figure 2). We have also investigated the effect of glucosamine, a known inhibitor of glucokinase [18], on the glucose-induced activation of glycogen synthase. Figure 2 shows that the addition of 10 or 50 mM glucosamine to hepatocytes that had been preincubated with glucose decreased the concentration of Glc-6-P, and that this decrease was associated with a matching, partial inactivation of glycogen synthase.

Ciudad et al. [19] have previously observed an excellent correlation between the concentrations of Glc-6-P and of glycogen synthase *a* in isolated hepatocytes incubated with various sugars and gluconeogenic precursors, and with the glucokinase inhibitor mannoheptulose. Our data show that this correlation also applies when the concentration of Glc-6-P is modified after the previous inactivation of phosphorylase, and suggest that Glc-

Figure 2 Correlation between the extent of the glucose-induced activation of glycogen synthase and the concentration of Glc-6-P

The open circles show, for each experiment represented in Figure 1, the level of Glc-6-P (mean of the concentrations determined at 32, 35 and 40 min after the addition of glucose) and the percentage of glycogen synthase in the *a*-form at 40 min. The linear regression line applies to these data (correlation coefficient $r = 0.85$; $P < 0.001$). The triangles with horizontal and vertical bars represent the means $\pm$ S.E.M. for five preparations of hepatocytes incubated with 50 mM glucose for 30 min, and for the final 10 min in the additional presence of 0, 10 mM or 50 mM glucosamine as indicated by the arrows. Both concentrations of glucosamine caused statistically significant decreases in both parameters ($P < 0.013$ or smaller).

6-P, or a metabolite in equilibrium with Glc-6-P, controls directly the interconversion between synthase *a* and synthase *b*. The actual mediator appears to be Glc-6-P, since activation of glycogen synthase has also been obtained in hepatocytes, leucocytes and adipocytes with glucose analogues that are not further metabolized after phosphorylation on carbon 6 [3,20–22].

Glucose does not cause inhibition of glycogen-synthase kinases in hepatocytes

The glucose-induced activation of glycogen synthase could be explained by increased synthase phosphatase activity and/or decreased synthase kinase activity. It is noteworthy that Villar-Palasi has demonstrated antagonistic effects of Glc-6-P on protein phosphatases [23] and protein kinase A [24] with purified muscle glycogen synthase as substrate. We have used inhibitors of protein kinases and protein phosphatases to distinguish between these possible mechanisms in intact hepatocytes. In one series of experiments, cells were incubated for 45 min with F_2 -Glc to inactivate phosphorylase (results not shown) plus 15 mM each of glutamine and fructose to achieve a partial activation of glycogen synthase (Figure 3A). Then a wide range of intracellular Glc-6-P concentrations was created within 2 min by the addition of 0, 20 mM or 60 mM glucose (Figure 3B). Addition of 5 μ M microcystin-LR, 2 min later, induced the re-inactivation of glycogen synthase (Figure 3A). This is explained by the complete inhibition of protein phosphatases-1 and -2A, which constitute all the synthase phosphatase activity in the liver [25]. Thus, the inactivation rate of glycogen synthase in the presence of a saturating concentration of microcystin can be taken as an *in situ* measurement of the synthase kinase activity. We found that the prior addition of 20 or 60 mM glucose, while causing a slight further activation of glycogen synthase, had no effect on the subsequent rate of synthase inactivation (Figure 3A). In view of the large and sustained differences in intracellular Glc-6-P (Figure

Figure 3 Glucose concentration does not affect the rate of glycogen synthase inactivation induced by microcystin in isolated hepatocytes

Hepatocytes were preincubated for 45 min with 10 mM F_2 -Glc, 15 mM fructose (Fru) and 15 mM glutamine (Gln). Subsequently, the indicated concentrations (0, 20, 60 mM) of glucose were added. Two minutes later 5 μ M microcystin was added for all but one set of conditions (○). Samples were taken at the indicated time points for the assay of glycogen synthase (A) and Glc-6-P (B). The results represent the means $\pm$ S.E.M. for four experiments.

3B), these data argue against an effect of glucose or its metabolites on hepatic synthase kinase activity.

In another series of experiments, hepatocytes were first incubated for 30 min with 10 mM F_2 -Glc and no glucose, resulting in a pronounced inactivation of phosphorylase (results not shown) without attendant activation of glycogen synthase (Figure 4A). Subsequent addition of ITU, a general inhibitor of protein kinases in hepatocytes [26], caused partial activation of glycogen synthase, even in the absence of glucose. However, the important point here is that the incremental synthase activation in response to 20 mM and 60 mM glucose was the same in the presence and in the absence of ITU (Figure 4A). Since inhibition of glycogen-synthase kinases by ITU obviously did not decrease the magnitude of the effect of glucose, these results constitute further evidence that the effect of glucose or its metabolites cannot be attributed to inhibition of the synthase kinase activity in intact hepatocytes. In Figure 4(B) it is shown that ITU did not interfere with the glucose-induced accumulation of Glc-6-P.

Glc-6-P stimulates the dephosphorylation of liver glycogen synthase

The above data (Figures 3 and 4) suggest that Glc-6-P promotes the activation of glycogen synthase through stimulation of the synthase phosphatase reaction. There has been a previous report

Figure 4 Additive effects of ITU and glucose on the activation of glycogen synthase in isolated hepatocytes

Hepatocytes were preincubated for 30 min with 10 mM F_2 -Glc, followed by incubation in the absence (open symbols) or presence (filled symbols) of 50 μ M ITU. At 35 min, the indicated concentrations of glucose were added. Samples were taken for the assays of glycogen synthase (A) and Glc-6-P (B). The results represent the means $\pm$ S.E.M. for four experiments.

that the activation of glycogen synthase in an isolated liver glycogen fraction was enhanced by Glc-6-P [27]. The activation of purified liver glycogen synthase by PP-1_c was also stimulated by Glc-6-P, although only in the presence of 10 mM $MgCl_2$ [28]. It was recently discovered in this laboratory that, upon incubation in the presence of Glc-6-P, apparently homogeneous liver glycogen synthase *b* undergoes a 'pseudo-activation', of unknown nature, that does not involve dephosphorylation [29]. It is also significant that, in an earlier study using muscle glycogen synthase *b* as substrate, Kato and Bishop [30] found that 0.1 mM Glc-6-P stimulated about 2-fold the rate of activation of muscle glycogen synthase without affecting the rate of dephosphorylation. For these reasons we adopted a stringent assay procedure where any added Glc-6-P was oxidized by Glc-6-P dehydrogenase before the assay of synthase *a*; moreover additional checks were made that the effect of the highest concentration of Glc-6-P was blocked by the protein-phosphatase inhibitor, microcystin (e.g. Figures 5A and 5B).

About 75% of the glycogen-synthase phosphatase activity in the liver stems from PP-1G, the type-1 protein phosphatase associated with the glycogen particles [22]. We found that the addition of 2.5 mM Glc-6-P increased the activity of PP-1G significantly with any substrate tested (Table 1). However, the stimulation was rather modest when PP-1G was assayed on phosphocasein (+23%) and phosphohistone IIA (+54%). The latter effects appear to be phosphatase-directed since the dephos-

Figure 5 Effect of the Glc-6-P concentration on the glycogen-synthase phosphatase activity of PP-1G

Purified glycogen synthase *b* was incubated with a freshly prepared glycogen fraction as a source of PP-1G, in the presence of the indicated concentrations of Glc-6-P, without (full lines) or with (broken lines) 1 μ M microcystin, and in the presence of 3 mM AMP plus 5 mM $MgCl_2$ (A), or 150 mM KCl (B), or 150 mM KCl plus 1 mg/ml of phosphorylase *a* (C). At the indicated times samples were withdrawn for the assay of synthase *a* as described in the Experimental section. The results represent the means $\pm$ S.E.M. for three experiments.

phorylation of these substrates by the free catalytic subunit (PP-1_c) was not stimulated by Glc-6-P (Table 1). Larger stimulatory effects of 2.5 mM Glc-6-P (at least 2.2-fold) were apparent when PP-1G acted on glycogen synthase and phosphorylase (Table 1); since Glc-6-P is a known allosteric regulator of phosphorylase [31] and glycogen synthase [32], the major part of its effect on the synthase phosphatase and phosphorylase phosphatase activities of PP-1G is probably substrate-directed. This interpretation is supported by further data: (i) the cytosolic synthase phosphatase activity, due to PP-1S, which accounts for nearly 20% of the hepatic synthase phosphatase activity [14], was also stimulated by 2.5 mM Glc-6-P (+76 $\pm$ 1%; n = 3); (ii) the dephosphorylation of phosphorylase, whether by PP-1_c or by PP-1G, was stimulated to the same extent (2.2-fold) by 2.5 mM Glc-6-P

Table 1 Substrate-dependence of the stimulation of PP-1G by glucose-6-P

Each substrate was incubated in the absence of KCl with either PP-1G or PP-1_C in the absence and presence of 2.5 mM glucose-6-P, as described in the Experimental section. The results represent the activities in the presence of Glc-6-P as a percentage of the corresponding controls without Glc-6-P (means $\pm$ S.E.M.; $n = 4-6$). Liver glycogen synthase *b* is not a substrate for PP-1C [14]. *Significantly different from the control without Glc-6-P ($P < 0.023$ or smaller).

Substrate	Activity of PP-1G (% of control)	Activity of PP-1 _C (% of control)
Glycogen synthase	221 $\pm$ 24*	—
Phosphorylase	229 $\pm$ 5*	219 $\pm$ 6*
Casein	123 $\pm$ 7*	101 $\pm$ 5
Histone IIA	154 $\pm$ 8*	128 $\pm$ 14

Figure 6 Effects of various sugar phosphates on the synthase phosphatase activity of PP-1G

The incubations and assays were performed as described for Figure 5(B), with either no added sugar phosphate (○) or plus 2.5 mM Glc-6-P (▼), glucose 1-P (▲), fructose 6-P (●) or fructose 1-P (■). The results represent the means $\pm$ S.E.M. for four experiments.

(Table 1); and (iii) the stimulatory effect of 2.5 mM Glc-6-P on the phosphorylase phosphatase reaction was only observed in the presence of caffeine, another known ligand of phosphorylase [31].

Glc-6-P clearly stimulated the synthase phosphatase activity of PP-1G at concentrations of 0.1–0.5 mM (Figure 5A), which are in the physiological range. Interestingly, we noted that Glc-6-P stimulated the synthase phosphatase activity of PP-1G to the same final extent in the presence of 150 mM KCl which, however, nearly completely blocked the expression of the basal synthase phosphatase activity of PP-1G (Figure 5B). The inhibition by KCl appears to be a salt effect, since similar results were obtained with 150 mM NaCl, sodium acetate or NaHCO₃ (results not shown). We conclude therefore that the synthase phosphatase activity of PP-1G is virtually entirely Glc-6-P dependent at physiological ionic strength. It is also important to note that the stimulatory effect of Glc-6-P (0.5 mM and 2.5 mM) was not antagonized by P_i at concentrations up to 3 mM (results not shown), which have previously been shown to antagonize the stimulation of glycogen synthase *b* by Glc-6-P [33].

As had previously been demonstrated for the basal synthase phosphatase activity of PP-1G (see Introduction), its Glc-6-P-

dependent synthase phosphatase activity was virtually abolished by the addition of phosphorylase *a* (Figure 5C). This implies that the inactivation of phosphorylase is a prerequisite for the Glc-6-P-mediated activation of glycogen synthase by PP-1G.

The addition of 2.5 mM glucose-1-P, fructose-1-P or fructose-6-P, either in the presence of 150 mM KCl (Figure 6) or without KCl (results not shown), did not affect the synthase phosphatase activity of PP-1G, while the same concentration of Glc-6-P caused a many-fold stimulation. Xylulose-5-P, albeit tested only once, was also ineffective (results not shown). The total inefficiency of fructose-6-P was unexpected, in view of its reported potency in stimulating the dephosphorylation [23] and activation [34] of muscle glycogen synthase. This suggests that fructose-6-P does not bind efficiently to liver glycogen synthase *b*, but the question does not seem to have received attention after the initial report by Leloir and Goldemberg [35], who observed that fructose-6-P stimulated some 10-fold the activity of a partially purified enzyme from rat liver which, however, was said to be contaminated with phosphohexose isomerase. We found that fructose-6-P at up to 10 mM did not stimulate the activity of the purified glycogen synthase *b* from dog liver that was used as a substrate for glycogen-synthase phosphatase in the present work (results not shown).

CONCLUSIONS

It has previously been proposed that Glc-6-P is responsible for the synergistic effect of glucose on the insulin-induced dephosphorylation of glycogen synthase in skeletal muscle [36] and in adipose tissue [22]. Using intact hepatocytes, as well as isolated enzymes, we have provided evidence for an essential role of Glc-6-P in the (insulin-independent) glucose-induced activation of glycogen synthase in the liver. As shown in Figure 5(B), the activation of glycogen synthase by PP-1G was virtually entirely dependent on Glc-6-P at physiological ionic strength, and was stimulated by concentrations of Glc-6-P above 0.1 mM (approximately the intracellular level in the liver of a fasted rat).

Our observation that the Glc-6-P-dependent synthase phosphatase activity of PP-1G (Figure 5C), like the basal activity [2], is inhibited by phosphorylase *a*, suggests a two-step mechanism for the glucose-induced activation of glycogen synthase in the liver. According to this view, the expression of the synthase phosphatase activity of PP-1G requires both the removal of the allosteric inhibitor, phosphorylase *a*, and the generation of the activator, Glc-6-P. In addition, Glc-6-P may also cause 'pseudo-activation' of glycogen synthase *b*, which does not involve dephosphorylation [29].

In a broader perspective, our work provides additional evidence for the concept that the function of hepatic protein phosphatases is regulated by physiological changes in metabolite concentrations. The effect of such metabolites can be substrate-directed or phosphatase-directed, or both. Examples besides the current work are the well-known stimulation by glucose of the inactivation of phosphorylase [2], the stimulation by glutamate of the dephosphorylation of acetyl-CoA carboxylase [37], and the stimulation by xylulose-5-P of the protein phosphatase-2A that dephosphorylates fructose-2,6-bisphosphatase [38,39].

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