Biochem. J. (1992) 288, 497–501 (Printed in Great Britain)

Role of glucose 6-phosphate in the translocation of glycogen synthase in rat hepatocytes

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Incubation of rat hepatocytes with glucose induces the translocation of glycogen synthase from soluble fractions to fractions which sediment at 10000 *g*. Incubation of the cells with fructose, galactose, 2-deoxyglucose or 5-thioglucose, which activate glycogen synthase, also resulted in the translocation of the enzyme, whereas 3-*O*-methylglucose, 6-deoxyglucose and 1,5-anhydroglucitol, which do not cause the activation of the enzyme, were ineffective. Adenosine and carbonyl cyanide *m*-chlorophenylhydrazone, although activating glycogen synthase, did not induce its translocation. Mannoheptulose, which inhibits glucose phosphorylation, impaired the translocation of glycogen synthase induced by glucose. Furthermore, the extent of the translocation of the enzyme triggered by glucose and other sugars showed a high positive correlation with the intracellular concentration of glucose 6-phosphate. Microcystin, which blocks the activation of glycogen synthase by glucose, but not the accumulation of glucose 6-phosphate, did not affect the translocation of the enzyme. These results indicate that glucose 6-phosphate plays a role in the translocation of glycogen synthase in rat hepatocytes.

INTRODUCTION

Glycogen synthase is the key enzyme in the control of glycogen metabolism. In rat hepatocytes, glucose induces the activation of the enzyme, which is reflected in an increase in the −glucose 6-phosphate/+glucose 6-phosphate activity ratio [1]. In a recent paper [2] we have reported that glucose, in addition to activating rat hepatocyte glycogen synthase, triggers changes in the intracellular distribution of this enzyme, measured as the amount both of total glycogen synthase activity and of immunoreactive enzyme sedimenting at about 10000 *g*. This translocation of glycogen synthase was shown to be independent of glycogen synthesis [2].

One of the working hypotheses derived from our previous experiments was that glycogen synthase translocation might be linked to the activation of the enzyme. In order to gain further insight into the mechanism by which glucose triggers these changes in the distribution of glycogen synthase in the cell, we performed a series of studies using other effectors that are able to modify the activation state of this enzyme. Our results suggest that translocation of glycogen synthase is not a necessary consequence of the activation of the enzyme, but is related to the intracellular levels of glucose 6-phosphate.

EXPERIMENTAL

Hepatocytes from 24 h-starved male Sprague–Dawley rats were prepared and incubated as previously described [2]. Fructose, galactose, 2-deoxyglucose, 6-deoxyglucose, 5-thioglucose, 3-*O*-methylglucose, 1,5-anhydroglucitol, glucose and mannoheptulose were dissolved in water at 300 mM. Adenosine and microcystin were dissolved in water at 10 mM and 10 μM respectively. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was dissolved in dimethyl sulphoxide at 5 mM (with a maximal final concentration of 1% dimethyl sulphoxide in the incubation medium). Various volumes of these solutions were added to the cell suspension to give the desired final concentrations. At the end of the incubations, cells were centrifuged (3000 *g*, 20 s) and the cell pellet was immediately homogenized in ice-cold buffer containing 10 mM-Tris/HCl (pH 7.0), 0.6 M-sucrose, 150 mM-KF, 15 mM-EDTA, 25 μg of leupeptin/ml, 0.5 mM-phenylmethanesulphonyl fluoride and 50 mM-β-mercaptoethanol, by using a Polytron homogenizer with a PT-7 rotor at setting 6 for 20 s. Homogenates were centrifuged at 9200 *g* for 15 min at 4 °C. Pellets were recovered and resuspended in 640 μl of the above buffer. Samples of both supernatants and pellets were taken and immediately used for glycogen synthase activity measurements or processed for SDS/PAGE.

Measurement of glycogen synthase activity, determination of protein concentration and immunoblot experiments were performed as described in [2]. To determine intracellular concentrations of glucose 6-phosphate, pellets from 2 ml of cell suspension were homogenized with $HClO_4$ (final concn. 0.87 M). Extracts were neutralized with 5 M-K_2CO_3 with the aid of universal indicator (Merck). Glucose 6-phosphate was determined enzymically with glucose-6-phosphate dehydrogenase [3].

Statistical significance of differences was assessed by Student's *t* test.

Suppliers

Fructose, galactose, 2-deoxyglucose, 6-deoxyglucose, 5-thioglucose, 3-*O*-methylglucose, 1,5-anhydroglucitol, glucose, mannoheptulose, adenosine, CCCP and microcystin were obtained from Sigma Chemical Co. The origin of the other reagents is given in [2].

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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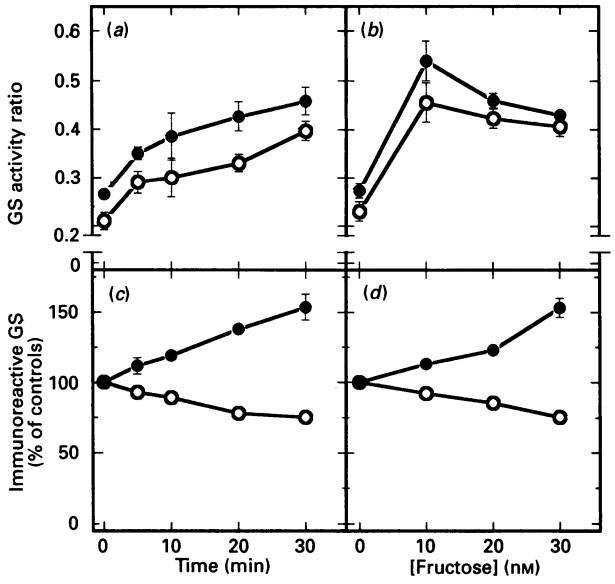

Fig. 1. Effects of fructose on the activation state and the translocation of glycogen synthase

Hepatocytes were incubated with 30 mM-fructose for different times (*a* and *c*) or with increasing concentrations of fructose for 30 min (*b* and *d*). After incubation, homogenates were prepared and 9200 *g* pellet (●) and supernatant (○) fractions were obtained. Glycogen synthase (GS) activity ratio (−glucose 6-phosphate/+glucose 6-phosphate) (*a* and *b*) and the amount of immunoreactive glycogen synthase (*c* and *d*) were measured. Data on immunoreactive glycogen synthase are expressed as percentages of the control, which corresponds to 100%. Results are means±S.D. for at least four independent experiments.

RESULTS AND DISCUSSION

Effect of different sugars on glycogen synthase translocation

Our laboratory has recently reported [2] that, in rat hepatocytes, glucose triggers the translocation of glycogen synthase from soluble fractions to fractions which sediment at relatively low centrifugal forces (about 10000 *g* for 15 min). As suggested by several lines of evidence, the translocation of glycogen synthase is not the result of glycogen accumulation.

The results obtained from our previous experiments raised two key questions: (1) Are these effects specific to glucose? and (2) is the activation of glycogen synthase a necessary requisite for its translocation? and if not, is there a factor responsible for the translocation of the enzyme? In this paper several experimental approaches addressing these questions are presented.

We first studied whether these effects could also be caused by other sugars. When hepatocytes were incubated for different times with increasing concentrations of fructose, a clear-cut activation of glycogen synthase in both supernatants and pellets was observed. At the same time, this enzyme was translocated from supernatants to 9200 *g* pellets. This phenomenon was observed both by measuring changes in the distribution of glycogen synthase activity and by determining immunoreactive protein in supernatant and 9200 *g* pellets by Western-blot analysis and scanning of autoradiograms (Fig. 1). Total specific activity (measured in the presence of glucose 6-phosphate) decreased in the supernatants from 7.2±0.1 to 6.5±0.1 m-units/mg of protein, whereas it rose in the pellets from 2.1±0.2 to 2.8±0.1 m-units/mg of protein. Galactose, 2-deoxyglucose and 5-thioglucose also produced the activation and the translocation of the enzyme, although they were less potent than glucose (Table 1). In contrast, when cells were incubated with 3-*O*-methylglucose, 6-deoxyglucose or 1,5-anhydroglucitol, which did not activate glycogen synthase, no translocation of this enzyme was observed (results not shown).

These experiments demonstrate that certain glucose analogues and other hexoses are able to trigger the translocation of glycogen synthase. Therefore, translocation is not specific to glucose. Interestingly, those agents that triggered the translocation of glycogen synthase were also able to elicit its activation [4,5], whereas those sugar derivatives which do not activate glycogen synthase [4] were also unable to trigger the translocation of the enzyme. These results support the idea that activation and translocation of glycogen synthase are closely related phenomena.

Effects of adenosine and CCCP on glycogen synthase translocation

Further information about the relationship between activation and translocation of synthase came from experiments performed with adenosine or the metabolic inhibitor CCCP. These agents are known to activate glycogen synthase in rat hepatocytes, presumably through an increase in the intracellular concentration of AMP, without modifying glucose 6-phosphate levels [6,7]. As shown in Fig. 2, although adenosine and CCCP activated glycogen synthase they did not induce changes in the intracellular

Table 1. Effects of different sugars on the activation state and the localization of glycogen synthase

Hepatocytes were incubated with the sugars indicated at 30 mM for 30 min. Homogenates were prepared and centrifuged at 9200 *g* for 15 min. Glycogen synthase activity ratio (−glucose 6-phosphate/+glucose 6-phosphate), total glycogen synthase specific activity (m-units/mg of protein) and immunoreactive protein (Immun. prot.; expressed as percentage of the control) were determined in supernatants and pellets. Results are means±S.D. for four to seven independent experiments: $^bP < 0.01$, $^cP < 0.001$ versus control.

		Supernatant			Pellet		
	Time (min)	Activity ratio	Sp. activity	Immun. prot.	Activity ratio	Sp. activity	Immun. prot.
Glucose	0	0.21±0.02	7.2±0.1	100	0.26±0.03	2.1±0.2	100
	30	0.47±0.03^c	6.1±0.1^c	65±4^c	0.56±0.03^c	3.3±0.2^c	203±6^c
Galactose	0	0.23±0.01	7.2±0.1	100	0.26±0.01	2.1±0.2	100
	30	0.38±0.02^c	6.6±0.1^c	78±4^c	0.39±0.03^c	2.7±0.1^c	140±5^b
2-Deoxyglucose	0	0.22±0.03	7.2±0.1	100	0.26±0.02	2.1±0.1	100
	30	0.41±0.03^c	6.4±0.1^c	72±4^c	0.41±0.03^c	2.8±0.1^c	146±9^b
5-Thioglucose	0	0.23±0.02	7.2±0.1	100	0.27±0.02	2.2±0.1	100
	30	0.45±0.03^c	6.5±0.1^c	75±5^c	0.45±0.03^c	2.8±0.1^c	148±8^b

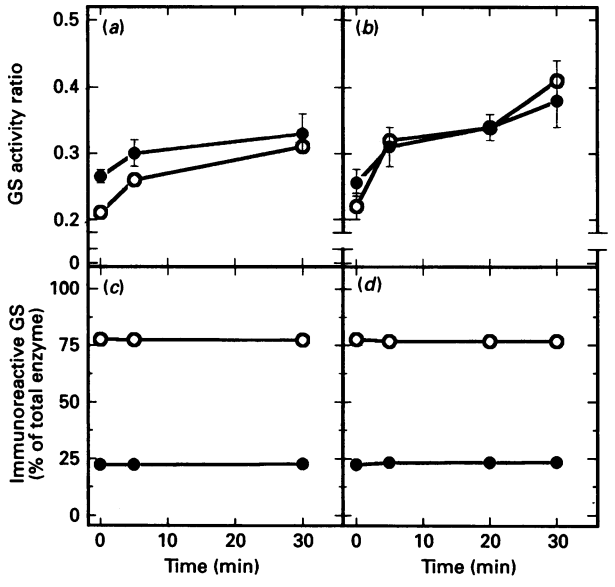

Fig. 2. Effects of adenosine and CCCP on the activation state and the translocation of glycogen synthase

Cells were incubated with 0.5 mM-adenosine (*a* and *c*) or 100 μM-CCCP (*b* and *d*) for different times. Glycogen synthase (GS) activity ratio (−glucose 6-phosphate/+glucose 6-phosphate) and the relative distribution of immunoreactive enzyme were determined in both 9200 *g* pellets (●) and supernatants (○). Results are means ± S.D. for three to eight independent experiments.

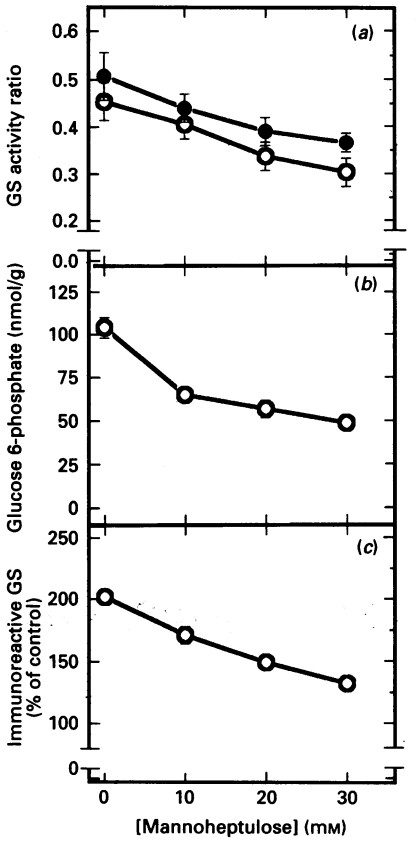

Fig. 3. Dose-dependent effects of mannoheptulose on the glucose-triggered translocation of glycogen synthase

Hepatocytes were incubated for 20 min with increasing concentrations of mannoheptulose and then with 30 mM-glucose for an additional period of 30 min. Panel (*a*) shows the activity ratio (−glucose 6-phosphate/+glucose 6-phosphate) of glycogen synthase (GS) in 9200 *g* pellets (●) and supernatants (○). Panel (*b*) shows the intracellular concentration of glucose 6-phosphate. Panel (*c*) displays the amount of immunoreactive protein in pellets, expressed as percentage gain over untreated cells. Results are means ± S.D. for three to five independent experiments.

localization of the enzyme. These results indicate that the translocation of the enzyme is not a necessary consequence of its activation and that, at least in some circumstances, activation and translocation are separate events.

Modification of the effects of glucose by mannoheptulose

The most striking feature shared by all sugars tested able to trigger the translocation of glycogen synthase was their ability to induce an increase in glucose 6-phosphate levels, such as glucose, fructose or galactose [5], or their ability to give phosphate esters at C-6, such as 2-deoxyglucose or 5-thioglucose [4]. Furthermore, the extension of the translocation showed strong positive correlation with the intracellular levels of glucose 6-phosphate achieved after incubation with different agents, suggesting that glucose phosphorylation could be involved in the mechanism of translocation of glycogen synthase.

To test this hypothesis, hepatocytes were incubated in the absence and in the presence of mannoheptulose, a powerful inhibitor of glucokinase [8], and then with glucose. As shown in Table 2, mannoheptulose alone did not modify glycogen synthase activity, its intracellular localization or the glucose 6-phosphate concentration. However, when the hepatocytes were incubated with glucose in the presence of mannoheptulose, both the activation and the translocation of glycogen synthase by glucose were decreased. Glucose 6-phosphate levels were also decreased in cells treated with mannoheptulose (Table 2). In another set of experiments, cells were incubated with increasing concentrations

Table 2. Modification by mannoheptulose of the effects of glucose on the translocation of glycogen synthase

Hepatocytes were incubated for 20 min in the absence or in the presence of 20 mM-mannoheptulose and then with 30 mM-glucose for a further 30 min. Results are means ± S.D. for four independent experiments: $^{b}P < 0.01$, $^{c}P < 0.001$ versus control. Abbreviation: G6P, glucose 6-phosphate.

Time (min)	Mannoheptulose	Activity ratio (−G6P/+G6P) Supernatant	Activity ratio (−G6P/+G6P) Pellet	G6P (nmol/g)	Immunoreactive protein in pellets
0	−	0.22 ± 0.01	0.28 ± 0.02	22 ± 1	100
	+	0.22 ± 0.01	0.28 ± 0.02	20 ± 1	102 ± 4
30	−	0.47 ± 0.03^{c}	0.54 ± 0.03^{c}	103 ± 6^{c}	203 ± 7^{c}
	+	0.35 ± 0.03^{c}	0.41 ± 0.03^{c}	58 ± 5^{b}	150 ± 6^{c}

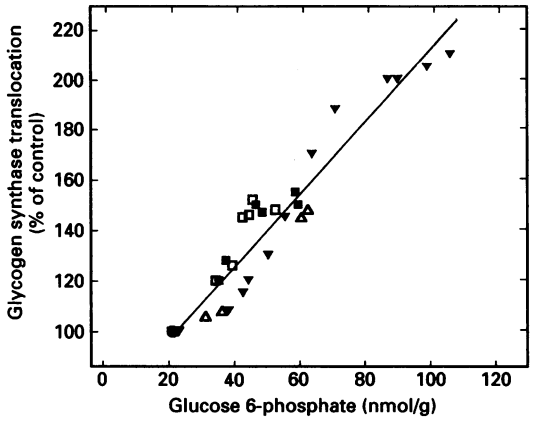

Fig. 4. Correlation between translocation of glycogen synthase and intracellular concentration of glucose 6-phosphate in rat hepatocytes

Cells were incubated with glucose (▼), glucose + mannoheptulose (△), fructose (□) or galactose (■) under different conditions (time and concentration). The intracellular concentration of glucose 6-phosphate was measured and plotted versus the corresponding amount of immunoreactive glycogen synthase found in 9200 *g* pellets (expressed as percentage of control cells). The correlation coefficient was $r = 0.95$. Points corresponding to cells incubated with 3-*O*-methylglucose (●), 6-deoxyglucose (○) and 1,5-anhydroglucitol (★) are clustered at the bottom of the line.

of mannoheptulose and then with 30 mM-glucose. As shown in Fig. 3, the activation state of glycogen synthase, the amount of immmunoreactive glycogen synthase present in the 9200 *g* pellets and the glucose 6-phosphate concentration progressively decreased in parallel as the concentration of mannoheptulose increased. However, preincubation with mannoheptulose did not modify the effects of fructose (results not shown). Mannoheptulose did not alter glucose 6-phosphate concentration in cells incubated with fructose (50 ± 4 nmol/g in cells incubated with mannoheptulose plus fructose, versus 51 ± 3 nmol/g in cells incubated with fructose alone). These results suggest that glucose phosphorylation may be involved in the mechanism of the glucose-triggered translocation of glycogen synthase.

The hypothesis that glucose 6-phosphate could play a role in the translocation of glycogen synthase was reinforced when the levels of this compound were measured in cells incubated under different conditions with various metabolizable sugars. When the glucose 6-phosphate levels were plotted against the amount of immunoreactive glycogen synthase present in the 9200 *g* pellets, a high positive linear correlation ($r = 0.95$) was found, which indicates a close relationship between the intracellular concentration of glucose 6-phosphate and the translocation of glycogen synthase (Fig. 4). However, since the activation state of glycogen synthase also changes in parallel with the levels of glucose 6-phosphate, it could still be argued that the decrease in translocation was a consequence of the lesser activation of the enzyme.

Modification of the effects of glucose by microcystin

In order to discern the relative roles of the activation state of glycogen synthase and glucose 6-phosphate levels in the translocation of the enzyme, cells were preincubated with or without microcystin, a powerful inhibitor of protein phosphatases [9], before glucose addition. Microcystin completely blocked the activation of glycogen synthase induced by glucose, as reported in [10], but did not alter the accumulation of glucose 6-phosphate. When the glucose-induced translocation of glycogen synthase was determined, no difference was observed between cells incubated in the presence or in the absence of microcystin (Table 3). These results indicate that, under conditions in which glucose 6-phosphate accumulation is not blocked, translocation of glycogen synthase may occur even without activation of the enzyme. It is then clear that the activation of glycogen synthase is not a requirement for the translocation of the enzyme and that an increase in glucose 6-phosphate levels is sufficient to provoke this event. Furthermore, adenosine and CCCP, which do not increase the concentration of glucose 6-phosphate, are not able to translocate glycogen synthase despite inducing the activation of the enzyme.

All the evidence so far obtained in our laboratory ([4,5,11]; the present paper) supports the hypothesis that glucose 6-phosphate is responsible for both the activation and the translocation of glycogen synthase. Glucose 6-phosphate would then be a key signal in the control of glycogen synthase activation state and intracellular localization in rat hepatocytes

We thank Ms. Catalina Relaño and Ms. Margarita Hernández for skilful technical assistance, Mr. Robin Rycroft for his excellent assistance in preparing the English manuscript and Dr. Carlos Ciudad for helpful suggestions. This work was supported by grant 91/0314 from the Fondo de Investigación Sanitaria (FIS).

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Table 3. Modification by microcystin of the effects of glucose on the translocation of glycogen synthase

Cells were incubated for 15 min in the absence or in the presence of 100 nM-microcystin and then with 30 mM-glucose for 30 min. Results are means ± S.D. for three independent experiments: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ versus control. Abbreviation: G6P, glucose 6-phosphate.

	Activity ratio (−G6P/+G6P)		Total sp. activity (m-units/mg of protein)		G6P (nmol/g)
	Supernatant	Pellet	Supernatant	Pellet	
Control	0.21 ± 0.03	0.30 ± 0.04	7.5 ± 0.3	2.4 ± 0.2	19 ± 2
Microcystin	0.16 ± 0.03	0.29 ± 0.02	7.3 ± 0.5	2.0 ± 0.3	15 ± 2
Glucose	0.44 ± 0.04^{c}	0.52 ± 0.04^{c}	6.1 ± 0.4^{b}	3.1 ± 0.3^{b}	103 ± 10^{c}
Microcystin + glucose	0.17 ± 0.03	0.32 ± 0.02	6.2 ± 0.6^{a}	2.9 ± 0.3^{b}	106 ± 12^{c}

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Received 16 March 1992/26 May 1992; accepted 2 June 1992