Glucose induces the translocation of glycogen synthase to the cell cortex in rat hepatocytes

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After incubation with glucose a dramatic change in the intracellular distribution of glycogen synthase was observed in rat hepatocytes. Confocal laser scanning microscopy showed that glycogen synthase existed diffusely in the cytosol of control cells, whereas in cells incubated with glucose it accumulated at the cell periphery. Colocalization analysis between glycogen synthase immunostaining and actin filaments showed that the change in glycogen synthase distribution induced by glucose correlated with a marked increase in the co-distribution of the two proteins, indicating that, in response to glucose, glycogen synthase moves to the actin-rich area close to the membrane. When glycogen synthase was immunostained with rabbit anti-(glycogen synthase) and Protein A–colloidal gold, few particles were observed close to the membrane in control cells. In contrast, in cells incubated with glucose most of the gold particles were found near the membrane, confirming that glycogen synthase had moved to the cell cortex. Furthermore, in agreement with the glycogen synthase distribution, glycogen deposition appeared to be more active at the periphery of the cell.

INTRODUCTION

Glycogen synthase is the key enzyme in the control of glycogen synthesis. It is accepted that its activity is regulated by its state of phosphorylation. Only dephosphorylated forms are assumed to be active *in vivo* [1]. However, it is difficult to explain how glycogen synthase can be responsible for the deposition of glycogen *in vivo* as pointed out by Nutall and Gannon [2].

We have shown that glucose, in addition to activating hepatic glycogen synthase [3–6], triggers changes in the intracellular distribution of the enzyme both in rat hepatocytes [7,8] and during the transition from food deprivation to refeeding in rats [9]. In response to glucose, the enzyme, measured as both total glycogen synthase activity and immunoreactive protein, is translocated from the soluble fraction to the fraction that sediments at 10000 ***g***. Incubation with glucose induces the aggregation of synthase molecules, suggesting that the translocation of the enzyme is associated with the formation of clusters. Evidence obtained in our laboratory supports the general hypothesis that glucose 6-phosphate constitutes the signal responsible for both the activation [10,11] and translocation [12] of glycogen synthase induced by glucose.

In order to gain insight into the physiological significance of glycogen synthase translocation we performed a series of immunocytochemical studies in rat hepatocytes. Our results indicate that, in response to glucose, glycogen synthase moves to the periphery of the cell. This is precisely the cell location in which glycogen synthesis begins. Translocation of glycogen synthase may therefore constitute a novel mechanism of regulation of glycogen synthesis.

EXPERIMENTAL

Animals and preparation of hepatocytes

Male Sprague–Dawley rats (Charles River, Barcelona, Spain; 200–220 g) were used. Rats were maintained on a 12:12 h light/dark cycle with free access to standard laboratory rat chow pellets (Panlab) and water. Animals were deprived of food for 24 h before hepatocyte isolation.

Suspensions of isolated parenchymal liver cells were prepared from 24 h starved animals as previously described [8]. Cells were resuspended in Krebs–Ringer–bicarbonate buffer, pH 7.4. Viability was checked by the Trypan Blue-exclusion method. Preparations with viability below 90 % were not used. Samples (4 ml) of these suspensions, containing 6×10^6–8×10^6 cells/ml, were incubated at 37 °C with gassing and continuous shaking (100 strokes/min). At the end of the incubations, cells were centrifuged (3000 ***g***, 20 s) and the cell pellet was immediately fixed and used as described below.

Immunofluorescence labelling and confocal microscopy

Isolated hepatocytes incubated with or without 30 mM glucose for 30 min were fixed in 3 % paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 60 min and permeabilized with 0.05 % saponin in PBS for 10 min. For immunofluorescence staining, cells were blocked with 20 mM glycine/1 % BSA in PBS (buffer A) for 10 min and incubated for 1 h at room temperature with rabbit anti-(glycogen synthase) antibody at 1:75 dilution. After three washes in PBS for 10 min, cells were incubated for 45 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins at 1:50 dilution (Dako Corp., Santa Barbara, CA, U.S.A.) and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (for actin filament staining) (Sigma Chemical Co., St. Louis, MO, U.S.A.) diluted in buffer A. After three washes in PBS, the hepatocytes were placed on glass slides and mounted with immunofluorescence medium (ICN Biomedicals, Costa Mesa, CA, U.S.A.). Immunofluorescence images were obtained with a Leica TCS 4D (Leica Lasertechnik, Heidelberg, Germany) confocal scanning laser microscope adapted to an inverted Leitz DMIRBE microscope and a 63× (NA 1.4 oil) Leitz Plan-Apo objective. The light source was an argon/krypton laser (75 mW). FITC (used as a marker for glycogen synthase) and TRITC (used as a marker for actin filaments) were excited simultaneously at 488 and 568 nm respectively with the laser. Optical sections (0.1 μm) were obtained and colocalization analysis was performed with Multicolor software (version 2.0

Abbreviations used: FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

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Leica Lasertechnik) as described by Pagan et al. [13]. The confocal colocalization, defined as the topographical overlapping of fluorescent markers [FITC (green) and TRITC (red)] for two cellular components (glycogen synthase and actin filaments), was represented in a cytofluorogram, in which the area where the markers overlap was indicated in yellow. By image treatment using the confocal system and to illustrate the cellular sites at which the two proteins colocalized, we generated new images in which colocalization is indicated in white (see Figures 1G and 1H). Quantification of white pixels in Figure 1 was performed by using the Multicolor software mentioned above.

Immunoelectron microscopy

For electron microscopy, isolated hepatocytes incubated with or without 30 mM glucose for 30 min were washed in PBS and fixed with 2% paraformaldehyde/0.1% glutaraldehyde (Agar Scientific, Stansted, Essex, U.K.). Cell pellets were then cryoprotected for 10 h with 2.1 M sucrose (Merck), mounted on sample carriers and frozen in liquid nitrogen. Cryoultrasections were obtained in a Reichert-Jung ultramicrotome equipped with the FC4 system. Sections were transferred to a 100-mesh grid with carbon-coated Formvar film. For immunolabelling on ultrathin cryosections, we followed the procedure described by Griffiths et al. [14] with slight modifications. Grids were washed 3×5 min on drops of 20 mM glycine in PBS and blocked with PBS/glycine/1% BSA for 20 min. Incubation (30 min) with the primary antibody rabbit anti-(glycogen synthase) at 1:75 dilution in the blocking solution was performed, followed by 3×5 min washes in PBS/glycine. The grids were incubated for 20 min in a solution of Protein A labelled with 15 nm gold particles at 1:50 dilution. After three washes in PBS of 5 min each and six washes in double-distilled water of 2.5 min each, the sections were contrasted in 0.3% uranyl acetate (Merck) in methyl cellulose (Sigma) for 10 min on ice. Finally, grids were examined on a Hitachi 600 AB electron microscope (Hitachi, Tokyo, Japan). For quantification analysis, immunodetected cryosections of 20000× magnification (ten photographs each for control and glucose-treated cells) were obtained on a Hitachi 600 AB electron microscope and the distance from each colloidal gold particle to the membrane was measured.

Glycogen staining

Isolated hepatocytes incubated without and with 30 mM glucose for 15, 30 or 60 min were fixed in 2% paraformaldehyde/2.5% glutaraldehyde in PBS for 1 h, dehydrated in graded acetone and embedded in spurr resin. Ultrathin sections were obtained in a Reichert-Jung ultramicrotome and placed on gold grids. Glycogen was stained by Thiéry's method [15]. Grids were floated on aq. 1% periodic acid for 30 min. After being washed in distilled water, sections were floated on 0.2% thiocarbohydrazide in 20% acetic acid for 24 h. Sections were rinsed with three changes of 10% acetic acid, then with 5% and 1% acetic acid, and finally with distilled water. Grids were floated on aq. 1% silver proteinate solution for 30 min in the dark at room temperature. Sections were finally washed in distilled water. An Hitachi 600 AB electron microscope was used.

RESULTS

Effects of glucose on the intracellular localization of glycogen synthase

Hepatocytes from 24 h starved rats were incubated without (control) or with 30 mM glucose for 30 min, fixed and processed for immunofluorescence detection of glycogen synthase. Actin filaments were simultaneously stained with TRITC–phalloidin. In control cells, glycogen synthase was scattered through the cytoplasm with no apparent localization (Figure 1A). Densito-

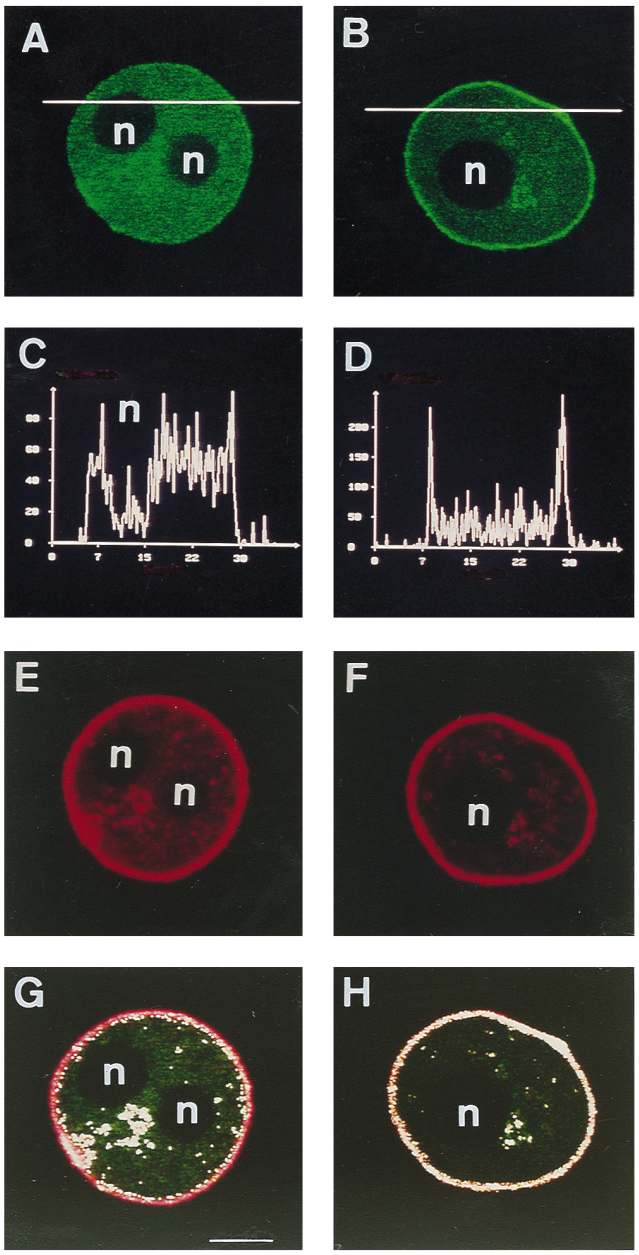

Figure 1 Effects of glucose on glycogen synthase distribution in hepatocytes

Isolated hepatocytes were incubated without (**A**,**E**,**G**) or with 30 mM glucose for 30 min (**B**,**F**,**H**) and processed as indicated in the Experimental section. Glycogen synthase immunofluorescence (**A**,**B**) was performed using rabbit anti-(rat glycogen synthase) and FITC-conjugated anti-rabbit immunoglobulin. Actin (**E** and **F**) was detected by TRITC-conjugated phalloidin. In glucose-treated hepatocytes most of the glycogen synthase is near the cell cortex. Note that the scale of fluorescence intensity values is higher in (**D**) than in (**C**). (**C**) and (**D**) show the densitogram profile of the fluorescence intensity distribution along the lines in (**A**) and (**B**) respectively. (**G**) and (**H**) show the combined image of (**A**) and (**E**) and (**B**) and (**H**), respectively. Colocalization pixels (where green and red fluorescence overlap) are shown in white. Note that in glucose-treated hepatocytes colocalization is much higher than in untreated hepatocytes. n, nucleus. Bar, 10 μm.

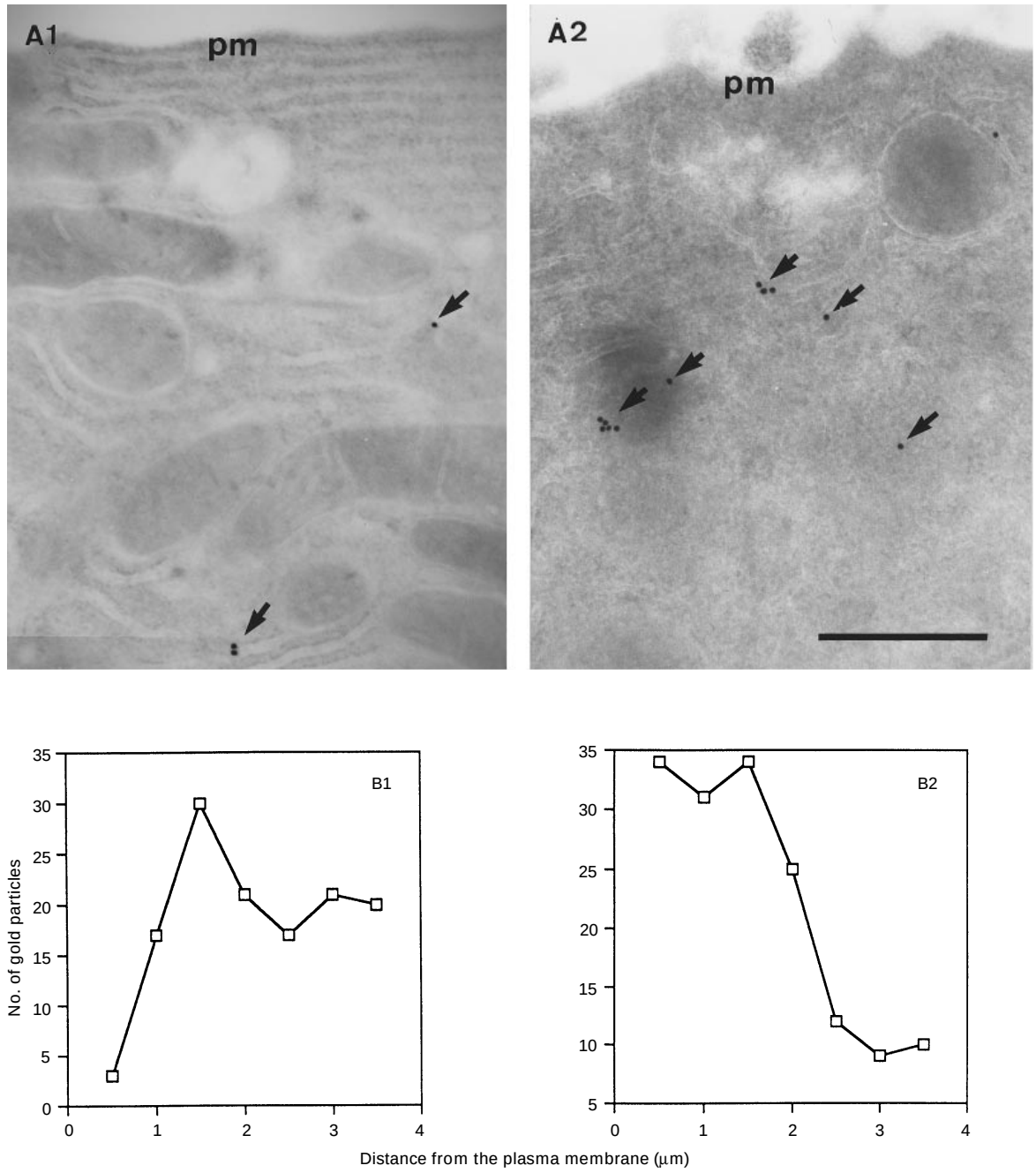

Figure 2 (A) Immunogold detection of glycogen synthase in hepatocytes incubated without (A1) or with 30 mM glucose for 30 min (A2) and (B) radial distribution of gold particles in hepatocytes incubated without (B1) or with 30 mM glucose for 30 min (B2)

(**A**) Cells were prepared for cryoultramicrotomy. Glycogen synthase was detected using rabbit anti-(glycogen synthase) antibody and Protein A–colloidal gold (15 nm). In control cells enzyme is spread throughout the cytoplasm (arrows) whereas in glucose-treated cells it appears in clusters and closer to the cell cortex. pm, plasma membrane. Bar, 0.5 μm. In (**B**) the distance of gold particles from the plasma membrane was calculated from ten photographs for each condition. Gold particle distribution was plotted at 0.5 μm intervals from the plasma membrane.

metric analysis of the immunofluorescence intensity (Figure 1C) confirmed this random pattern of distribution. Glucose induced a dramatic change in the intracellular distribution of glycogen synthase. In cells incubated with glucose, glycogen synthase immunofluorescence was mainly seen at the cell periphery (Figure 1B). This feature was also illustrated by the densitogram profile of fluorescence intensity, in which two clear-cut sharp peaks can be observed at the edges of the cell section (Figure 1D).

Since only one planar optical section of the cell is observed by confocal microscopy, in hepatocytes, as in most epithelium-derived cells, actin filaments are mainly visible at the cell cortex (Figure 1E). No apparent change in actin filament distribution was observed in glucose-treated cells (Figure 1F). Colocalization analysis of glycogen synthase immunostaining and actin filaments revealed that the change in the distribution of glycogen synthase induced by glucose treatment correlated with a marked increase

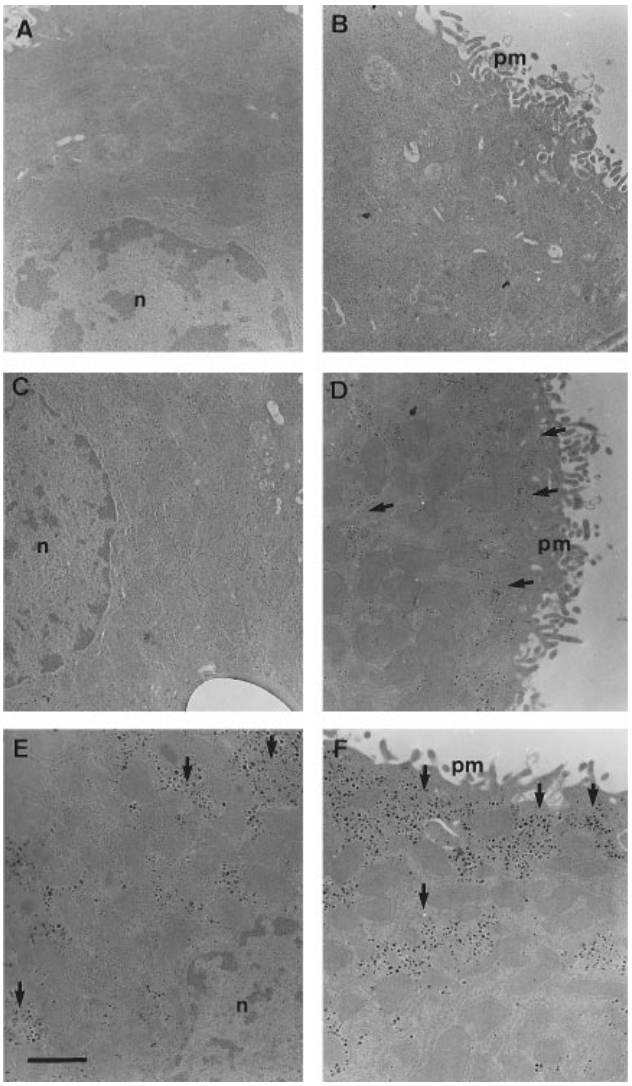

Figure 3 Glycogen distribution in glucose-treated hepatocytes

Hepatocytes treated with glucose for 15 (**A**,**B**), 30 (**C**,**D**) or 60 (**E**,**F**) min were fixed, dehydrated and embedded in resin as described in the Experimental section. Ultrathin sections were prepared so as to reveal glycogen deposition. Representative pictures of the perinuclear area (**A**, **C**, **E**) and the cell cortex (**B**, **D**, **F**) were taken. Note that after 15 min of treatment no glycogen was detected in the cytoplasm near the nucleus (**A**) and a small amount of glycogen was present in the cell cortex (arrows) (**B**). At 30 min perinuclear deposition of glycogen (**C**) was still low, but glycogen deposition was high near the cell cortex (**D**). After incubation with glucose for 1 h, glycogen was detected within the perinuclear area, but in higher proportions at positions close to the plasma membrane. pm, plasma membrane; n, nucleus. Bar, 1 μm.

in the co-distribution of these two proteins. This can be clearly observed in the overlay images (Figures 1G and 1H) in which white pixels indicate colocalization. The number of white pixels in the cell cortex was much higher (4.4-fold) in glucose-incubated cells (Figure 1H) than in control cells (Figure 1G). These results indicate that, in response to glucose, glycogen synthase moves to the actin-rich area close to the membrane called ectoplasm.

In order to study the intracellular distribution of glycogen synthase at higher resolution, hepatocytes were processed by cryoultramicrotomy, and glycogen synthase was immunostained with rabbit anti-(glycogen synthase) and Protein A–colloidal gold. In control cells (Figure 2A1), the enzyme was spread out in the cytoplasm. In cells incubated with glucose (Figure 2A2) more gold particles appeared towards the periphery of the cell, and clusters of immunoreactive protein aggregates were also observed (Figure 2A2). In order to quantify these observations, ten photographs (20000 × ; combined area 11.2 μm^2) were obtained for each sample, the gold particles were counted and the distance of each individual particle from the plasma membrane was measured. The radial distribution of these gold particles was plotted at 0.5 μm intervals. In control cells, few particles were present close to the membrane (Figure 2B1). In contrast, in glucose-treated cells (Figure 2B2), most of the gold particles were found near the membrane, thus confirming that glycogen synthase had been translocated to the cell cortex in response to glucose.

Pattern of glycogen synthesis

In order to ascertain the physiological function of glycogen synthase translocation we attempted to determine whether it had an impact on glycogen synthesis. To this end, we studied the location of glycogen after different periods of glucose incubation (Figure 3). After 15 min incubation with glucose (Figures 3A and 3B), a small amount of glycogen was observed and it was all at the periphery of the cell. After 30 min incubation (Figures 3C and 3D) glycogen deposition was more evident, although it was still essentially confined to the cell periphery. At 1 h (Figures 3E and 3F), the total amount of glycogen has increased markedly but it was now detected towards the cell centre, although the major clusters were still found at the periphery of the cell.

DISCUSSION

These results offer new insights into the mechanism by which glycogen synthesis is turned on by glucose. Here we show that translocation of glycogen synthase to the actin-rich region near the plasma membrane may be involved in this process. Furthermore, the results indicate that, in agreement with glycogen synthase distribution, glycogen deposition starts at the periphery of the cell. In fact, some observations suggest that glycogen deposition is more intense in the outer part of the cell [16]. However, most measurement of glycogen deposition has been in fasted and re-fed rats, measuring glycogen levels 1 h or more after refeeding. This may explain why glycogen synthesis has not previously been recognized as beginning at the cell periphery.

We have reported that glucose, in addition to activating glycogen synthase, promotes changes in the distribution of enzyme activity from soluble fractions to fractions that sediment at about 10000 ***g*** [7]. It was suggested that the enzyme may be bound to structures that are sedimented at relative low centrifugal forces. Our results support the hypothesis that the increase in glycogen synthase in the pelleted fraction is a consequence of an intracellular change in enzyme distribution.

We have previously demonstrated that glucose 6-phosphate is responsible for both the activation and translocation of glycogen synthase [10–12]. It appears that the stimulatory effect of glucose 6-phosphate on the dephosphorylation (and thus activation) of the enzyme is specific for glycogen synthase and is probably due to a change in the allosteric configuration of this enzyme which facilitates its dephosphorylation, rather than to stimulation of the phosphatases [17]. One may speculate that the same change induces the translocation toward the periphery of the cell. Dephosphorylation itself is probably unnecessary since we have shown that, under conditions in which glucose 6-phosphate accumulates but glycogen synthase activation is blocked with phosphatase inhibitors, translocation of glycogen synthase from the soluble to the insoluble fraction may occur even without activation of the enzyme [12].

Translocation of glycogen synthase may enhance the efficiency of glycogen deposition, which would help to explain the physiological dilemma mentioned in [2]. Access of glycogen synthase to its substrate, which would be 'channelled' into glycogen, would be facilitated by its translocation to the cell cortex. This hypothesis is favoured by the observed formation of clusters of glycogen synthase. Moreover, it has been reported that, in response to glucose, glucokinase leaves its nuclear-bound receptor and moves towards the membrane [18–21]. Such translocation may thus facilitate glycogen synthesis in the periphery of the cell.

The possibility that glycogen synthase could be associated with subcellular structures such as the cytoskeleton has also previously been considered [7]. The results presented here indicate that, in response to glucose, glycogen synthase strongly colocalizes with the cortical actin cytoskeleton. We performed a sequence analysis of liver glycogen synthase and found that the hepatic isoform, from both rat [22] and human [23], but not the skeletal-muscle isoform [24,25], has the sequence KKKLHG (694–699). This sequence is found in the C-terminal region of the molecule near the phosphorylation sites. A similar sequence, KKKLTG, is present in cofilin, a protein that binds to actin and is also phosphorylated at multiple sites [26,27]. Interestingly, cofilin, when activated by dephosphorylation, is also translocated to the plasma membrane [28]. Further biochemical studies are needed to clarify whether there is a direct interaction between glycogen synthase and actin.

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