Glucose induces the translocation and the aggregation of glycogen synthase in rat hepatocytes

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Incubation of rat hepatocytes with glucose results in a decrease in the amount of glycogen synthase activity found in supernatants obtained after centrifugation of cell homogenates at 9200 *g*. The enzymic activity was quantitatively recovered in the sediments. This effect of translocation was dose- and time-dependent and correlated with the amount of immunoreactive enzyme determined by immunoblotting in both fractions. Hydrolysis by α -amylase of glycogen accumulated upon incubation with the sugar did not affect the translocation pattern. Translocation was also observed when cells were incubated with 2-deoxyglucose, which did not result in accumulation of glycogen. Immunocytochemical evidence indicates that glucose induces the aggregation of glycogen synthase molecules into clusters which are recovered in the sediments. These results indicate that glucose, in addition to activating glycogen synthase, may trigger changes in the localization of the enzyme in the cell.

INTRODUCTION

Glycogen synthase is the key enzyme in the control of glycogen synthesis. Its activity is regulated by phosphorylation/dephosphorylation reactions affecting multiple sites. Dephosphorylated forms are physiologically active, whereas the more phosphorylated forms are inactive. However, the activity of phosphorylated forms can be measured *in vitro* in the presence of high concentrations of the allosteric activator, glucose 6-phosphate. Therefore, changes in the activation state of the enzyme are reflected in the –glucose 6-phosphate/+glucose 6-phosphate activity ratio, whereas the activity measured in the presence of elevated concentrations of glucose 6-phosphate expresses the total activity of the enzyme [1,2].

Glycogen synthase has a high affinity for glycogen [3]. In fed rats the hepatic enzyme can be recovered in the particulate fraction sedimenting after centrifugation at 100 000 *g* for about 1 h, whereas in fasted rats the enzyme is mostly found in the cytosolic fraction. However, early differential-centrifugation studies performed in rat liver [4] demonstrated that a significant proportion of the enzyme activity can be found in the so-called ‘mitochondrial pellet’, that is, a fraction that sediments when centrifuged for 10 min at around 13 000 *g*. In spite of that observation, glycogen synthase activity found in those pellets has been largely ignored, and experiments involving determination of glycogen synthase activity, either in hepatocytes or in whole liver, have often been performed in supernatants obtained after centrifugation of homogenates for 10–15 min at relative centrifugal forces ranging from 8000 to 15 000 *g*.

In rat hepatocytes it has been demonstrated that changes in the activation state of the enzyme induced by different hormones correlate with the phosphorylation state of the enzyme subunit [5,6]. Since glucose induces the activation of hepatic glycogen synthase [7–10], we proceeded to incubate hepatocytes from fasted rats with glucose in order to test whether or not the increase in the activity ratio of the enzyme was accompanied by its dephosphorylation. Surprisingly, we found that the amount of immunoprecipitable enzyme from cytosolic supernatants was lower in glucose-treated cells than in control cells. As a result of

this observation we considered the possibility that glucose, in addition to its well-known ability to activate glycogen synthase, could induce some kind of redistribution of this enzyme inside the cell.

In this paper we present evidence indicating that the amount of glycogen synthase that sediments in the ‘mitochondrial fraction’ is increased upon incubation of the hepatocytes with glucose, indicating that glucose may trigger the translocation of this enzyme. Furthermore, incubation with glucose induces the aggregation of synthase molecules into clusters, suggesting that the translocation of the enzyme may be a consequence of its aggregation.

MATERIALS AND METHODS

Preparation of hepatocyte homogenates

Hepatocyte suspensions were prepared from 24 h-starved male Sprague–Dawley rats as previously described [11]. Cells were resuspended in Krebs–Ringer bicarbonate buffer (pH 7.4). Samples (4 ml) of these suspensions, containing $(6\text{--}8) \times 10^6$ cells/ml, were incubated at 37 °C with gassing and continuous shaking (100 strokes/min). Glucose and 2-deoxyglucose were dissolved in water at 300 mM. Variable 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 320 μ l of ice-cold buffer containing 10 mM-Tris/HCl (pH 7.0), 0.6 M-sucrose, 150 mM-KF, 15 mM-EDTA, 25 μ g 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* (unless otherwise stated) for 15 min at 4 °C. Pellets were recovered and resuspended in 640 μ l 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.

Glycogen synthase activity measurement

Glycogen synthase activity was measured at 30 °C as described

Abbreviation used: PBS, phosphate-buffered saline.

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Table 1. Time-dependent effects of glucose on the localization of glycogen synthase activity

Hepatocytes were incubated with 30 mM-glucose for different times. Homogenates were prepared and centrifuged at 9200 *g* for 15 min. Glycogen synthase activity in the presence and absence of 6.6 mM-glucose 6-phosphate (G6P) was measured in supernatants and pellets. Results are means $\pm$ S.D. for 7–20 independent experiments: **P* < 0.05, °*P* < 0.001 versus control at zero time.

Cells	Time (min)	Activity ratio (– G6P/+ G6P)		Total activity (m-units/mg of protein)	
		Supernatant	Pellet	Supernatant	Pellet
Control	0	0.21 $\pm$ 0.02	0.26 $\pm$ 0.03	7.2 $\pm$ 0.1	2.1 $\pm$ 0.2
	30	0.20 $\pm$ 0.02	0.26 $\pm$ 0.03	7.2 $\pm$ 0.1	2.1 $\pm$ 0.1
30 mM-Glucose	5	0.33 $\pm$ 0.03°	0.46 $\pm$ 0.03°	7.1 $\pm$ 0.1 ^a	2.3 $\pm$ 0.1 ^a
	10	0.35 $\pm$ 0.04°	0.50 $\pm$ 0.04°	6.8 $\pm$ 0.1°	2.7 $\pm$ 0.2°
	20	0.42 $\pm$ 0.04°	0.53 $\pm$ 0.04°	6.4 $\pm$ 0.1°	3.0 $\pm$ 0.2°
	30	0.47 $\pm$ 0.03°	0.56 $\pm$ 0.03°	6.1 $\pm$ 0.1°	3.3 $\pm$ 0.2°

in [12]. One unit of enzyme activity is the amount of enzyme that incorporates 1 μ mol of [¹⁴C]glucose from UDP-[¹⁴C]glucose into glycogen/min. Glycogen synthase activity ratio was determined by measuring the activity in the absence and in the presence of 6.6 mM-glucose 6-phosphate in the reaction mixture.

Other determinations

Glycogen was measured as follows: 0.25 ml of 10% (w/v) HClO₄ was added to 0.1 ml of homogenate, and the mixture was centrifuged (25000 *g* for 15 min). The acid supernatant was adjusted to pH 5 with K₂CO₃. Glycogen was converted into glucose by incubation with α -amylglucosidase and the resulting sugar was measured with a Gluco-quant kit (Boehringer Mannheim). Protein concentration was measured by the biuret method as described in [13], or by Bradford's method [14] with bovine serum albumin as standard.

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

Immunochemical and immunocytochemical techniques

Immunoblot experiments were performed as follows. Cell homogenates were centrifuged and pellets resuspended as described above. Samples were taken and immediately boiled in the presence of 25 mM-Tris/HCl (pH 6.8), 2.5% (v/v) β -mercaptoethanol and 1% (w/v) SDS. Samples were electrophoresed in 10%-polyacrylamide slab gels in the presence of SDS [15] and transferred to membranes (Immobilon PVDF, Millipore) as described previously [16,17]. Membranes were incubated for 1 h at 4 °C with a polyclonal antibody raised in rabbits against homogeneous rat liver glycogen synthase [5]. Immunoreactive proteins were detected after autoradiography by using ¹²⁵I-protein G. The amount of radioactive protein was quantified by scanning the developed films.

For immuno-electron microscopy, the procedure of Griffiths [18] was followed. Isolated hepatocytes or sediments obtained by centrifugation of homogenates at 9200 *g* for 15 min were fixed in 2% paraformaldehyde/0.1% glutaraldehyde in 200 mM-Hepes (pH 7.4) and kept in that solution at 4 °C. For cryosectioning, cells or sediments were placed in 2.1 M-sucrose in phosphate-buffered saline (PBS; 100 mM-sodium phosphate/154 mM-NaCl, pH 7.4) for 15 min, mounted on the specimen holder and frozen in liquid nitrogen. Cryo-ultrasections were obtained in a Reichert-Jung ultramicrotome. Sections were retrieved with a copper loop containing 2.3 M-sucrose in PBS, transferred to a 100-mesh grid with forward film and carbon-coated and placed in PBS until they were processed for immunodetection.

For the labelling procedure, grids were incubated with 1%

ovalbumin in PBS containing 200 mM-glycine for 10 min. Then grids were incubated with the antibody solution [anti-(glycogen synthase) diluted 1/50 with 1% ovalbumin/200 mM-glycine in PBS] for 30 min at room temperature, then washed four times in pools of PBS/200 mM-glycine for 10 min. Afterwards, grids were incubated in Protein A-gold (16 nm) diluted in 5% (v/v) foetal-calf serum/200 mM-glycine in PBS for 20 min, rinsed four times in PBS for 10 min and with distilled water six times for 20 min (total) and contrasted in 0.3% uranyl acetate in methylcellulose for 10 min on ice. Then the grids were removed with a copper loop, and the excess of fluid was removed on a filter paper.

Materials

Glucose, 2-deoxyglucose, glucose 6-phosphate, rabbit liver glycogen, UDP-glucose, α -amylglucosidase and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. Analytical-grade reagents came from Merck. Collagenase and Gluco-quant were purchased from Boehringer Mannheim. Protein A-Gold, ¹²⁵I-protein G, [U-¹⁴C]glucose and 2-deoxy[U-¹⁴C]glucose were obtained from Amersham. UDP-[U-¹⁴C]glucose was prepared by the method described in [19].

RESULTS

Effects of glucose on the localization of glycogen synthase activity in hepatocytes

Hepatocytes were incubated for different times with 30 mM-glucose. Homogenates were prepared and centrifuged at 9200 *g* for 15 min, and glycogen synthase activity was measured in pellets and supernatants in the presence (+) and absence (–) of glucose 6-phosphate. Glycogen synthase – glucose 6-phosphate/+ glucose 6-phosphate activity ratio was, as expected, increased by glucose in the supernatants and, to approximately the same degree, in the pellets. In control cells, about 25% of total glycogen synthase activity present in the homogenates was recovered in the pellets, whereas approx. 75% was found in the supernatants. However, upon incubation with 30 mM-glucose, total glycogen synthase activity was found to decrease in supernatants in a time-dependent manner, and the activity lost in the supernatants was quantitatively recovered in the pellets (Table 1). The effect of glucose on the distribution of total glycogen synthase activity in the pellets and supernatants was also dose-dependent. When hepatocytes were incubated for 30 min with increasing concentrations of the sugar (Table 2), changes were already detected at 10 mM-glucose. These results indicate that,

Table 2. Dose-dependent effects of glucose in the localization of glycogen synthase activity

Hepatocytes were incubated for 30 min with different concentrations of glucose, and homogenates were prepared and centrifuged at 9200 *g* for 15 min. Glycogen synthase activity was measured. Results are means $\pm$ S.D. for 3–9 independent experiments: ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 versus control.

Glucose (mM)	Activity ratio (–G6P/ +G6P)		Total activity (m-units/mg of protein)	
	Supernatant	Pellet	Supernatant	Pellet
0	0.20 $\pm$ 0.02	0.25 $\pm$ 0.03	7.2 $\pm$ 0.1	2.1 $\pm$ 0.1
5	0.21 $\pm$ 0.03	0.26 $\pm$ 0.03	7.1 $\pm$ 0.1 ^a	2.1 $\pm$ 0.1
10	0.25 $\pm$ 0.02 ^a	0.30 $\pm$ 0.03 ^a	6.9 $\pm$ 0.1 ^b	2.4 $\pm$ 0.1 ^b
20	0.37 $\pm$ 0.03 ^c	0.43 $\pm$ 0.03 ^c	6.5 $\pm$ 0.1 ^c	2.9 $\pm$ 0.1 ^c
30	0.47 $\pm$ 0.03 ^c	0.55 $\pm$ 0.03 ^c	6.1 $\pm$ 0.1 ^c	3.3 $\pm$ 0.1 ^c

Fig. 1. Time-dependent effect of glucose on the localization of immunoreactive glycogen synthase

Hepatocytes were incubated with 30 mM-glucose for different periods of time. Homogenates were prepared and centrifuged at 9200 *g* for 15 min. Immunoreactive glycogen synthase from pellets (●), supernatants (○) and whole homogenates (▽) was detected and quantified. Data are presented as percentages of the amount of immunoreactive glycogen synthase in control cells and are means $\pm$ S.D. for 7–17 independent experiments. Inserts show the immunoblots corresponding to the pellets (A) and supernatants (B) from a representative experiment.

after 30 min of incubation with 30 mM-glucose, total glycogen synthase in the pellets increased by about 55%.

Changes in localization of immunoreactive glycogen synthase correlate with changes in enzyme activity

Since glycogen synthase activity measured in the presence of glucose 6-phosphate reflects the total amount of enzyme, we considered the possibility that the observed changes could also be detected by immunochemical techniques. For that reason, immunoblot experiments using a polyclonal antibody raised against homogeneous rat liver glycogen synthase were performed.

Fig. 2. Dose-dependent effect of glucose on the localization of immunoreactive glycogen synthase

Hepatocytes were incubated for 30 min with different concentrations of glucose. Conditions were identical with those described in the legend of Fig. 1. Results are means $\pm$ S.D. from five experiments performed on different days. Inserts show the immunoblots corresponding to the pellets (A) and supernatants (B) from a representative experiment.

Table 3. Distribution of glycogen synthase after centrifugation of homogenates at different relative centrifugal forces

Hepatocytes were incubated with 30 mM-glucose for 30 min and homogenates were prepared. Samples were centrifuged at the indicated relative centrifugal force (RCF) for 15 min, and total glycogen synthase activity (expressed as m-units/mg of protein) and immunoreactive protein were determined in supernatants and pellets. Data for immunoreactive glycogen synthase are expressed as percentages of the control and are shown in parentheses. Results are means $\pm$ S.D. for 4–7 independent experiments: ^c*P* < 0.001 versus control.

RCF	Supernatant		Pellet	
	Control	Glucose	Control	Glucose
2000 <i>g</i>	7.2 $\pm$ 0.1 (100)	7.2 $\pm$ 0.2 (101 $\pm$ 2)	2.2 $\pm$ 0.1 (100)	2.2 $\pm$ 0.1 (100 $\pm$ 2)
9200 <i>g</i>	7.2 $\pm$ 0.1 (100)	6.1 $\pm$ 0.1 ^c (67 $\pm$ 4) ^c	2.1 $\pm$ 0.1 (100)	3.3 $\pm$ 0.2 ^c (208 $\pm$ 8) ^c
23000 <i>g</i>	7.1 $\pm$ 0.1 (100)	6.0 $\pm$ 0.2 ^c (63 $\pm$ 5) ^c	2.1 $\pm$ 0.2 (100)	3.4 $\pm$ 0.2 ^c (230 $\pm$ 7) ^c

As shown in Fig. 1, incubation of rat hepatocytes with 30 mM-glucose for different times results in a decrease in the amount of immunoreactive enzyme in 9200 *g* supernatants. Concomitantly, an increase in the amount of glycogen synthase detected by the antibodies was found in the pellets. The amount of immunoreactive material in the homogenates (that is, in samples taken before centrifugation) remained unchanged. Dose-response experiments were performed and showed that changes in the location of glycogen synthase were only significant at concentrations above 10 mM-glucose (Fig. 2). This fact is in agreement with the activity data shown in Table 2. Therefore, the results

Table 4. Effects of glycogen depletion by α -amylase in the localization of glycogen synthase

Hepatocytes were incubated with 30 mM-glucose for different times, and homogenates were prepared. Samples of the homogenates were incubated with α -amylase or with buffer. Then samples were centrifuged at 9200 g for 15 min, and glycogen and immunoreactive glycogen synthase were determined in the supernatants and pellets. Immunoreactive glycogen synthase values are presented as percentages of control cells. Glycogen is expressed in mg/g wet wt. of cells (n.d., not determined). Results are means $\pm$ s.d. for 3–5 independent experiments. Immunoreactive glycogen synthase values in supernatants and pellets were not significantly altered by α -amylase treatment of homogenates.

Time (min)	α -Amylase ...	Immunoreactive glycogen synthase (%)			
		Supernatant		Pellet	
		–	+	–	+
0		100	100	100	100
5		98 $\pm$ 2	97 $\pm$ 3	125 $\pm$ 7	121 $\pm$ 5
20		76 $\pm$ 1	76 $\pm$ 2	181 $\pm$ 2	183 $\pm$ 7
30		67 $\pm$ 2	68 $\pm$ 2	202 $\pm$ 6	203 $\pm$ 7

Time (min)	α -Amylase ...	Glycogen			
		Supernatant		Pellet	
		–	+	–	+
0		1.6 $\pm$ 0.1	1.3 $\pm$ 0.1	1.2 $\pm$ 0.2	1.0 $\pm$ 0.1
5		2.0 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1
20		(n.d.)	(n.d.)	(n.d.)	(n.d.)
30		3.4 $\pm$ 0.3	1.3 $\pm$ 0.1	1.4 $\pm$ 0.2	1.0 $\pm$ 0.1

presented suggest that glucose triggers the translocation of glycogen synthase from locations recovered in 9200 g supernatants to pelletable fractions.

In order to gain insight into this effect, we repeated these experiments at different relative centrifugation forces. Table 3 shows that translocation of glycogen synthase activity was not detected after centrifugation for 15 min at 2000 g. The changes observed upon centrifugation at 23 000 g for 15 min were quantitatively similar to those observed at 9200 g for 15 min. The same results were obtained from immunoblot experiments (Table 3).

Translocation of glycogen synthase is not a result of glycogen synthesis

Since it is known that glycogen synthase binds to glycogen and that incubation of hepatocytes with glucose leads to glycogen synthesis, it was logical to assume that the increased amount of enzyme recovered in the pellets might be a result of the sedimentation of a complex between the enzyme and the polysaccharide synthesized *de novo*. However, when hepatocytes were incubated with glucose, glycogen accumulated mainly in the 9200 g supernatants (Table 4), whereas glycogen synthase decreased in the supernatants and increased in the pellets (Fig. 1 and Table 1), thus suggesting that translocation of glycogen synthase could be unrelated to the synthesis of glycogen.

To study further the role of glycogen, two different sets of experiments were performed. First, hepatocytes were incubated with 30 mM-glucose for different times, and homogenates prepared and divided in two. One aliquot was incubated with α -amylase, whereas the second was used as a reference. Conditions

Fig. 3. Time-dependent effect of 2-deoxyglucose on the localization of immunoreactive glycogen synthase

Hepatocytes were incubated with 30 mM-2-deoxyglucose for different periods of time. Conditions were as described in the legend of Fig. 1. Results are means $\pm$ s.d. from four experiments performed on different days.

Fig. 4. Immunolocalization of glycogen synthase on hepatocyte cryo-sections

After incubation, cells were fixed and processed for cryo-ultramicrotomy and immuno-electron microscopy. Glycogen synthase on hepatocytes incubated for 30 min with 30 mM-glucose (a) and control hepatocytes (b) was immunolocalized with anti-(glycogen synthase) antibody and Protein A–gold particles (arrows). Insert in (a) shows a magnification of the clusters. Abbreviations: m, mitochondria; n, nucleus; re, rough endoplasmic reticulum. Bars: 0.25 μ m.

Fig. 5. Immunolocalization of glycogen synthase on cryosections from 9200 g pellets

Pellets obtained by centrifugation of homogenates at 9200 *g* for 15 min were fixed and processed. Glycogen synthase was immunolocalized in pellets from hepatocytes incubated for 30 min with 30 mM-glucose (a) and in pellets from control hepatocytes (b). Insert in (a) shows a magnification of the clusters. Bars: 0.15 μ m.

were established to ensure that after digestion with α -amylase the concentration of glycogen in homogenates obtained from cells incubated with glucose was as low as that measured in control cells. After incubation with the enzyme, samples were centrifuged at 9200 *g* for 15 min, and the amounts of glycogen and of immunoreactive glycogen synthase were measured in pellets and supernatants. As shown in Table 4, depletion of glycogen from both supernatants and pellets did not impair the translocation of the enzyme.

A second line of evidence suggesting that glycogen synthesis is not responsible for the translocation of glycogen synthase was obtained from experiments where hepatocytes were incubated with 2-deoxyglucose. It is known that this sugar is phosphorylated [20] but is not further metabolized [21]. Nonetheless, incubation of the cells with 30 mM-2-deoxyglucose for different times resulted in a translocation of the enzyme in a way similar to that observed with glucose (Fig. 3). Under these conditions we did not detect incorporation of 2-deoxy[U- 14 C]glucose into glycogen (results not shown). Therefore, translocation of glycogen synthase does not appear to be related to the biosynthesis of glycogen.

Incubation of hepatocytes with glucose results in aggregation of glycogen synthase into clusters

It is generally accepted that glycogen synthase is mostly found as a cytosolic protein in the liver of fasted rats, suggesting that the enzyme is not bound to any cellular organelle. The discovery

that glucose allows an increased recovery of the enzyme in low-speed pellets prompted us to undertake immunocytochemical studies in order to understand the mechanisms of the translocation of the enzyme. Immunolocalization of glycogen synthase in cryosections of control hepatocytes shows that the enzyme is scattered in the cytoplasm. The same experiment performed with hepatocytes incubated with 30 mM-glucose for 30 min shows that the immunoreactive protein aggregates into clusters (Fig. 4). Evidence that the clusters are recovered after centrifugation at 9200 *g* for 15 min is given in Fig. 5. As shown, pellets obtained from control hepatocytes contain randomly scattered glycogen synthase. On the contrary, clusters similar to those observed in glucose-incubated hepatocyte cryosections can be observed in preparations of pellets from glucose-treated cells.

DISCUSSION

In this paper we report the effect of glucose on the intracellular localization of glycogen synthase in rat hepatocytes. We show that glucose is able to promote changes in the distribution of the enzyme activity from soluble to fractions which sediment at about 10000 *g*.

The effect of glucose on the activation state of hepatic glycogen synthase has been known for many years [7]. Although some experiments aimed to study the control of this enzyme by sugars or hormones have been performed by measuring the activity of the enzyme in whole homogenates [6,9,21], often supernatants obtained by centrifugation of homogenates at approx. 10000 *g* have been used. Although it has been known since 1968 that more than 20% of the enzyme activity is not recovered in supernatants obtained from livers of 24 h-fasted rats [4], the presence of a significant amount of enzyme activity in the pellets has been systematically disregarded. Furthermore, most work has been done by determining the ratio of activities in the presence and in the absence of glucose 6-phosphate, instead of measuring the specific activity of the enzyme. These facts may explain why the phenomena described in the present paper have not been observed previously.

The observed changes in glycogen synthase activity in the two fractions studied do not originate from an artifact of the method used to measure the activity of this enzyme, but they correspond to actual changes in the amount of protein, as determined by immunoblot experiments. Accordingly, the changes in the localization of glycogen synthase determined by enzymic measurements or immunochemical techniques are quantitatively very similar. Moreover, the increases in activity and immunoreactive protein observed in the pellets correspond very closely to the decreases determined in the supernatant fractions. Furthermore, there is no change in the total amount of immunoreactive protein when measured in whole homogenates. These facts indicate that the increments in glycogen synthase observed in the pellets are due not to a newly synthesized protein but instead to the glucose-triggered translocation of pre-existing enzyme molecules.

The translocation produced by glucose is both time- and concentration-dependent. Comparison between changes in the activation state of glycogen synthase (Fig. 2) and translocation of the enzyme (Table 2) shows a strong parallelism, suggesting that glycogen synthase translocation might be linked to the activation of the enzyme.

Since glucose triggers the activation of glycogen synthase [22] and the accumulation of glycogen [10,23], one might argue that the newly synthesized glycogen is responsible for the observed translocation. The fact that glycogen synthase binds to the polysaccharide [3] could support this hypothesis. However, we present several lines of evidence suggesting that translocation is independent of the synthesis of glycogen: (1) upon incubation

with glucose, newly synthesized glycogen is recovered essentially in the 9200 *g* supernatants, whereas glycogen synthase is enriched in the pellets; (2) translocation is still detected after depletion of newly synthesized glycogen by α -amylase in extracts obtained from glucose-treated cells; and (3) it is observed after incubation of the cells with 2-deoxyglucose, which does not result in glycogen accumulation.

The experiments performed at different centrifugal forces indicate that translocation is not detected when homogenates are centrifuged for 15 min at 2000 *g*, but the phenomenon is quantitatively very similar after centrifugation for 15 min at 9000 *g* or 23000 *g*. The observation points to the fact that the enzyme has to be bound to structures able to sediment at quite low relative centrifugal forces. An important conclusion from this observation is that previous experiments on the effect of glucose on the phosphorylation state of rat liver glycogen synthase must be reconsidered. Thus it has been reported [24] that upon incubation of ³²P-labelled hepatocytes from fasted rats with 30 mM-glucose the amount of radioactive phosphate incorporated into glycogen synthase was decreased by about 60 %. Since in these experiments glycogen synthase was immunoprecipitated from cytosolic supernatants, it is very likely that the decrease in phosphate content observed could reflect, at least in part, a decreased amount of enzyme in supernatants obtained from glucose-treated hepatocytes as compared with control cells.

We performed immunocytochemical studies using electron microscopy in order to determine a possible association of the enzyme to subcellular organelles. Our experiments show that in control cells the enzyme is scattered through the cytoplasm, apparently in a random way, and that glucose induces the formation of clusters of immunoreactive protein. However, these clusters, which are also observed when pellets from glucose-treated cells are examined, do not seem to be associated with any subcellular organelles. In any case, it is very unlikely that the aggregates of glycogen synthase by themselves would have mass enough to sediment at the relatively low centrifugal forces used in our study. Therefore, the possibility that glycogen synthase could be associated with subcellular structures such as the cytoskeleton has to be considered. In this context, it is noteworthy that some enzymes involved in the glycolytic pathway are associated with microfilaments [25,26].

Although the understanding of the physiological significance of the phenomena described in this paper requires further studies, an attractive hypothesis would be that the activation of glycogen synthase triggers the formation of clusters which are bound to

subcellular structures. In the light of this hypothesis, translocation of glycogen synthase would be a consequence of the aggregation of the enzyme into clusters. The association of glycogen synthase molecules into clusters may constitute an early step in the process of hepatic glycogen biosynthesis.

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