# Intracellular distribution of glycogen synthase and glycogen in primary cultured rat hepatocytes

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Changes in the intracellular distribution of liver glycogen synthase (GS) might constitute a new regulatory mechanism for the activity of this enzyme at cellular level. Our previous studies indicated that incubation of isolated hepatocytes with glucose activated GS and resulted in its translocation from a homogeneous cytosolic distribution to the cell periphery. These studies also suggested a relationship with insoluble elements of the cytoskeleton, in particular actin. Here we show the translocation of GS in a different experimental model that allows the analysis of this phenomenon in long-term studies. We describe the reversibility of translocation of GS and its effect on glycogen distribution. Incubation of cultured rat hepatocytes with glucose activated GS and triggered its translocation to the hepatocyte periphery. The relative amount of the enzyme concentrated near the plasma membrane increased with time up to 8 h of incubation with glucose, when the glycogen stores reached their maximal value. The lithium-induced covalent activation of GS was not sufficient to cause its translocation to the cell periphery. The intracellular distribution of GS closely resembled that of glycogen. Our results showed an interaction between GS and an insoluble element of the hepatocyte matrix. Although no co-localization between actin filaments and GS was observed in any condition, disruption of actin cytoskeleton resulted in a significantly lower percentage of cells in which the enzyme translocated to the cell periphery in response to glucose. This observation suggests that the microfilament network has a role in the translocation of GS.

Key words: confocal microscopy, cytoskeleton, immunofluorescence, translocation.

## INTRODUCTION

Glycogen synthase (GS) is the enzyme responsible for the synthesis of $\alpha$-1,4-linked glucose chains in glycogen. It is the rate-limiting enzyme in the synthesis of the polysaccharide, and its activity is highly regulated through phosphorylation at multiple sites and also by allosteric effectors, mainly glucose 6-phosphate (G6P). However, only the dephosphorylated forms are active *in vivo* [1,2]. It is well established that glucose causes a concentration- and time-dependent covalent activation of GS in liver and isolated rat hepatocytes [3–5]. Activation of GS can also be achieved through incubation of the cells with LiCl, presumably owing to the dephosphorylation of some specific sites of the enzyme [6]. In experimental animals, oral administration of $Li_2CO_3$ also results in the covalent activation of hepatic GS [7].

The cytoskeleton has also been implicated in the modulation of glycogen synthesis in liver [8] and muscle [9]. Drugs such as cytochalasins B and D are potent inhibitors of glycogen synthesis in cultured hepatocytes. It was suggested that the mechanism by which such inhibition was exerted implied actin depolymerization and that the microfilament network had a role in the control of glycogen synthesis [8].

We have previously reported that, besides its effect on the activation state of GS, glucose also triggers changes in the intracellular distribution of this enzyme in isolated hepatocytes. In response to glucose, GS translocates from a soluble fraction to a fraction that sediments at relatively low centrifugal forces [10–12]. More recently we have shown that incubation of isolated rat hepatocytes with glucose causes GS to be concentrated near the cell periphery, where a marked increase in the co-localization with cortical actin filaments occurs [13]. We have now studied these phenomena in primary cultured hepatocytes because this system allows longer-term experiments than those feasible with isolated hepatocytes.

Our results indicate that, in cultured hepatocytes, GS translocates from the cytosol to the cell periphery in response to glucose. GS translocation is a reversible process, and the covalent activation of the enzyme is not sufficient to induce its translocation. Initially, glycogen is synthesized close to the plasma membrane and deposits of the polysaccharide grow from the periphery towards the interior of the cell. No clear co-localization between actin filaments and translocated GS was observed, but our findings suggest that the actin cytoskeleton has a role in the glucose-induced translocation of GS to the hepatocyte periphery.

## MATERIALS AND METHODS

### Hepatocyte isolation and culture

Male Wistar rats weighing 180–300 g were used (Harlan Interfauna Ibérica, Barcelona, Spain). All animal experiments were performed in compliance with Spanish regulations on animal handling. Hepatocytes were isolated by perfusion with collagenase as described previously [14]. The viability of isolated cells was over 90% as determined by the exclusion of Trypan Blue (Sigma-Aldrich Química, Madrid, Spain). Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM; Biochrom KG, Berlin, Germany) supplemented with 10 mM glucose, 10% (v/v) foetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100 nM insulin (Sigma-Aldrich Química), 100 nM dexamethasone (Sigma-Aldrich Química) and penicillin/streptomycin (Biological Industries), and seeded on

Abbreviations used: GS, glycogen synthase; G6P, glucose 6-phosphate; DMEM, Dulbecco's modified Eagle's medium; TRITC, tetramethylrhodamine isothiocyanate.

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plastic plates 60 mm in diameter pretreated with 0.1 % gelatin (Sigma-Aldrich Química) at a final density of $8 \times 10^4$ cells/cm$^2$. After 3–5 h at 37 °C in humidified air/$CO_2$ (19:1), unattached cells were removed by being washed with PBS (Biological Industries), the culture medium was replaced by serum and glucose-free DMEM, and the incubation was continued for 16–18 h. In some cases the overnight incubation was done with serum-free DMEM containing 30 mM glucose or 20 mM LiCl. The experiments were performed 24 h after the isolation of the hepatocytes as detailed in the text and Figure legends. At the end of each experiment, cell monolayers were washed with PBS, frozen in liquid nitrogen and stored at −80 °C until biochemical analysis.

### Metabolite determinations

To measure glycogen, cells were scraped with 30 % (w/v) KOH; the extract was then boiled for 15 min and the resulting solution was spotted on chromatography paper 31 ET (Whatman, Maidstone, Kent, U.K.). Glycogen was precipitated by immersing the papers in ice-cold 66 % (v/v) ethanol. After two washes in ethanol, the papers were air-dried and incubated with amyloglucosidase (Sigma-Aldrich Química) as described by Chan and Exton [15]. The resulting glucose was measured with a Gluco-quant kit (Roche Diagnostics GmbH, Mannheim, Germany).

### Enzyme activity assays

To measure enzyme activities, 100 $\mu$l of ice-cold homogenization buffer consisting of 10 mM Tris/HCl (pH 7), 150 mM KF, 15 mM EDTA, 15 mM 2-mercaptoethanol, 0.6 M sucrose, 10 $\mu$g/ml leupeptin, 1 mM benzamidine and 1 mM PMSF was added to the frozen plates; cells were collected with a plastic scraper. Homogenization was performed at 4 °C with a Polytron homogenizer. Homogenates were centrifuged at 10000 ***g*** for 15 min at 4 °C; supernatants and sediments were recovered for determinations. Sediments were resuspended in 100 $\mu$l of the same buffer. Protein concentration was measured by the Pierce BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL, U.S.A.). GS activity was measured in the presence or absence of 6.6 mM G6P [16]. GS activity in the absence of G6P represents the active form of the enzyme; the activity in the presence of 6.6 mM G6P is a measure of total activity. The ratio of these two activities (−G6P/+G6P) is an estimate of the activation state of the enzyme. Lactate dehydrogenase activity was measured with an assay kit (Roche Diagnostics GmbH, Mannheim, Germany) in a Cobas-Bio autoanalyser.

### Treatment of cell monolayers with Triton X-100

To analyse the possible association between GS and the insoluble elements of the cellular matrix, cell monolayers were washed at room temperature with PBS and a cytoskeleton stabilization buffer containing 0.1 M Pipes, pH 6.9, 2 M glycerol, 5 mM $MgCl_2$, 2 mM EGTA and 10 mM NaF. The cells were then incubated for 10 min at room temperature with the same buffer plus 0.15 % (v/v) Triton X-100, 20 $\mu$g/ml leupeptin, 20 $\mu$g/ml aprotinin and 1 mM PMSF. After two washes, cells were frozen in liquid nitrogen and stored at −80 °C until biochemical analysis.

### Antibodies

GS immunochemical and immunocytochemical experiments were performed with two polyclonal antibodies specific for rat liver GS, one of which was used in previous studies on the translocation of GS in isolated hepatocytes [13,17]. The second polyclonal antibody (L1) was generated against a peptide at the C-terminus of the enzyme. The peptide containing the 14 C-terminal amino acids of rat liver GS ($I^{690}$PKGKKKLHGEYKN$^{703}$; single-letter amino acid codes) was synthesized (Chiron Technologies, Clayton, Victoria, Australia) and coupled to keyhole limpet haemocyanin by using Imject® Activated Immunogen Conjugation Kits from Pierce. On days 0, 30 and 60, one rabbit was immunized by subcutaneous injections of 100 $\mu$g of the conjugate, which was dissolved in 200 $\mu$l of PBS and mixed with 200 $\mu$l of Freund's adjuvant (Sigma-Aldrich Química), complete in the first injection and incomplete in subsequent ones. The animal was bled at day 70 and the antiserum was collected.

The L1 antibody was effective in immunoprecipitating 80–90 % of the soluble GS activity in homogenates from rat livers or cultured rat hepatocytes. In Western blots, L1 recognized a band of 87 kDa, which corresponded to the molecular mass of the enzyme. When L1 was used for immunoprecipitation, the 87 kDa protein recognized by the antibody disappeared from the supernatants and was recovered in the immunoprecipitates. Incubation of the antiserum with the peptide used for immunization led to the disappearance of the GS band from Western blots.

A monoclonal antibody was used for glycogen immunodetection. This antibody has been shown to bind specifically to glycogen from chondrocytes, hepatocytes and muscle cells, as well as to purified glycogen [18]. We further verified its specificity by showing a lack of detectable immunofluorescence in hepatocytes incubated in the absence of glucose; hepatocytes store negligible amounts of glycogen. A fluorescent signal was observed in hepatocytes that stored measurable quantities of glycogen but disappeared when the cells were pretreated with 22 units/ml $\alpha$-amylase (Sigma-Aldrich Química) in PBS for 30 min before incubation with the anti-glycogen antibody. A unit of $\alpha$-amylase will liberate 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20 °C.

### Immunofluorescence analyses

Hepatocytes seeded on gelatin-coated glass coverslips (10 mm × 10 mm) were washed with PBS and fixed for 30 min in PBS containing 4 % (w/v) paraformaldehyde. After fixation, cells were incubated with $NaBH_4$ (1 mg/ml) to decrease autofluorescence, then permeabilized with 0.2 % (v/v) Triton X-100 in PBS and blocked with 3 % (w/v) BSA in PBS. Next, cells were incubated with the L1 antibody for 1 h at room temperature, and then washed in PBS and treated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated pig anti-rabbit immunoglobulin (Dako Diagnosticos, Barcelona, Spain). Glycogen was detected with the monoclonal anti-glycogen antibody and a TRITC-conjugated goat anti-mouse IgM secondary antibody (Chemicon International, Temecula, CA, U.S.A.). To visualize actin filaments, the fixed cells were exposed to 2–4 $\mu$M FITC-conjugated phalloidin (Sigma-Aldrich Química) for 30 min. Monoclonal anti-($\alpha$-tubulin) and anti-($\beta$-tubulin) (Amersham Pharmacia Biotech, Uppsala, Sweden) or anti-(pan cytokeratin) (Sigma-Aldrich Química) antibodies and FITC-conjugated anti-mouse immunoglobulin (Dako Diagnosticos) were used to observe microtubules and intermediate filaments respectively. Coverslips were finally air-dried and mounted on glass microscope slides with Immuno Floure Mounting Medium (ICN Biomedicals, Costa Mesa, CA, U.S.A.). Controls included examination of cultures for autofluorescence and incubation of the fixed cells with only the labelled secondary antibody.

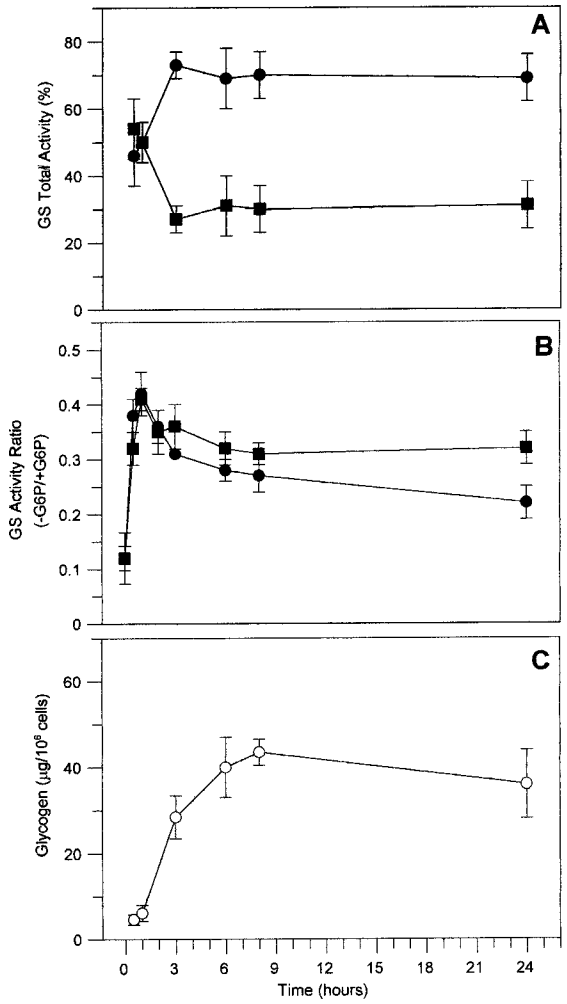


**Figure 1 Time course of GS distribution, GS activity ratio and glycogen accumulation in cultured hepatocytes incubated in the presence of glucose**

Cells were treated with 30 mM glucose for different periods; homogenates were prepared as described in the Materials and methods section. Total GS activity (**A**) and GS activity ratio (**B**) were determined in the supernatant (■) and in the pellet fractions (●) from centrifugation at 10000 ***g*** for 15 min. Total glycogen content (**C**) was determined in whole cells. In (**A**) GS activity is expressed as the percentage of total activity present in each fraction. Results are means ± S.E.M. for seven independent experiments.

Fluorescence images were obtained with a Leica TCS 4D confocal scanning laser microscope (Leica Lasertechnik, Heidelberg, Germany) adapted to an inverted Leitz DMIRBE microscope and a 63× (numerical aperture 1.4 oil) Leitz Plan-Apo objective. The light source was an argon/krypton laser (75 mW), and optical sections (0.1 $\mu$m) were obtained.

## RESULTS

### Changes in GS distribution in response to glucose

We studied the effect of glucose on the distribution of total GS activity in cultured rat hepatocytes. Cells were incubated with 30 mM glucose for different durations or with several concentrations of glucose for 1 h; homogenates were centrifuged at 10000 ***g*** for 15 min and total GS activity was then measured in supernatants and sediments. In the absence of glucose, 59 ± 8 % (mean ± S.E.M.) of total GS activity was found in the soluble fraction and the remaining 41 ± 8 % in the pelletable faction. When glucose was added, total GS activity decreased in the supernatants and was quantitatively recovered in the pellets in a time-dependent (Figure 1A) and dose-dependent (results not shown) manner. After approx. 2 h of incubation with 30 mM glucose, the initial percentages were inverted; after 3 h, 73 ± 4 % of total GS activity was found in the sediments. This percentage did not change substantially after this time (Figure 1A). The activity ratio of the enzyme (−G6P/+G6P) increased in both fractions, reaching a maximum of approx. 0.4 in both the supernatant and pellet fractions after a 1 h incubation with 30 mM glucose (Figure 1B). These results show that glucose activated and translocated GS in primary cultured hepatocytes in a manner similar to that in isolated hepatocytes [10] and in the liver of whole animals [12]. However, the extent of translocation was greater in cultured hepatocytes than in suspension cells.

### Changes in subcellular localization of GS in response to glucose

To study whether the treatment of cultured hepatocytes with glucose produced a change in the subcellular localization of GS, we performed immunocytochemical analyses (Figure 2). Hepatocytes were incubated with 30 mM glucose for different durations; they were then fixed and finally immunostained with the L1 antibody against GS described in the Materials and methods section. In the absence of glucose, the enzyme was distributed uniformly throughout the cytosol (Figure 2A); however, treatment with glucose led to a time-dependent accumulation of GS near the cell periphery. The enzyme formed uneven clumps, in contrast with the appearance described for isolated hepatocytes, when it produced a narrow crown near the plasma membrane [13]. This accumulation was apparent in some cells after 1 h of incubation (Figure 2B) and in most of the cells after 3 h (Figure 2C). The relative amount of enzyme that was concentrated near the cell periphery increased up to 8 h of incubation with glucose (Figures 2D and 2E); however, after 24 h, immunofluorescence arising from GS substantially decreased and showed a more diffuse pattern (Figure 2F). It should be noted that hepatocytes incubated for 24 h showed clear morphological changes and became larger and more extended than cells kept in culture for only 8 h.

### Reversibility of GS translocation

To check whether the glucose-triggered accumulation of GS at the cell cortex is a reversible process, primary cultured rat hepatocytes were first incubated overnight with 30 mM glucose and then transferred to a glucose-free medium for different periods. Total GS activity remained constant during the first 8 h of incubation without glucose (6.3 ± 0.2 m-units/$10^6$ cells) but decreased to half of its initial value after 24 h (results not shown) [GS units are standard EC ($\mu$mol/min) units]. Glycogen content gradually decreased with incubation time and was undetectable after 24 h (Figure 3A). Figure 3(B) shows the distribution of total GS activity in the supernatants and sediments from the centrifugation of the homogenates at 10000 ***g***. After incubation overnight with 30 mM glucose, 77 ± 4 % of total GS activity was found in the pelletable fraction. The activity recovered in the sediments decreased with time as the hepatocytes were incubated in the absence of glucose; after 24 h, only 35 ± 7 % remained in the insoluble fraction.

Immunostaining of GS in hepatocytes incubated overnight with 30 mM glucose showed the presence of large patches of immunoreactive enzyme at the cell periphery (Figure 4A). The

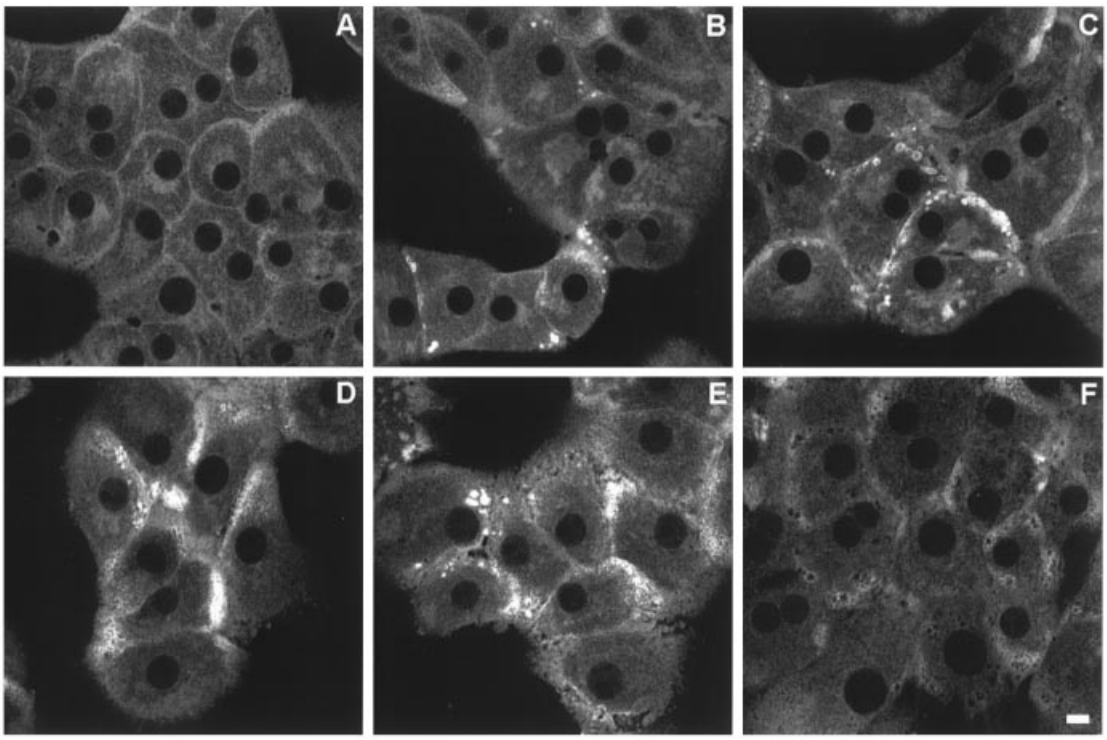


**Figure 2 Glucose-induced changes in the intracellular distribution of GS**

Confocal microscopy images of cultured hepatocytes incubated with 30 mM glucose for 0 h (**A**), 1 h (**B**), 3 h (**C**), 6 h (**D**), 8 h (**E**) and 24 h (**F**). At the end of the incubation, cells were processed for immunofluorescence analysis with the L1 anti-GS antibody as indicated in the Materials and methods section. Scale bar, 10 $\mu$m.

subsequent incubation of hepatocytes in the absence of glucose produced a decrease in the size of these aggregates (Figures 4B–4E) and after 24 h no accumulation of GS at the cell periphery was detected (Figure 4F).

### GS activation is not sufficient to translocate the enzyme

Lithium ions have been shown to increase the GS activity ratio in isolated hepatocytes [6] and in livers of whole animals [7]. We studied the ability of this reagent to activate GS in primary cultured hepatocytes. Incubation of rat hepatocytes with 20 mM LiCl in the absence of glucose for different periods did not produce any change in the levels of total GS activity (results not shown). Nevertheless, this treatment led to a considerable increase in the GS activity ratio, which rose from approx. 0.12 to 0.30 after 16 h of incubation, in both the supernatant and the pellet fractions from a 10000 ***g*** centrifugation of cell homogenates. An equivalent concentration of NaCl had no effect on the activation state of the enzyme. However, the $Li^+$-induced activation of GS did not change the distribution of the enzyme between the soluble and pelletable fractions, which remained the same as in the control cells (results not shown).

In another set of experiments, hepatocyte cultures were incubated overnight with DMEM plus 20 mM LiCl in the absence of glucose. The culture medium was then replaced by a medium with or without 30 mM glucose, also containing 20 mM LiCl, and the incubation was continued for another 3 h. In hepatocytes treated only with LiCl, GS showed a uniform cytosolic distribution, as in control cells (Figure 5A), whereas incubation of cultured hepatocytes with glucose in the presence of LiCl led to the accumulation of GS at the plasma membrane (Figure 5B). These results show that the covalent activation of GS is not sufficient to promote its translocation to the insoluble fraction or its accumulation in the periphery of the hepatic cell.

### Role of the cytoskeleton in the glucose-induced translocation of GS

The possibility that hepatic GS could be associated with subcellular structures such as the cytoskeleton has previously been considered. In isolated hepatocytes, the glucose-triggered accumulation of GS near the plasma membrane was shown to coincide with a marked increase in the co-localization of the enzyme with cortical actin filaments [13]. Furthermore, the translocation of the enzyme to the pelletable fraction of the cell homogenates on incubation with glucose suggests an interaction with an insoluble element of the cell matrix.

The possible involvement of the cytoskeleton in the glucose-induced changes in GS distribution was first studied by analysing the ability of Triton X-100 to extract the enzyme from cell monolayers. In this kind of experiment, cytosolic proteins are readily removed by treatment with the detergent, whereas insoluble proteins and those bound to insoluble cellular components remain in the interior of the cells. Cultured hepatocytes were incubated for 3 h in the absence (control) or in the presence of 30 mM glucose, and the percentage of total GS activity not extracted by treatment with Triton X-100 was measured. In control cells, only $30 \pm 5\,\%$ ($n = 3$) of total GS activity remained bound to the hepatocyte monolayer, whereas in cells incubated with glucose this percentage rose to $70 \pm 5\,\%$. In both cases, lactate dehydrogenase, an archetypical cytosolic enzyme, was completely extracted (results not shown). The percentages of ex-

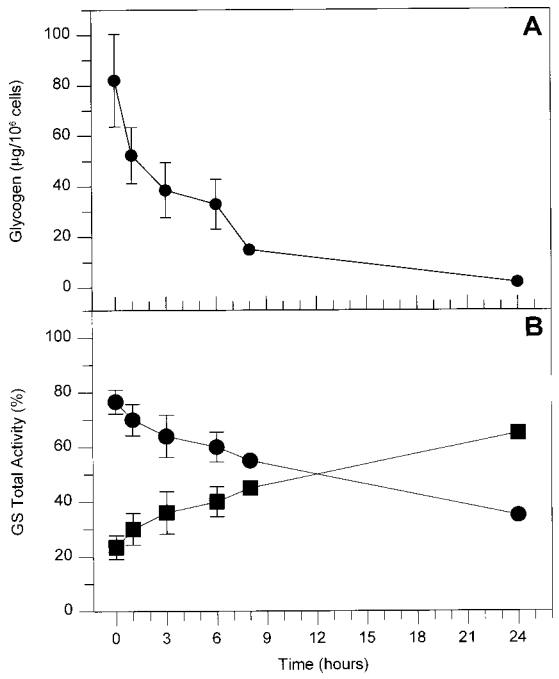


**Figure 3 Time course of the effect of glucose depletion on glycogen content and GS distribution**

Primary cultured hepatocytes were incubated overnight in DMEM containing 30 mM glucose; the culture medium was then replaced with glucose-free DMEM and the incubation was continued for different periods of time. (**A**) The gradual decrease of glycogen stores after glucose depletion. (**B**) The percentage of total GS activity found in the supernatant (■) and in the pellet fractions (●) of the cellular homogenates after centrifugation at 10000 ***g*** for 15 min as indicated in the Materials and methods section. Results are means ± S.E.M. for four independent experiments.

tractable and non-extractable GS activity were comparable with the relative amounts of the enzyme found in the soluble and pelletable fractions after centrifugation at 10000 ***g***, in cells incubated both in the absence and in the presence of glucose (Figure 1A). Interestingly, in cells incubated with glucose for 3 h, $85 \pm 5\%$ of the total glycogen was also resistant to extraction by the detergent and remained in the cell.

We also studied the possible interaction between GS and some cytoskeletal elements in double-labelling immunofluorescence experiments. Figure 6 shows the distribution of GS (red) and actin microfilaments (green) in cultured hepatocytes incubated in the absence of glucose (Figure 6A), in the presence of 30 mM glucose for 8 h (Figure 6B) or 24 h (Figure 6C). No clear co-localization of GS and actin filaments was observed in any condition. Similar results were obtained when the distribution of GS and other components of the cytoskeleton, such as microtubules and intermediate filaments, was studied by double immunostaining of cultured hepatocytes (results not shown).

Finally, we analysed whether an intact cytoskeleton was required for the glucose-induced translocation of GS. Cultured hepatocytes were treated for 1 h with 5 $\mu$M cytochalasin D and the drug was maintained in the subsequent 3 h incubation with 30 mM glucose. Staining with phalloidin showed that the actin microfilaments were depolymerized before the addition of glucose (results not shown). These cells stored $32 \pm 7\%$ less glycogen than control hepatocytes, and GS immunolocalization revealed a significant decrease in the number of cells that showed a clear accumulation of GS at the plasma membrane.

### Pattern of glycogen deposition

When cells were incubated with 30 mM glucose, the total amount of glycogen gradually increased up to 8 h of incubation, reaching a level of $43 \pm 3$ $\mu g/10^6$ cells and remained essentially constant after this time (Figure 1C).

We studied the intracellular pattern of glycogen deposition by using a specific monoclonal antibody [18]. Immunostaining for glycogen showed that glycogen deposits were initially formed near the plasma membrane, where they mostly accumulated in a time-dependent manner up to 8 h of incubation (Figure 7). After 24 h there was an apparent increase in the total fluorescence attributable to glycogen and the label was found throughout the cytosol of hepatic cells (Figure 7F). Because total glycogen content did not substantially vary after 8 h of glucose treatment (Figure 1C), the greater intensity of glycogen immunolabelling can be explained only by assuming that the epitopes recognized by the antibody were more exposed after 24 h. Alternatively, there might be a maturation process of the newly synthesized glycogen, such as the introduction of branching points, which generates new epitopes for the antibody. The distribution of the polysaccharide throughout the cytoplasm suggests, as in the GS immunofluorescence assays, that there is a large intracellular reorganization associated with morphological changes in hepatocytes incubated for 24 h.

Double immunolabelling experiments with the L1 anti-GS and the anti-glycogen antibodies were performed, with negative results. Independently of the antibody used first, it apparently blocked the binding of the other antibody, thus impeding the confocal analysis of a possible co-localization. Nevertheless, this observation, together with the fact that the intracellular distribution of GS closely resembled that of the immunoreactive glycogen, suggested that after the initial translocation of the enzyme to the cell periphery, GS remained bound to its substrate and product.

## DISCUSSION

In this paper we report on the glucose-induced changes in the cellular distribution of GS and the pattern of glycogen deposition in cultured hepatocytes. Glucose triggers a change in the subcellular localization of GS from a uniform cytosolic distribution to the cell periphery. In isolated hepatocytes, GS forms a narrow ring at the cell cortex [13], whereas in primary cultured cells it forms large and uneven aggregates. However, it must be noted that, owing to intrinsic limitations of the system, experiments performed with isolated hepatocytes can be extended only up to 1 h of incubation with glucose; the GS aggregates detected at the cell periphery in cultured cells are clearly seen only after incubations of more than 1 h. Additionally, these two experimental models present clear differences in cellular morphology that could also contribute to the distinct features of GS translocation. As the primary culture system allows long-term experiments, we have been able to show that GS translocation is a reversible process. Glucose depletion, which causes glycogen degradation, also results in the redistribution of the enzyme back to a uniform cytosolic pattern.

The glucose-induced translocation of GS to the cell cortex has functional implications. Glycogen is initially synthesized near the plasma membrane and the deposits of the polysaccharide grow from the periphery towards the interior of the cell. On incubation with glucose, GS distribution closely resembles that of glycogen, strongly suggesting that after the initial movement to the cellular cortex and while the deposition of glycogen is active, GS remains bound to its substrate and product. Two observations support this hypothesis. First, the impossibility of simultaneously

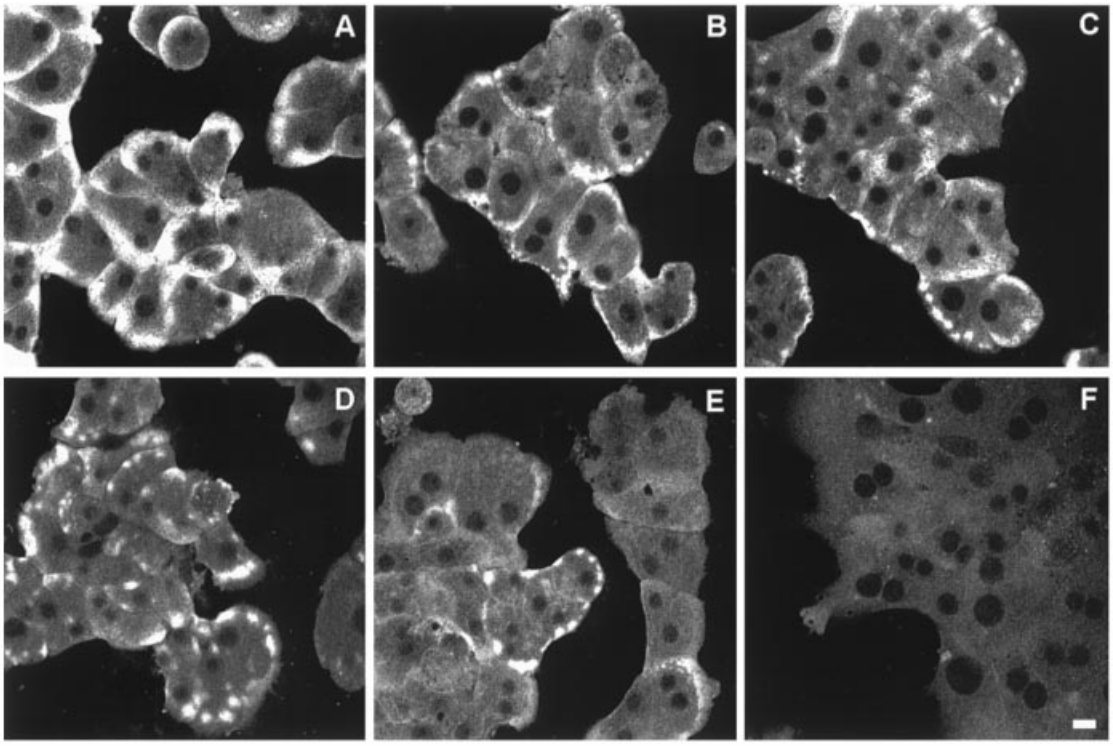


**Figure 4 Reversibility of GS translocation**

Confocal microscopy images of cultured hepatocytes incubated overnight with 30 mM glucose and then kept in a glucose-free culture medium for different durations. Cells were fixed and prepared for GS immunofluorescence analysis with the L1 anti-GS antibody as indicated in the Materials and methods section. GS immunostaining is shown of hepatocytes maintained overnight in a medium containing 30 mM glucose (**A**) and after 1 h (**B**), 3 h (**C**), 6 h (**D**), 8 h (**E**) and 24 h (**F**) of additional incubation without glucose. Scale bar, 10 $\mu$m.

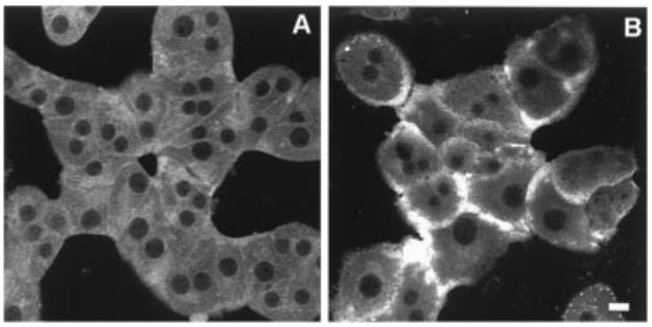


**Figure 5 Effect of LiCl on GS translocation**

Confocal microscopy images of cultured hepatocytes treated overnight in a glucose-free medium containing 20 mM LiCl and then incubated for 3 h in the same medium with no glucose added (**A**) or containing 30 mM glucose (**B**). Cells were fixed and processed for GS immunofluorescence analysis with the L1 anti-GS antibody as indicated in the Materials and methods section. Scale bar, 10 $\mu$m.

immunolabelling GS and glycogen suggests that the two antigens are in close contact. Secondly, GS is concentrated at the cell cortex while there is glycogen in the cell and returns to a homogeneous cytosolic distribution when the hepatocyte is depleted of glycogen. From the experimental evidence currently available, it is impossible to determine whether glycogen synthesis in the hepatocyte is active only at the cell periphery and the newly synthesized glycogen pushes the old one towards the interior of the cell, or whether glycogen deposition becomes active at more sites after the initial phase at the cell cortex. However, the observation that GS immunoreactive patches grow together with glycogen towards the cellular interior supports the latter hypothesis.

Glycogen degradation also seems to take place in an orderly fashion. When cultured hepatocytes with full glycogen reserves were depleted of glucose, GS patches gradually became smaller but remained at the cell periphery, suggesting that the remaining undegraded glycogen was also localized there. This 'ordered' deposition and degradation of glycogen might represent a functional advantage in the metabolism of the polysaccharide or it might simply enable the hepatocyte to store large amounts of glycogen. Molecular order in the synthesis and degradation of glycogen in the liver has previously been proposed on the basis of biochemical and morphometric analyses of particulate glycogen [19,20].

The molecular mechanisms that control the subcellular distribution of GS are unknown but here we show that the simple covalent activation of the enzyme is not sufficient to trigger its accumulation at the cell periphery. The activation state of GS is controlled by the phosphorylation state of several serine residues. The relative contribution of each of these residues has been analysed in detail for rabbit muscle GS [21]. It was shown that control of the GS activation state resided almost exclusively in sites 2 and 2a (at the N-terminus of the enzyme) and 3a and 3b (in the C-terminal region). Although a similar analysis has not been performed for liver GS, it is reasonable to assume that an analogous mechanism operates in the liver isoform, because these phosphorylation sites are also present [22,23]. In this report we show that the treatment of cultured hepatocytes with $Li^+$ ions

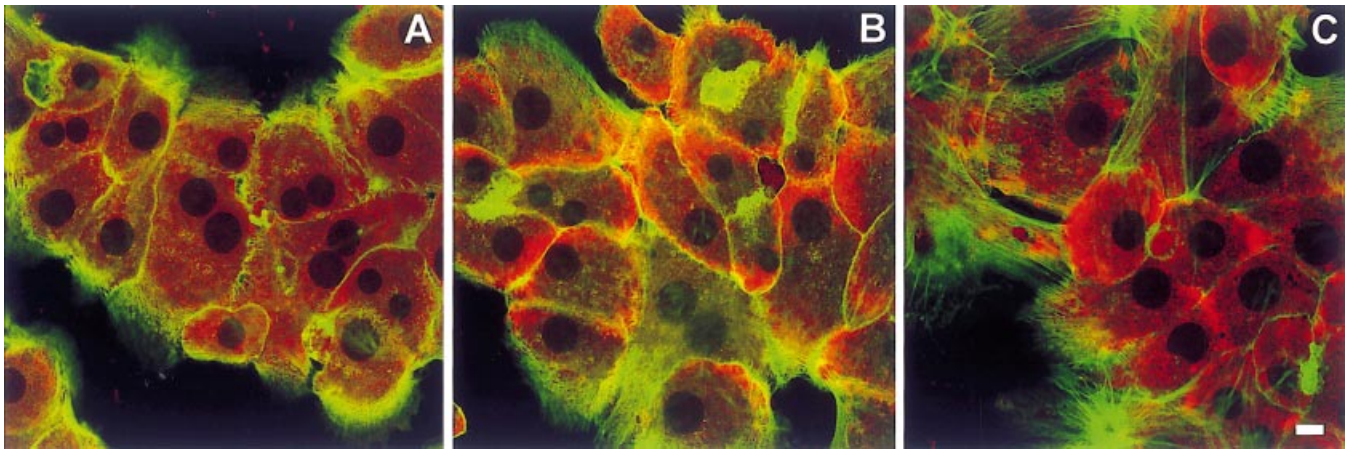


**Figure 6 Double fluorescence microscopy analysis of GS and actin microfilaments in cultured hepatocytes**

Confocal microscopy images of primary cultured hepatocytes showing the double labelling of GS and actin filaments. GS immunostaining was performed with the L1 anti-GS antibody and a TRITC-conjugated pig anti-rabbit immunoglobulin (red); actin filaments were stained with FITC-conjugated phalloidin (green). Hepatocytes were incubated in the absence of glucose (**A**) or with 30 mM glucose for 8 h (**B**) or 24 h (**C**). The distribution of GS does not follow the pattern of the actin microfilaments (yellow indicates overlap). Note that the hepatocytes incubated for 24 h show a more extended morphology and a large number of stress fibres. Scale bar, 10 $\mu$m.

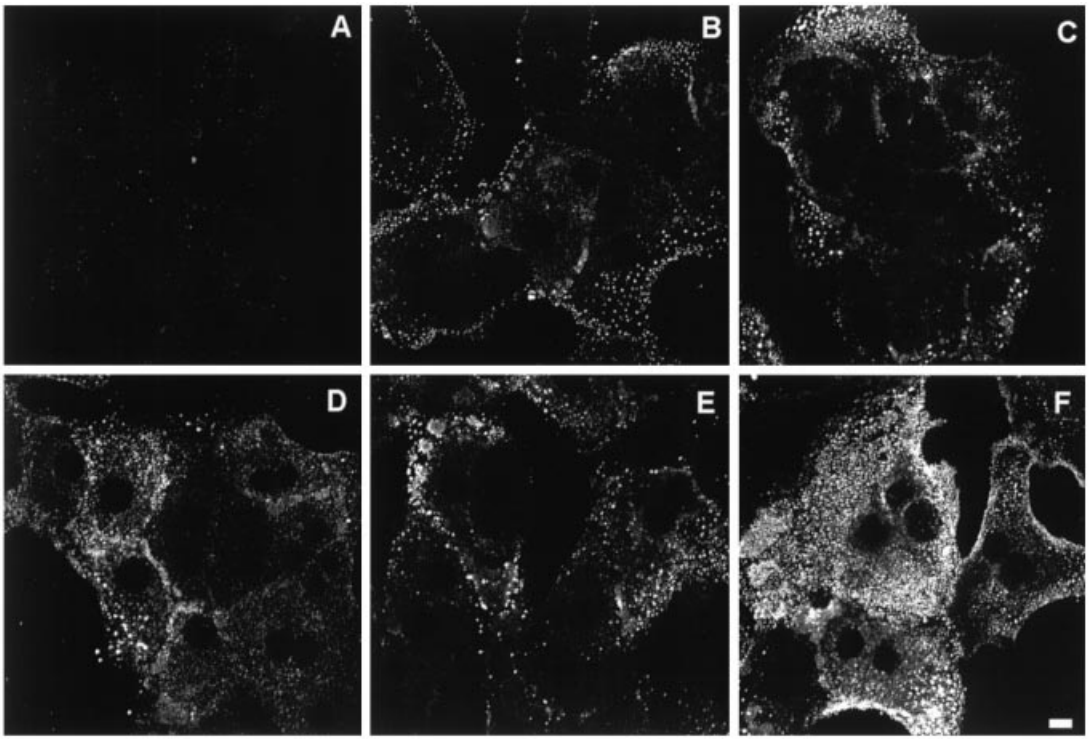


**Figure 7 Intracellular distribution of glycogen**

Confocal microscopy images of primary cultured hepatocytes incubated with 30 mM glucose for 0 h (**A**), 1 h (**B**), 3 h (**C**), 6 h (**D**), 8 h (**E**) and 24 h (**F**). At the end of the incubation the cells were processed for immunofluorescence analysis with the anti-glycogen monoclonal antibody as indicated in the Materials and methods section. Scale bar, 10 $\mu$m.

resulted in the activation of the enzyme but had no effect on its intracellular distribution. It remains to be determined whether other GS phosphorylation sites are involved in the control of its intracellular localization or whether this control is brought about by a different mechanism.

Incubation of cultured hepatocytes with glucose also results in a change in the distribution of GS between the soluble and pelletable fractions of the cellular homogenates. This phenomenon, although similar in its nature to what we have previously reported for isolated hepatocytes [10,11], exhibits significant differences. In cultured hepatocytes, GS distribution varies from approx. 40% in the pellet fraction in the absence of glucose to approx. 75% after a 3 h incubation with 30 mM glucose. This represents a larger change than that observed in isolated hepato-

cytes [10]; however, it is comparable with that determined in livers from fasted and refed rats [12]. It is worth noting that in cells incubated in the absence of glucose almost half of the enzyme is already pelletable. This cannot be due to glycogen binding because these cells contain no detectable glycogen. However, once glycogen deposition has started, the relative amounts of pelletable GS correlate with the extent of the accumulation of the enzyme at the cell periphery and with the total glycogen content of the cells, suggesting that these phenomena are related. As observed with the subcellular localization of GS, the simple covalent activation of the enzyme did not lead to its translocation to the insoluble fraction. Similar results have recently been obtained with GS from cultured 3T3-L1 adipocytes. Treatment with insulin stimulated GS activation in these cells, but extracellular glucose, and therefore glycogen deposition, was an absolute requirement to induce GS translocation to the insoluble fraction [24].

The resistance of GS to extraction with detergents from cell monolayers suggests the association with an insoluble element of the cell matrix. In isolated hepatocytes the concentration of the enzyme at the cell periphery coincides with a marked increase in co-localization with cortical actin filaments [13]. In cultured hepatocytes there is no clear co-localization between GS and any component of the cytoskeleton. The explanation for these apparently contradictory observations might lie in the fact that the morphology of isolated hepatocytes differs greatly from that of primary cultured cells, thus implying large differences in their cytoskeletal organizations. The differences in the pattern of the translocated GS in isolated and cultured hepatocytes probably reflect this. It has previously been shown that drugs such as cytochalasin D, which depolymerize actin filaments, have an inhibitory effect on glycogen synthesis in cultured hepatocytes. This effect is not due to a direct interaction between the drug and GS, to a decrease in ATP levels or to an inhibition of glucose transport [8]. Here we have confirmed that disruption of the actin cytoskeleton renders the cultured hepatocytes less capable of storing glycogen. After a 3 h incubation with 30 mM glucose, cells treated with cytochalasin D accumulated approx. 30% less glycogen than their corresponding controls. These cells also showed a partly blocked GS translocation to the plasma membrane, indicating that the actin network has a role in the glucose-induced redistribution of hepatic GS. However, the absence of co-localization between GS and actin microfilaments suggests that the association between the enzyme and this cellular structure is transient. These results suggest further that the translocation of GS is required for efficient glycogen synthesis in hepatic cells.

To conclude, the control of GS localization in cultured hepatocytes constitutes the basis for the subcellular organization of glycogen metabolism. This phenomenon probably represents an additional regulatory mechanism of hepatic glycogen biosynthesis, superimposed on the allosteric and covalent control of the enzyme activity.

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