Shared control of hepatic glycogen synthesis by glycogen synthase and glucokinase

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We have used recombinant adenoviruses (AdCMV-RLGS and AdCMV-GK) to overexpress the liver isoforms of glycogen synthase (GS) and glucokinase (GK) in primary cultured rat hepatocytes. Glucose activated overexpressed GS in a dose-dependent manner and caused the accumulation of larger amounts of glycogen in the AdCMV-RLGS-treated hepatocytes. The concentration of intermediate metabolites of the glycogenic pathway, such as glucose 6-phosphate (Glc-6-P) and UDP-glucose, were not significantly altered. GK overexpression also conferred on the hepatocyte an enhanced capacity to synthesize glycogen in response to glucose, as described previously [Seoane, Gómez-Foix, O'Doherty, Gómez-Ara, Newgard and Guinovart (1996) *J. Biol. Chem.* **271**, 23756–23760], although, in this case,

they accumulated Glc-6-P. When GS and GK were simultaneously overexpressed, the accumulation of glycogen was enhanced in comparison with cells overexpressing either GS or GK. Our results are consistent with the hypothesis that liver GS catalyses the rate-limiting step of hepatic glycogen synthesis. However, hepatic glycogen deposition from glucose is submitted to a system of shared control in which the 'controller', GS, is, in turn, controlled by GK. This control is indirectly exerted through Glc-6-P, which 'switches on' GS dephosphorylation and activation.

Key words: adenovirus, glucose, hepatocyte, liver metabolism.

INTRODUCTION

Glycogen synthesis is the major determinant of non-oxidative glucose metabolism. This polysaccharide is found in most mammalian cells, but the liver and skeletal muscle are the tissues that can accumulate glycogen to the largest extent, and play the most crucial role in maintaining blood glucose homeostasis. Owing to its greater mass, skeletal muscle, the major site of insulin-stimulated glucose uptake, contains the largest amount of glycogen. However, after a meal, about one-third of the ingested glucose is cleared by the liver [1], which contains the largest concentration of the polysaccharide.

Glycogen synthase (GS) catalyses the addition of glucose residues to the non-reducing end of a nascent glycogen chain via α -1,4-glycosidic bonds, using UDP-glucose (UDP-Glc) as substrate. GS activity is highly regulated via phosphorylation and allosteric effectors, mainly glucose 6-phosphate (Glc-6-P), and it is generally accepted that the reaction catalysed by GS is rate-limiting for glycogen synthesis in all organs [2]. However, a recent report has shown that, in muscle fibres, the control of glycogen synthesis is shared between glucose transport and GS [3].

To date, two isoforms of mammalian GS have been described. Most tissues express the muscle form, whereas the liver isoenzyme appears to be tissue-specific [2]. Although the two forms are 70% identical in amino acid sequence, the N- and C-termini, which contain the phosphorylation sites that regulate the activity of the enzyme, show a much lower degree of homology [4]. These differences and their dissimilar intracellular distribution [5,6] suggest that muscle and liver GS may have distinct capacities in the control of glycogen synthesis in the respective organs.

The adenovirus-mediated overexpression of key enzymes of glucose metabolism in various cell types has been shown to constitute a valuable tool to modulate the capacity of the cell to take up and utilize glucose [7]. For instance, the overexpression of glucokinase (GK), but not of hexokinase (HK) I, greatly enhances glycogen deposition in hepatocytes, although this also results in an increase in lactate production. In the present work, we have used a recombinant adenovirus to efficiently overexpress the liver isoform of GS and GK in primary cultured hepatocytes. We have found that overexpression of GS has a metabolic impact, which is restricted to a large enhancement of the hepatocyte capacity to accumulate glycogen in response to glucose. This effect is enhanced further when GK is simultaneously overexpressed. Our results confirm the key role of GS in the control of hepatic glycogen synthesis. However, GS activation is controlled via Glc-6-P levels by GK. Therefore both enzymes contribute to the control of glycogen synthesis from glucose in the hepatocyte.

MATERIALS AND METHODS

Preparation of recombinant adenovirus

The AdCMV-RLGS adenovirus containing an expression unit with the cytomegalovirus promoter, a 2.3 kb fragment coding for rat liver glycogen synthase (RLGS) and the simian virus 40 polyadenylation signal was constructed as described by Becker et al. [8]. AdCMV-GK has already been described in previous reports [9]. AdCMV- β GAL, a virus coding for the bacterial enzyme β -galactosidase, was used as a negative control in the metabolic impact studies.

Abbreviations used: GS, glycogen synthase; RLGS, rat liver GS; UDP-Glc, UDP-glucose; Glc-6-P, glucose 6-phosphate; GK, glucokinase; HK, hexokinase; DMEM, Dulbecco's modified Eagle's medium; G6Pase, glucose-6-phosphatase.

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Hepatocyte isolation and treatment with recombinant adenovirus

Hepatocytes were isolated by collagenase perfusion from male Wistar rats (180–225 g) fasted for 24 h, as described previously [10]. Cells were suspended in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 mM glucose, 10% (v/v) fetal-bovine serum, 100 nM insulin (Roche Diagnostics, Barcelona, Spain) and 100 nM dexamethasone (Sigma-Aldrich Quimica S. A., Madrid, Spain), and seeded on to plastic plates of diameter 60 mm, treated with 0.1% (w/v) gelatin (Sigma) at a final density of 6×10^4 cells/cm². After attachment (4 h at 37 °C), hepatocytes were treated for 2 h with AdCMV-RLGS at a multiplicity of infection of 10. AdCMV-GK was used at a multiplicity of infection of 4. When cells were simultaneously infected with both viruses, the multiplicity of infection of both 4 and 10 was used. The medium was replaced with DMEM, and cells were kept at 37 °C for an additional 16–18 h. Hepatocytes were then incubated in DMEM containing 100 nM insulin and several concentrations of glucose, as explained in the text and Figure legends. At the end of each manipulation, cell monolayers were washed in PBS, and frozen in liquid N₂ until analysis.

Metabolite determinations

To measure glycogen content, cell monolayers were scraped into 30% (v/v) KOH; the extract was then boiled for 15 min, and centrifuged at 5000 *g* for 15 min. Glycogen was measured in the cleared supernatants as described previously [11]. The intracellular concentrations of Glc-6-P and UDP-Glc were measured using spectrophotometric assays [12,13]. L-Lactate concentration in the incubation medium was measured as described previously [14].

Enzyme activity assays

Frozen cell monolayers from 60-mm-diam. plates were scraped using 100 μ l of homogenization buffer, which consisted of 10 mM Tris/HCl (pH 7.0), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 10 μ g/ml leupeptin, 1 mM benzamidine and 1 mM PMSF. Cell lysis was induced by thawing. Protein concentration was measured as described by Bradford [15] using a Bio-Rad assay reagent. Glucose-phosphorylating activity was measured spectrophotometrically in the supernatant fraction of hepatocyte extracts centrifuged at 10000 *g* for 15 min, using 1 mM or 100 mM glucose at 30 °C, as described previously [16]. Total GS activity was measured in homogenates in the presence of 6.6 mM Glc-6-P, as described in [17]. Active GS (I or *a* form) was measured in the absence of Glc-6-P.

Electrophoresis and immunoblotting

GS and glycogen phosphorylase protein levels were measured by Western blotting. The electrotransfer of proteins from the gel to the nitrocellulose blot was performed at 200 V (constant) at room temperature for 2 h in a Bio-Rad miniature transfer apparatus, as described by Towbin et al. [18]. The nitrocellulose blot was incubated overnight at 4 °C in blocking buffer [1% (w/v) BSA/0.05% (v/v) Tween 20 in PBS]. It was then incubated for 1 h at room temperature with a rabbit antibody raised against rat liver GS or glycogen phosphorylase, and was then washed and incubated for 1 h with a secondary anti-rabbit antibody conjugated to horseradish peroxidase. Immunoreactive bands were detected using the ECL[®] kit (Amersham, Arlington, IL, U.S.A.), following the manufacturer's instructions.

Statistical analysis

Data are expressed as the means $\pm$ S.E.M. Statistical significance was determined by unpaired two-tailed Student's *t* test analysis. Statistical significance was assumed at *P* < 0.05.

RESULTS

GS overexpression in cultured rat hepatocytes

A recombinant adenovirus containing the RLGS cDNA under the control of the cytomegalovirus promoter (AdCMV-RLGS) was constructed. The ability of the AdCMV-RLGS adenovirus to direct the expression of functional RLGS was verified by treating primary cultured hepatocytes with several doses of the virus, and by measuring both total GS activity and immunoreactive protein in the extracts (Figure 1). In untreated cells and in hepatocytes infected with AdCMV- β Gal, total GS activity (measured in the presence of 6.6 mM Glc-6-P) was approx. 3 m-units/10⁶ cells, whereas in hepatocytes infected with the AdCMV-RLGS adenovirus at a multiplicity of infection of 10, this activity was higher than 18 m-units/10⁶ cells, 18 h post-treatment. The

Figure 1 RLGS expression in cultured rat hepatocytes treated with AdCMV-RLGS

Hepatocytes were treated with AdCMV-RLGS (filled bars in **A**) at a multiplicity of infection of 2.5, 5 or 10 (i, ii and iii in **B** respectively), or with a single dose of AdCMV- β gal at a multiplicity of infection of 10 (hatched bar in **A**), or left untreated (UT) (open bar in **A**) as described in the Materials and methods section. After 16 h in DMEM, cells were incubated for 2 h with 25 mM glucose. Cells were then collected and homogenates were assayed for total GS activity (**A**) or analysed by Western blotting, using an antibody raised against RLGS (**B**).

Table 1 GK and HK activity in RLGS, GK and RLGS/GK-overexpressing hepatocytes

Rat hepatocytes were treated with AdCMV-RLGS, AdCMV-GK and AdCMV-GS plus AdCMV-GK or left untreated and incubated for 16 h with DMEM without glucose. Cells were then exposed for 2 h to 25 mM glucose, and collected for measurement of GK and HK activities as described in the Materials and methods section. The activity is expressed in m-units/ 10^6 cells. Data represent the means $\pm$ S.E.M. for eight independent experiments in untreated and AdCMV-RLGS-treated cells, or the means $\pm$ S.E.M. for six independent experiments in AdCMV-GK- and AdCMV-RLGS/GK-treated cells.

	GK	HK
Untreated	12.0 $\pm$ 1.8	4.5 $\pm$ 0.6
AdCMV-GK	38.6 $\pm$ 5.3	3.6 $\pm$ 0.3
AdCMV-RLGS	13.5 $\pm$ 1.6	5.02 $\pm$ 0.7
AdCMV-RLGS/GK	41.9 $\pm$ 5.7	3.4 $\pm$ 0.2

immunoreactive protein showed an increase that paralleled that of the GS activity (Figure 1). The use of larger doses of adenovirus or longer post-infection periods yielded higher levels of functional GS (results not shown). However, later experiments performed at a multiplicity of infection of 10 (a dose that transduced over 95% of the cells when using AdCMV- β Gal) and with a post-treatment period of 18 h yielded an approx. 6-fold increase in total GS activity.

Glucose-phosphorylating activity and glycogen phosphorylase in RLGS-overexpressing hepatocytes

In some instances, the overexpression of a given protein results in an altered expression level of a related enzyme. For example, the adenovirus-mediated overexpression of rabbit muscle glycogen phosphorylase in cultured human muscle fibres resulted in an increased level of total GS [19]. Transgenic mice that overexpress muscle GS also showed increased levels of glycogen phosphorylase in muscle fibres [3,20].

In our study, the overexpression of liver GS in hepatocytes did not affect the expression of liver glycogen phosphorylase. Densitometric analyses of Western blots of cell homogenates revealed that the levels of glycogen phosphorylase remained constant, independent of the glucose concentration in the medium and, more importantly, there were no significant differences between cells overexpressing RLGS and control hepatocytes (results not shown).

In addition, we measured the total glucose-phosphorylating capacity of the cultured hepatocytes. GK and HK activities were essentially the same in cells overexpressing RLGS and in untreated hepatocytes, independent of the glucose concentration in the medium (Table 1). However, hepatocytes treated with AdCMV-GK at a multiplicity of infection of 4 showed a 3-fold increase in GK activity, but no differences in HK levels were observed (Table 1).

Glycogen accumulation in hepatocytes overexpressing RLGS, GK or RLGS/GK in response to glucose

The ability of cultured hepatocytes to accumulate glycogen in response to glucose was greatly increased in cells overexpressing RLGS (Figure 2). In the absence of glucose, both treated and untreated hepatocytes contained low amounts of glycogen. After a 2 h incubation with increasing glucose concentrations, the levels of glycogen synthesized by the cells showed, in both cases, a linear relationship with the concentration of glucose present in the medium. However, at all glucose concentrations, RLGS-overexpressing hepatocytes showed a 4-fold increase in the

Figure 2 Effects of GS, GK and GK/GS overexpression on glycogen accumulation

Hepatocytes were treated with AdCMV-RLGS (filled bars), with AdCMV-GK (widely spaced hatched bars), with AdCMV-RLGS plus AdCMV-GK (closely spaced hatched bars) or left untreated (open bars) as described in the Materials and Methods section. After 16 h in DMEM, hepatocytes were incubated for 2 h with several concentrations of glucose. Cells were flash-frozen and assayed for glycogen content. Data represent the means $\pm$ S.E.M. for eight independent experiments for untreated and AdCMV-RLGS-treated cells, the means $\pm$ S.E.M. for six independent experiments for AdCMV-GK and AdCMV-RLGS/GK infections. The letters indicate statistical significance at the following levels: a, $P < 0.01$; b, $P < 0.001$ for comparisons between the AdCMV-RLGS-treated group and untreated control; c, $P < 0.001$ for comparisons between the AdCMV-GK-treated group and untreated control; d, $P < 0.001$; and e, $P < 0.001$ for comparisons between the AdCMV-GS/GK-treated cells and the untreated control group or AdCMV-GK-treated group.

amount of glycogen compared with untreated cells, and at 25 mM glucose reached a value of approx. 200 μ g of glycogen/ 10^6 cells (Figure 2). When the incubation period with a high glucose concentration was prolonged to 24 h, the differences between treated and untreated cells became even more pronounced. Under these conditions, untreated hepatocytes accumulated approx. 50 μ g of glycogen/ 10^6 cells, whereas the glycogen content in treated cells was higher than 400 μ g of glycogen/ 10^6 cells.

The ability of cultured hepatocytes to accumulate glycogen was also improved with GK overexpression. In this case, a 2.5–3-fold increase in the levels of the enzyme led to a 3-fold increase in the amount of glycogen accumulated, reaching 170 μ g of glycogen/ 10^6 cells after a 2 h incubation with 25 mM glucose (Figure 2). An additive effect was observed when both enzymes were co-expressed, yielding a 6-fold increase in glycogen content when incubated with 10 and 25 mM glucose. After a 2 h incubation with 25 mM glucose, a level of 300 μ g of glycogen/ 10^6 cells was attained (Figure 2), comparable with that found in liver from rats fed *ad libitum* [21].

GS activity in RLGS-, GK- and RLGS/GK-overexpressing hepatocytes

Total GS activity in untreated hepatocytes was 3.0 ± 0.3 m-units/ 10^6 cells. GK overexpression did not alter total GS activity. Total GS activity increased 6-fold when cells were treated with AdCMV-RLGS, reaching values of 18.5 ± 1.4 m-units/ 10^6 cells. In these experiments, it was not appropriate to use the $-\text{Glc-6-P}/+\text{Glc-6-P}$ activity ratio to measure changes in the capacity of the cell to synthesize glycogen, since the levels of total GS were

Figure 3 Effects of glucose on GS activation

Hepatocytes were treated with AdCMV-RLGS (filled bars), with AdCMV-GK (widely spaced hatched bars), with AdCMV-RLGS plus AdCMV-GK (closely spaced hatched bars) or left untreated (open bars) as described in the Materials and methods section. After 16 h in DMEM, hepatocytes were incubated for 2 h with several glucose concentrations. Cells were then collected and GS activity was measured in the homogenates. Active GS was measured in the absence of Glc-6-P. Data represent the means $\pm$ S.E.M. for eight independent experiments for untreated and AdCMV-RLGS-treated cells, or means $\pm$ S.E.M. for six independent experiments for AdCMV-GK and AdCMV-RLGS/GK infections. The letters indicate statistical significance at the following levels: a, $P < 0.01$; b, $P < 0.001$ for comparisons between the AdCMV-RLGS-treated cells and the untreated control group; c, $P < 0.001$ for comparisons between the AdCMV-GK-treated group and untreated control; d, $P < 0.001$; e, $P < 0.001$ for comparisons between the AdCMV-GS/GK-treated cells and untreated control group or AdCMV-GK-treated group.

different. Therefore we measured the catalytic concentration of active GS instead (activity measured in the absence of Glc-6-P) (Figure 3). Incubation of cultured hepatocytes with glucose activated endogenous liver GS. Overexpressed GS also responded to glucose, and the activity measured in the absence of Glc-6-P gradually rose with increasing concentrations of glucose in the medium; the levels of active GS were approx. 4-fold higher in AdCMV-RLGS treated cells than in controls (Figure 3). In hepatocytes overexpressing GK alone, the increase in active GS was about 3-fold, as described previously [10]. When both adenoviruses were used simultaneously, an approx. 9-fold increase in the level of active GS was detected (Figure 3).

Glc-6-P and UDP-Glc levels in RLGS-, GK- and RLGS/GK-overexpressing hepatocytes

Glc-6-P is a key metabolite in the glycogen synthetic pathway. On the one hand, it is assumed to be in equilibrium with glucose 1-phosphate, the precursor of UDP-Glc, which in turn is the substrate of GS, acting as the glucosyl donor for the elongation of the glycogen molecule. On the other hand, Glc-6-P is an allosteric activator of GS, and, more importantly, it also promotes the covalent, permanent activation of GS by dephosphorylation [22].

To check whether the overexpression of GS resulted in changes in the levels of the precursors of glycogen synthesis, we measured Glc-6-P and UDP-Glc levels in both treated and untreated rat hepatocytes incubated in the presence of increasing concentrations of glucose. Our results show that the accumulation of

Figure 4 Effects of GS, GK and GS/GK overexpression on Glc-6-P levels

Hepatocytes were treated with AdCMV-RLGS (■), with AdCMV-GK (▲), with AdCMV-RLGS plus AdCMV-GK (●) or left untreated (□), as described in the Materials and methods section. After 16 h in DMEM, hepatocytes were incubated for 2 h with several glucose concentrations. Cells were then collected and intracellular Glc-6-P concentrations were measured. Data represent the means $\pm$ S.E.M. for eight independent experiments for untreated and AdCMV-RLGS-treated cells, or means $\pm$ S.E.M. for six independent experiments for AdCMV-GK and AdCMV-RLGS/GK infections. The letters indicate statistical significance at the following levels: a, $P < 0.01$; b, $P < 0.001$ for comparisons between the AdCMV-GK-treated cells and AdCMV-RLGS/GK-treated hepatocytes or the untreated control group.

Figure 5 Effects of GS, GK and GS/GK overexpression on UDP-Glc levels

Hepatocytes were treated with AdCMV-RLGS (filled bars), with AdCMV-GK (widely spaced hatched bars), with AdCMV-RLGS plus AdCMV-GK (closely spaced hatched bars) or left untreated (open bars), as described in the Materials and methods section. After 16 h in DMEM, hepatocytes were incubated for 2 h with several glucose concentrations. Cells were then collected and intracellular UDP-Glc concentrations were measured. Data represent the means $\pm$ S.E.M. for eight independent experiments for untreated and AdCMV-RLGS-treated cells, or means $\pm$ S.E.M. for six independent experiments for AdCMV-GK and AdCMV-RLGS/GK infections.

large amounts of glycogen in cells overexpressing liver GS occurred without affecting the intracellular concentration of these two metabolites. Glc-6-P increased in a glucose-dose-dependent manner (Figure 4), while UDP-Glc levels remained essentially constant at all glucose concentrations (Figure 5); however, no statistically significant differences in these levels

Figure 6 Effects of GS, GK and GS/GK overexpression on lactate production

Hepatocytes were treated with AdCMV-RLGS (filled bars), with AdCMV-GK (widely spaced hatched bars), with AdCMV-RLGS plus AdCMV-GK (closely spaced hatched bars) or left untreated (open bars), as described in the Materials and methods section. After 16 h in DMEM, hepatocytes were incubated for 2 h with several glucose concentrations. Media were then collected for lactate determination. Data represent the means $\pm$ S.E.M. for eight independent experiments for untreated and AdCMV-RLGS-treated cells, or means $\pm$ S.E.M. for six independent experiments for AdCMV-GK and AdCMV-RLGS/GK infections.

were observed between hepatocytes overexpressing RLGS and the corresponding controls.

The overexpression of GK alone in cultured hepatocytes resulted in an increase in Glc-6-P levels compared with untreated cells, as described in previous work [9]. No effect on UDP-Glc was observed.

When GK and GS were co-overexpressed, Glc-6-P and UDP-Glc levels were not significantly different from those of untreated hepatocytes (Figure 4) at any given glucose concentration.

Lactate production in RLGS-, GK- and RLGS/GK-overexpressing hepatocytes

To study whether the overexpression of GS affected the glycolytic rate of cultured hepatocytes, lactate production was measured. The amount of lactate released into the media increased with glucose concentration, but these values were not significantly different between adenovirus-treated and untreated cells (Figure 6).

The overexpression of GK alone and GK co-overexpressed with GS caused an increase in lactate production (Figure 6).

DISCUSSION

Here, we have studied the control of hepatic glycogen biosynthesis by glucose. It is generally accepted that GS is the enzyme that controls the rate-limiting step in this process. However, we have shown previously that GK overexpression strongly stimulates glycogen deposition in hepatocytes from both normal and diabetic rats. The present study shows that both enzymes are involved in the control of glycogen biosynthesis, in such a way that GK controls the GS activation state via Glc-6-P levels. The control mechanism can be explained as follows: the covalent GS activation is exerted through dephosphorylation of the enzyme; this dephosphorylation is catalysed by protein phosphatases, and is enhanced by Glc-6-P. This metabolite is thought to bind to

GS, and to cause a conformational change that activates the enzyme and renders it a better substrate for protein phosphatases [22].

GS overexpression alone leads to an increase in active GS, induced by endogenous GK activity, because more substrate is available for the Glc-6-P-dependent dephosphorylation of GS catalysed by phosphatases. When GK is overexpressed, the increase in Glc-6-P results in a higher activation of endogenous GS. When both enzymes are overexpressed, there is a combination of the two effects. Therefore the positive effect of Glc-6-P in glycogen deposition is not due to a 'push' that increases substrate concentration, because UDP-Glc levels are not significantly increased in GK-overexpressed cells. An increase in Glc-6-P produced by GK results in the dephosphorylation of GS molecules, and therefore in an increase in active GS, which in turn 'pulls' substrate into glycogen.

Our results confirm that the catalytic concentration of 'active' GS (in the I or a form) is the key factor in glycogen biosynthesis. When glucose is used as a substrate, this concentration is determined by the levels of both total GS and GK. In this context, it should be noted that the GS activity ratio is a valid indication of changes in the capacity of the cell to synthesize glycogen only when the amount of total GS is constant. When total GS is increased, as in AdCMV-RLGS-treated cells, a lower activity ratio may correspond to a higher GS 'I' activity. Therefore comparison of activity ratios between cells that contain different amounts of total GS may be misleading.

When GK is overexpressed in hepatocytes, both glycogen synthesis and glycolytic flux are markedly stimulated [9]. When GS is overexpressed, there is an increased flux through glycogen synthesis, but not through glycolysis. Moreover, intermediate metabolites between GK and GS, such as Glc-6-P and UDP-Glc, did not drop significantly during GS overexpression. Since GK activity is not inhibited by Glc-6-P, either directly or indirectly via the regulatory protein [23], flux through GK depends only on glucose concentration, and not on demand for Glc-6-P. Previous studies have shown that the free Glc-6-P pool of hepatocytes is increased by GK overexpression and decreased by glucose-6-phosphatase (G6Pase) overexpression [24]. A decrease in Glc-6-P (and UDP-Glc) during overexpression of GS might have been expected by analogy to the G6Pase studies [24].

A possible reason why this was not observed is that there may be channelling between Glc-6-P and GS. This would be consistent with the higher incorporation of [2-³H]glucose into glycogen with increased glucose concentration or GK translocation [25]. It would also be consistent with the hypothesis that Glc-6-P content is buffered primarily by G6Pase [23]. During overexpression of GS alone, the increased flux through glycogen synthesis may be compensated by a decreased flux through G6Pase. When GK and GS are overexpressed simultaneously, the increased flux through GK may be coupled, via channelling, to increased flux to GS, and this would explain why Glc-6-P does not accumulate. Furthermore, the measured levels of Glc-6-P are the same in GS-overexpressing cells as in hepatocytes overexpressing both GS and GK. Nevertheless, glycogen accumulation and, more interestingly, active GS levels are higher in the latter case. Once again, this suggests that there is compartmentalization and that the local Glc-6-P concentration 'sensed' by GS is higher in cells that simultaneously overexpress both enzymes.

The existence of Glc-6-P channelling was invoked to explain our previous observations that Glc-6-P produced by GK has a much higher impact on glycogen deposition than that produced by HK. Changes in the flux through GS in the absence of changes in Glc-6-P levels had previously been described in hepatocytes incubated in the presence of glutamine [26]. There is

also evidence in the literature for the compartmentalization of UDP-Glc [27].

Measurements from transgenic animals that overexpress a constitutively active form of rabbit muscle GS have shown that, in skeletal-muscle fibres, the control of glycogen synthesis is shared between glucose transport and GS [3,20]. In that study, the authors showed that muscle GS overexpression produced an increase in the glycogen content of various types of skeletal fibres. However, the levels of UDP-Glc substantially decreased as a result of the increased GS activity, and the lack of substrate limited glycogen accumulation. This situation clearly differs from that which occurs in hepatocytes. Glucose transport across the plasma membrane in hepatocytes is mainly carried out by GLUT-2, a low affinity, large capacity transporter [28]. Owing to the kinetic properties of this transporter, GLUT-2 essentially maintains intra- and extra-cellular glucose in equilibrium. Therefore, in the liver, glucose transport is not a controlling step, but rather it is GK that exerts the control [29].

In summary, we conclude that hepatic glycogen deposition from glucose is submitted to a system of control in which the 'controller', GS, is, in turn, controlled by GK. This system is different to phosphorylation cascades, in which an enzyme directly controls the activity of the following one in the series. In our system, the control is exerted indirectly via the level of Glc-6-P, which 'switches on' GS dephosphorylation, thus leading to its activation. Therefore Glc-6-P must be considered as both a precursor and a signal molecule to direct glucose incorporation into glycogen.

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REFERENCES

- Ferrannini, E., Bjorkman, O., Reichard Jr, G. A., Pilo, A., Olsson, M., Wahren, J. and DeFronzo, R. A. (1985) The disposal of an oral glucose load in healthy subjects. A quantitative study. *Diabetes* **34**, 580–588
- Roach, P. J., Cheng, C., Huang, D., Lin, A., Mu, J., Skurat, A. V., Wilson, W. and Zhai, L. (1998) Novel aspects of the regulation of glycogen storage. *J. Basic Clin. Physiol. Pharmacol.* **9**, 139–151
- Azpiaz, I., Manchester, J., Skurat, A. V., Roach, P. J. and Lawrence, J. C. (2000) Control of glycogen synthesis is shared between glucose transport and glycogen synthase in skeletal muscle fibers. *Am. J. Physiol. Endocrinol. Metab.* **278**, E234–E243
- Bai, G., Zhang, Z. J., Werner, R., Nuttall, F. Q., Tan, A. W. and Lee, E. Y. (1990) The primary structure of rat liver glycogen synthase deduced by cDNA cloning. Absence of phosphorylation sites 1a and 1b. *J. Biol. Chem.* **265**, 7843–7848
- Fernández-Novell, J. M., Bellido, D., Vilaró, S. and Guinovart, J. J. (1997) Glucose induces the translocation of glycogen synthase to the cell cortex in rat hepatocytes. *Biochem. J.* **321**, 227–231
- Ferrer, J. C., Baqué, S. and Guinovart, J. J. (1997) Muscle glycogen synthase translocates from the cell nucleus to the cytosol in response to glucose. *FEBS Lett.* **415**, 249–252
- Antinozzi, P. A., Berman, H. K., O'Doherty, R. M. and Newgard, C. B. (1999) Metabolic engineering with recombinant adenoviruses. *Annu. Rev. Nutr.* **19**, 511–544
- Becker, T., Noel, R., Coats, W. S., Gomez-Foix, A., Alam, T., Gerard, R. D. and Newgard, C. B. (1994) Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* **43**, 161–189
- Seoane, J., Gómez-Foix, A. M., O'Doherty, R. M., Gómez-Ara, C., Newgard, C. B. and Guinovart, J. J. (1996) Glucose 6-phosphate produced by glucokinase, but not hexokinase I, promotes the activation of hepatic glycogen synthase. *J. Biol. Chem.* **271**, 23756–23760
- Massagué, J. and Guinovart, J. J. (1977) Insulin control of rat hepatocyte glycogen synthase and phosphorylase in the absence of glucose. *FEBS Lett.* **82**, 317–320
- Chan, T. M. and Exton, J. H. (1976) A rapid method for the determination of glycogen content and radioactivity in small quantities of tissue or isolated hepatocytes. *Anal. Biochem.* **71**, 96–105
- Lang, G. and Michal, G. (1974) D-Glucose-6-phosphate and D-fructose-6-phosphate. In *Methods of Enzymatic Analysis*, vol. 3 (Bergmeyer, H. U., ed.), pp. 1238–1242, Academic Press, New York
- Keppeler, D. and Decker, K. (1974) Uridine-5'-diphosphoglucose. In *Methods of Enzymatic Analysis*, vol. 3 (Bergmeyer, H. U., ed.), pp. 2225–2228, Academic Press, New York
- Gutmann, I. and Wahlefeld, A. W. (1974) L-(+)-Lactate determination with lactate dehydrogenase and NAD. In *Methods of Enzymatic Analysis*, vol. 3 (Bergmeyer, H. U., ed.), pp. 1464–1468, Academic Press, New York
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
- Kuwajima, M., Newgard, C. B., Foster, D. W. and McGarry, J. D. (1986) The glucose-phosphorylating capacity of liver as measured by three independent assays. Implications for the mechanism of hepatic glycogen synthesis. *J. Biol. Chem.* **261**, 8849–8853
- Thomas, J. A., Schlender, K. K. and Larner, J. (1968) A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-¹⁴C-glucose. *Anal. Biochem.* **25**, 486–499
- Tobwin, H., Staehelin, T. and Gordon, J. (1992) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Bio/Technology* **24**, 145–149
- Baqué, S., Guinovart, J. J. and Gómez-Foix, A. M. (1996) Overexpression of muscle glycogen phosphorylase in cultured human muscle fibers causes increased glucose consumption and nonoxidative disposal. *J. Biol. Chem.* **271**, 2594–2598
- Manchester, J., Skurat, A. V., Roach, P., Hauschka, S. D. and Lawrence, J. C. (1996) Increased glycogen accumulation in transgenic mice overexpressing glycogen synthase in skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10707–10711
- Berman, H. K., O'Doherty, R. M., Anderson, P. and Newgard, C. B. (1998) Overexpression of protein targeting to glycogen (PTG) in rat hepatocytes causes profound activation of glycogen synthesis independent of normal hormone- and substrate-mediated regulatory mechanisms. *J. Biol. Chem.* **273**, 26421–26425
- Villar-Palasi, C. and Guinovart, J. J. (1997) The role of glucose 6-phosphate in the control of glycogen synthase. *FASEB J.* **7**, 554–558
- Aiston, S., Trinh, K. Y., Lange, A. J., Newgard, C. B. and Agius, L. (1999) Glucose-6-phosphatase overexpression lowers glucose 6-phosphate and inhibits glycogen synthesis and glycolysis in hepatocytes without affecting glucokinase translocation. Evidence against feedback inhibition of glucokinase. *J. Biol. Chem.* **274**, 24559–24566
- Seoane, J., Trinh, K., O'Doherty, R. M., Gómez-Foix, A. M., Lange, A. J., Newgard, C. B. and Guinovart, J. J. (1997) Metabolic impact of adenovirus-mediated overexpression of the glucose-6-phosphatase catalytic subunit in hepatocytes. *J. Biol. Chem.* **272**, 26972–26977
- Agius, L. and Peak, M. (1993) Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin. *Biochem. J.* **296**, 785–796
- Lavoine, A., Baquet, A. and Hue, L. (1987) Stimulation of glycogen synthesis and lipogenesis by glutamine in isolated rat hepatocytes. *Biochem. J.* **284**, 429–437
- Gustafson, L. A., Jumelle-Laclau, M. N., van Woerkom, G. M., van Kuilenburg, A. B. P. and Meijer, A. J. (1997) Cell swelling and glycogen metabolism in hepatocytes from fasted rats. *Biochim. Biophys. Acta* **1318**, 184–190
- Thorens, B., Cheng, Z. Q., Brown, D. and Lodish, H. F. (1990) Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. *Am. J. Physiol.* **259**, C279–C288
- O'Doherty, R. M., Lehman, D. L., Seoane, J., Gomez-Foix, A. M., Guinovart, J. J. and Newgard, C. B. (1996) Differential metabolic effects of adenovirus-mediated glucokinase and hexokinase I overexpression in rat primary hepatocytes. *J. Biol. Chem.* **271**, 20524–20530

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