A point mutation in the UDP-glucose pyrophosphorylase gene results in decreases of UDP-glucose and inactivation of glycogen synthase

Juan-Carlos HIGUITA*, Alberto ALAPE-GIRÓN*†, Monica THELESTAM* and Abram KATZ‡[1]

*Microbiology and Tumor Biology Center, Karolinska Institutet, S-171 77 Stockholm, Sweden, †Instituto Clodomiro Picado, Facultad de Microbiologia and Departamento de Bioquimica, Facultad de Medicina, Universidad de Costa Rica, San Jose, Costa Rica, and ‡Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden

The regulatory role of UDP-glucose in glycogen biogenesis was investigated in fibroblasts containing a point mutation in the UDP-glucose pyrophosphorylase gene and, consequently, chronically low UDP-glucose levels (Qc). Comparisons were made with cells having the intact gene and restored UDP-glucose levels (G3). Glycogen was always very low in Qc cells. [^{14}C]-Glucose incorporation into glycogen was decreased and unaffected by insulin in Qc cells, whereas insulin stimulated glucose incorporation by $\approx 50\,\%$ in G3 cells. Glycogen synthase (GS) activity measured *in vitro* was virtually absent and the amount of enzyme in Qc cells was decreased by about $50\,\%$. The difference in GS activity between cells persisted even when G3 cells were devoid of glycogen. Incubation of G3 cell extracts with either exogenous UDP-glucose or glycogen resulted in increases in GS activity. Incubation of Qc cell extracts with exogenous UDP-glucose had no effect on GS activity; however, incubation with glycogen fully restored enzyme activity. Incubation of G3 cell extracts with radioactive UDP-glucose resulted in substantial binding of ligand to immunoprecipitated GS, whereas no binding was detected in Qc immunoprecipitates. Incubation of Qc cell extracts with exogenous glycogen fully restored UDP-glucose binding in the immunoprecipitate. These data suggest that chronically low UDP-glucose levels in cells result in inactivation of GS, owing to loss of the ability of GS to bind UDP-glucose.

Key words: glucose uptake, glycogen synthesis, insulin, lactate, phosphorylase.

INTRODUCTION

Glycogen is the storage form of glucose in cells and serves as a 'sink' for removal of extracellular glucose. Glycogen formation is paramount for blood glucose homoeostasis, and defects in this process may underlie and contribute to the development of diseases such as non-insulin-dependent diabetes mellitus [1]. In mammalian cells, glycogen biogenesis is initiated by the self-glucosylating protein, glycogenin (GN) [2–4]. Glucosylated GN then serves as the substrate for glycogen synthase (GS), which together with branching enzyme catalyses the formation of mature glycogen. GS is present in phosphorylated and non-phosphorylated forms [5]. Under many conditions dephosphorylation of GS is associated with activation of the enzyme and hence glycogen synthesis [6,7].

UDP-glucose (UDP-Glc) is the glucosyl donor for GN and GS, as well as other glucosyl transferases [8–12]. Recent studies suggest that UDP-Glc has signalling properties, including the ability to activate a G-protein-coupled receptor and increase intracellular calcium concentrations [13,14]. Additionally, there is evidence for compartmentation of UDP-Glc in cells [15] and that UDP-Glc could affect glycogen synthesis by affecting the cellular localization of GS [16]. These findings raise the possibility that UDP-Glc may play a role in glycogen biogenesis other than at the substrate level. Recently, we developed a mammalian cell line that exhibits chronically low UDP-Glc contents [17] owing to a point mutation in the UDP-glucose pyrophosphorylase (UDPG:PP) gene [18]. This cell line has now been used as a model to investigate the effects of low UDP-Glc contents on the capacity to synthesize glycogen and GS activity.

EXPERIMENTAL

Materials

2-Deoxy-D-[1,2-^{3}H]glucose, D-[U-^{14}C]glucose, α-D-[U-^{14}C]-glucose 1-phosphate, UDP-[U-^{14}C]glucose and UDP-[6-^{3}H]glucose were from Amersham Biosciences. All reagents and enzymes were from either Sigma or Boehringer Mannheim. Cell-culture media and supplements were from Gibco-BRL. Polyclonal antibodies against mouse skeletal muscle GS were a gift from Dr J. C. Lawrence (University of Virginia, Charlottesville, VA, U.S.A.) and polyclonal antibodies against actin were from Affinity Bioreagents (East Rutherford, NJ, U.S.A.). Secondary antibodies conjugated to horseradish peroxidase were from Amersham Biosciences and Sigma. Human insulin (Actrapid) was purchased from Novo Nordisk (Bagsværd, Denmark).

Cells

We used diploid Chinese hamster lung fibroblasts containing a point mutation in the UDPG:PP gene that were stably transfected with the pCDNA3 vector (Invitrogen, Carlsbad, CA, U.S.A.) containing a wild-type bovine UDPG:PP gene (G3) or with the vector alone (Qc), as described elsewhere [17–19]. This

Abbreviations used: 2-DG, 2-deoxyglucose; GN, glycogenin; GS, glycogen synthase; PCA, perchloric acid; TCA, trichloroacetic acid; UDP-Glc, UDP-glucose; UDPG:PP, UDP-glucose pyrophosphorylase.

[1] To whom correspondence should be addressed (e-mail abram.katz@fyfa.ki.se).

ensured that the G3 and Qc cells had the same genetic background prior to transfection, but that only the cells transfected with the vector alone remained with low UDP-Glc levels (Qc). Cells were cultured in minimum essential medium (containing 5 mM glucose) supplemented with 10 % fetal bovine serum, 5 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and geneticin (0.4 mg/ml) and continuously gassed with 95 % O_2/5 % CO_2 at 37 °C. Cells received fresh medium every 24 h to avoid significant loss of substrate (glucose) and accumulation of intermediates (e.g. lactate; see below). After harvesting, cells were quickly frozen in liquid N_2 and stored at −80 °C until processed. Prior to analysis cells were freeze-dried and aliquoted.

Glycogen, GS and phosphorylase

Freeze-dried powder was resuspended in ice-cold homogenizing buffer (HxB; 50 μl/mg of dry weight) consisting of 10 mM EDTA, 50 mM KF and 30 % (v/v) glycerol, pH 7.0, and sonicated or homogenized on ice. The homogenate was used for subsequent analyses.

The homogenate was centrifuged at 23000 ***g*** for 15 min at 4 °C. For analysis of GS, supernatant was diluted with 4 vol. of ice-cold diluting buffer containing 20 mM EDTA, 130 mM KF and 50 mM Tris, pH 7.8. GS activity was measured with the filter-paper technique following the incorporation of labelled glucose into glycogen [20]. Activity was measured at 30 °C for 10 min in the presence of 0.14 mM UDP-[U-^{14}C]Glc, 5 mg/ml purified glycogen and 0.17 or 7.2 mM glucose 6-phosphate, pH 7.8 [21], unless stated otherwise. Activity measured in the presence of the high glucose 6-phosphate concentration represents the total activity of GS regardless of the phosphorylation state of the enzyme [5]. At the end of the incubation, filter papers were washed with 70 % ethanol, dried, and radioactivity was counted in a scintillation spectrometer. For analysis of phosphorylase, supernatant was diluted with 0.5 vol. of ice-cold buffer containing 100 mM KF and 100 mM Mes, pH 6.3. Phosphorylase activity was also measured with the filter-paper technique [22] by measuring the incorporation of glucose from α-D-[U-^{14}C]glucose 1-phosphate into glycogen in the absence and presence of 3.3 mM AMP at 30 °C for 20 min. Activity measured in the presence of AMP represents the total activity of phosphorylase regardless of the phosphorylation state of the enzyme. Additional details on the enzyme analyses are provided elsewhere [23].

For analysis of glycogen, KOH was added to an aliquot of homogenate resulting in a final concentration of 1 M KOH and heated at 90 °C for 20 min. The extract was neutralized, hydrolysed enzymically [24] and analysed for free glucose by a fluorimetric enzymic technique based on the production of NADPH [25]. Other metabolites were extracted by addition of ice-cold perchloric acid (PCA) to an aliquot of homogenate resulting in a final concentration of 0.5 M PCA [26]. PCA extracts were centrifuged as above, decanted and neutralized with 2.2 M $KHCO_3$ and centrifuged again. Supernatants were analysed for metabolites by fluorimetric enzymic techniques based on changes in NAD(P)H [25]. Extraction of metabolites from homogenates prepared with ice-cold enzyme extraction buffer yields values that are similar to those obtained from tissue that is directly deproteinized with PCA [26]. Protein concentration was determined with the DC Protein Assay Kit (Bio-Rad).

Glucose uptake and glycogen synthesis

Measurements of 2-deoxyglucose (2-DG) uptake and glycogen synthesis were performed essentially as described elsewhere [27]. Briefly, cells were first incubated at 37 °C for 60 min in glucose-free supplemented minimum essential medium containing 5 mM pyruvate with or without insulin (10 m-units/ml). Thereafter, 2-deoxy-D-[1,2-^{3}H]glucose (1 μCi/ml) or D-[U-^{14}C]glucose (2 μCi/ml) were added to the cells and 8 min later cells were washed three times with ice-cold PBS. For 2-DG uptake, cells were scraped into 5 M KOH, heated at 100 °C for 5 min and centrifuged at 23000 ***g***. Supernatant (100 μl) was added to scintillation cocktail (Ultima Gold) and counted for ^{3}H. For glycogen synthesis, cells were scraped into ice-cold 10 mM EDTA, 50 mM KF and 30 % (v/v) glycerol, pH 7.0. The extracts were homogenized and two 100 μl aliquots were spotted on to filter paper. One paper was washed in 70 % ethanol (3 × 20 min), dried and counted for ^{14}C. The other paper was washed in trichloroacetic acid (TCA; 10 % TCA, 1 × 10 min; 5 % TCA, 2 × 5 min) followed by 5 min in 95 % ethanol, dried and counted [23]. Glucose uptake and glycogen synthesis rates were linear under the conditions studied (results not shown).

Western blots

For Western blots, β-mercaptoethanol (5 %) was added to diluted supernatants used for enzyme analyses and heated at 100 °C for 5 min. Protein (30 μg) was electrophoresed under reducing conditions on 10 % (w/v) SDS/polyacrylamide gels and then electrotransferred to 0.45 μm nitrocellulose membranes. The membranes were incubated in PBS containing 5 % dried skimmed milk and 0.1 % Tween 20 (blocking solution) for 2 h at room temperature to block non-specific binding. Membranes were then incubated overnight with antibodies against GS and actin in blocking solution at 4 °C. Unbound antibodies were removed by washing three times (10 min each) with blocking solution before incubation with an anti-chicken (for GS) or anti-rabbit (for actin) IgG–horseradish peroxidase conjugate for 2 h at room temperature. Membranes were washed as above and the bound antibodies were detected with a chemiluminescence Western blot kit (Amersham Biosciences) according to the manufacturer's instructions. Immunoreactive bands were quantified with a densitometer using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Binding of UDP-Glc to GS

For studying the binding of UDP-Glc to GS, cells were cultured for 120 h as described above. Cells were washed with ice-cold PBS three times, scraped into 1 ml of ice-cold water, transferred to Eppendorf tubes and freeze-dried. A mixture of HxB and GS diluting buffer (1:4; 400 μl) was added to the cell powder, followed by homogenization and centrifugation at 23000 ***g*** for 10 min at 4 °C. An aliquot of the supernatant was used for measurement of protein. To 335 μl of the supernatant, 665 μl of KF buffer, pH 7.8 (0.3 M Tris, 0.12 M EDTA, 0.78 M KF, 0.025 % Triton X-100 and 0.05 % Nonidet P-40), and 70 μl of Protein L–agarose beads were added and the mixture was incubated on a rocking platform for 24 h at 4 °C. Samples were centrifuged at 23000 ***g***. UDP-[6-^{3}H]Glc (10 μCi) and unlabelled UDP-Glc (final concentration, 0.5 mM) were added to the supernatants, which were then incubated at 4 °C on a rocking platform for 48 h. Thereafter, 1 μl of GS antibody was added and incubated at 4 °C. After 24 h, 70 μl of Protein L–agarose beads was added and incubation was continued for another 24 h. Finally, samples were centrifuged and the pellet was collected, washed 3 × 1 min with ice-cold PBS and finally resuspended in 200 μl of PBS and counted.

GN

To study the incorporation of glucose from UDP-Glc into endogenous proteins/GN, cell powder was homogenized in 700 μl of ice-cold buffer containing 50 mM triethanolamine, pH 7.8, 150 mM KCl, 1 mM dithiothreitol, one-half of a Complete protein and protease inhibitor tablet (Boehringer Mannheim immunoprecipitation kit) and glycerol (30 % v/v), and centrifuged at 23000 ***g*** for 10 min at 4 °C. The supernatant was collected, protein was measured and 30 μg of protein was incubated with 0.1 μCi of UDP-[U-^{14}C]Glc with or without 5 mM $MnSO_4$ and/or 100 μM CDP for 90 min at 37 °C. β-Mercaptoethanol (5 %) and Bromophenol Blue (1 %) were added to the samples, which were then boiled for 5 min and loaded on a 10 % polyacrylamide gel. After SDS/PAGE, gels were dried overnight and exposed to a PhosphorImager screen for 1 week.

Statistics

Data are presented as means ± S.E.M. unless stated otherwise. Significant differences between means were determined with paired or unpaired *t* tests. Statistical significance was set at $P < 0.05$.

RESULTS

Metabolites

Metabolites reflecting the energy status of the cell were measured (Table 1). There were no noteworthy differences in glucose 6-phosphate or ATP contents between cell lines. However, lactate contents were higher and phosphocreatine contents lower in Qc than in G3 cells at 96–120 h after seeding. The concentration of glucose measured in the medium from the Qc cells after 120 h of incubation was lower (2.81 ± 0.02 mM in Qc; 3.82 ± 0.22 mM in G3; $n = 3$ dishes) and lactate higher (4.12 ± 0.08 mM in Qc; 3.04 ± 0.11 mM in G3; $n = 3$ dishes) than in medium from the G3 cells.

As expected, UDP-Glc contents were substantially less in Qc than in G3 cells (Figure 1). The relatively small amounts of UDP-Glc in the Qc cells probably derive from residual activity of UDPG:PP, which amounts to about 4 % in the mutant cells [18]. Glycogen was always below the level of detection in Qc cells, as well as in G3 cells during the first 48 h after seeding. Thereafter, glycogen increased in the G3 cells to 7 ± 3 ($n = 4$ dishes) and 14 ± 2 nmol of glucosyl units/mg of dry weight ($n = 6$ dishes), 72 and 120 h after seeding, respectively. While these glycogen levels are measurable, they are still low compared with other glycogen-producing tissues, such as skeletal muscle (200–700 nmol of glucosyl units/mg of dry muscle in rats and humans) [28,29].

Table 1 Metabolite contents in cells

Values are means ± S.E.M. from four dishes whose cells were harvested 96 ($n = 2$) and 120 h ($n = 2$) after seeding and are given in nmol/mg of dry weight. Values for 96 and 120 h were virtually identical and have therefore been pooled for statistical analysis. Protein concentrations in the G3 (1.1 ± 0.1 mg/ml extract) and Qc cells (1.1 ± 0.2 mg/ml extract) were not significantly different ($P > 0.05$). ***$P < 0.001$ versus G3.

	Metabolite content (nmol/mg of dry weight)			
Metabolite ...	Glucose 6-phosphate	Lactate	ATP	Phosphocreatine
G3	0.06 ± 0.00	3.4 ± 0.7	5.4 ± 0.2	3.00 ± 0.12
Qc	0.07 ± 0.00	14.9 ± 0.8***	5.2 ± 0.2	1.75 ± 0.10***

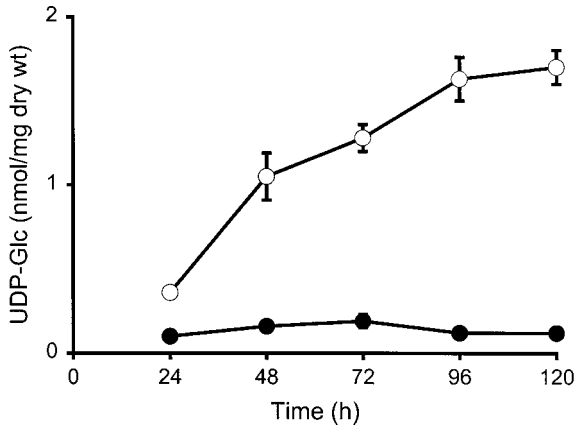

Figure 1 UDP-Glc contents are decreased in Qc cells

Values are means ± S.E.M. for 2–6 dishes. ○, G3 cells; ●, Qc cells.

Table 2 Glucose uptake and glycogen synthesis in cells

Values are means ± S.E.M. of six dishes studied at 37 °C 120 h after seeding and are given in d.p.m./dish over 8 min. For glycogen synthesis, extracts were precipitated with ethanol or TCA. Protein concentrations did not differ significantly between cells (1.0 ± 0.0 mg/dish in G3; 0.9 ± 0.1 in Qc; $P > 0.05$). **$P < 0.01$; ***$P < 0.001$.

	G3	Qc
2-DG uptake	22710 ± 464	27957 ± 514***
Glycogen synthesis		
Ethanol	638 ± 43	335 ± 36***
TCA	683 ± 51	395 ± 30**

2-DG uptake and glycogen synthesis

Lack of detectable glycogen in Qc cells could be due to low rates of glucose uptake, high rates of glycogenolysis owing to elevations in glycogen phosphorylase activity or low GS activity. The greater decrease in medium glucose concentration in Qc cells suggested that glucose uptake was not lowered. Indeed, Qc cells had a higher rate of 2-DG uptake than G3 cells (Table 2). Moreover, both cell lines were equally responsive to insulin (23 % increase in 2-DG uptake in Qc and G3, $P < 0.001$; results not shown). Cytochalasin B (10 μM) inhibited about 90 % of glucose uptake (results not shown), indicating that glucose transport was mediated by a transport protein(s). Furthermore, total phosphorylase activity was ≈ 50 % lower in Qc than in G3 cells (results not shown), suggesting that excessive glycogen degradation was not occurring in the Qc cells.

To examine whether the lack of glycogen in Qc cells was due to a decreased glycogen synthesis, incorporation of [^{14}C]glucose into glycogen/glycoproteins was measured. Consistent with the lower glycogen contents in the Qc cells, [^{14}C]glucose incorporation into ethanol-precipitable material (mature glycogen and endogenous glycoproteins) was decreased by about 40 % in Qc cells (Table 2). The incorporation of [^{14}C]glucose into TCA-precipitable material (endogenous glycoproteins) [23] was similar to the ethanol-precipitable results in both cell lines (Table 2), which indicates that there was little mature glycogen in both cell lines (see above). Probably, a greater proportion of the counts were incorporated into low-molecular-mass proteins in the Qc cells (i.e. GN), since one would expect GN to be more active in

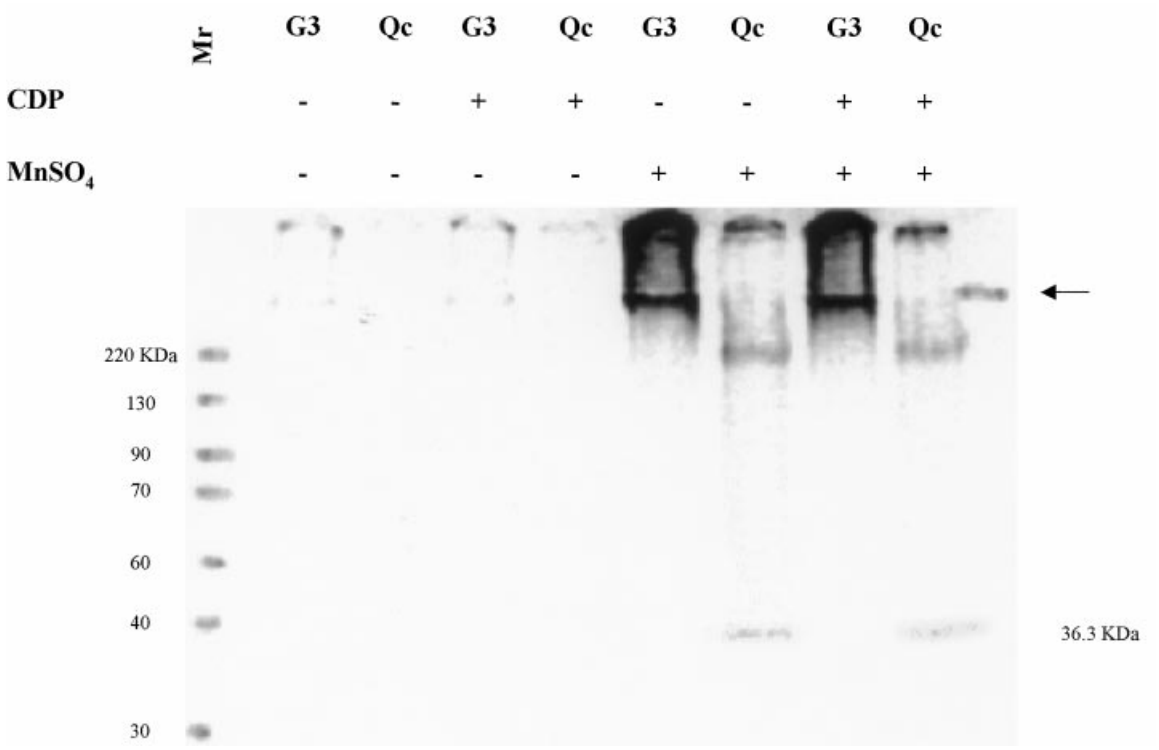

Figure 2 Functional GN in Qc cells

Protein (30 μg) from cell extracts prepared 120 h after seeding was incubated with UDP-[U-^{14}C]Glc for 90 min at 37 °C, electrophoresed and exposed to a PhosphorImager screen for 1 week. When present during incubations, $MnSO_4$ and CDP concentrations were 5 and 0.1 mM, respectively. Intensity of the band at 36 kDa in lane 7 (counting from the left) was decreased by 49% in lane 9. Similar results were obtained in two additional experiments, where CDP inhibited the Mn^{2+}-dependent band in Qc by 62 and 64% (results not shown). The arrow denotes the start of the running gel. Lane 1 contained molecular-mass standards.

glycogen-depleted cells [2–4]. Indeed, incubation of extracts from Qc cells with UDP-[U-^{14}C]Glc in the presence of Mn^{2+} resulted in the incorporation of label into an $\approx$ 36 kDa protein and the incorporation was inhibited by about 50 % by CDP (Figure 2, lanes 7 and 9 from left). Similar results were obtained in two other experiments. Since the molecular mass of GN is generally reported to be 37–38 kDa [3] and it is activated by Mn^{2+} and inhibited by CDP (see [23]), the results indicate that GN was active in the Qc cells. On the other hand, there was no evidence for GN activity in the G3 cells. In both Qc and G3 extracts, Mn^{2+} stimulated the incorporation of label into a high-molecular-mass material that barely entered the running gel and was not inhibited by CDP (Figure 2, lanes 6–9 from left). The extent of the Mn^{2+} effect on the high-molecular-mass material was greater in the G3 extracts [cf. density of stacking gels of lanes 6 (11286 arbitrary units) and 7 (5291 arbitrary units) from left]. On average, 89 % (range = 86–93 %, $n = 3$) of the total radioactivity (sum of stacking and running gel) in the G3 extracts was detected in the stacking gel, versus only 58 % (range, 49–64 %, $n = 3$) in the Qc extract, as determined by densitometry. This high-molecular-mass material was probably glycogen that was glucosylated to varying degrees, and the enzyme responsible for the glucose transfer was probably GS, since we recently showed that $MnSO_4$ stimulates GS-mediated glucosylation of endogenous glycoproteins. The latter reaction is not affected by CDP [23].

Insulin, which stimulates glycogen synthesis by increasing glucose uptake and activating GS [30,31], increased glycogen synthesis in G3 cells by about 50 % ($P < 0.01$), but had no significant effect in Qc cells ($P > 0.05$; results not shown). Therefore, the lower rates of basal [^{14}C]glucose incorporation and the inability of insulin to stimulate glycogen synthesis in Qc cells suggested a decreased activity of GS in these cells.

GS activity

Total GS activity was barely detectable in Qc cells, and at 120 h was < 5 % of that in G3 cells (Figure 3). Consistent with these findings, we found that incubating G3 cells for 120 h with 5 mM pyruvate instead of 5 mM glucose ($n = 4$) decreased cell UDP-Glc contents from 2.00 ± 0.08 (glucose) to 0.35 ± 0.02 nmol/mg of dry weight (pyruvate) and concomitantly GS activity from 1.29 ± 0.03 (glucose) to 0.52 ± 0.02 nmol/min per mg of dry weight (pyruvate). As expected, there was no detectable glycogen in G3 cells incubated with 5 mM pyruvate. Next, we determined whether the low GS activity was due to a lack of GS protein in the Qc extracts. The amount of GS protein in Qc cells was reduced (Figure 4), but the reduction amounted to only about 50 % (GS/actin ratio, 0.24 ± 0.05 in Qc cells and 0.53 ± 0.09 in G3 cells; $n = 4$ for each; $P < 0.05$). Differences in the phosphorylation state of GS also cannot explain the differences in activity between cell lines, since the assays were performed in the presence of saturating levels of glucose 6-phosphate. Furthermore, measurements of GS fractional activity (0.17/7.2 mM glucose 6-phosphate) in cell extracts (see below) showed values that were always low (< 0.08) in both cell lines (results not shown), suggesting that GS was highly phosphorylated in both cell lines. The possibility that Qc cells contain a soluble inhibitor of GS was examined by mixing extracts of Qc cells with extracts

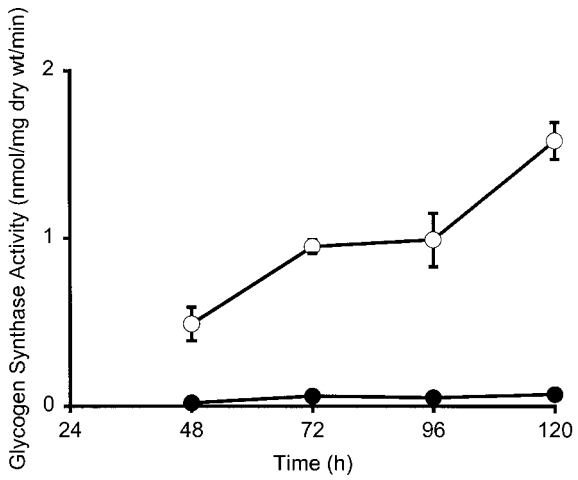

Figure 3 GS activity is decreased in Qc cells

Values are means ± S.E.M. for 2–12 dishes. ○, G3 cells; ●, Qc cells. Activities were measured in 23 000 ***g*** supernatants in the presence of 7.2 mM glucose 6-phosphate. Similar results were obtained on homogenates that were not centrifuged (not shown). Activities were also measured in the presence of 0.17 mM glucose 6-phosphate in the Qc cell extracts, but were so low that meaningful ratios (0.17/7.2 mM glucose 6-phosphate) could not be calculated.

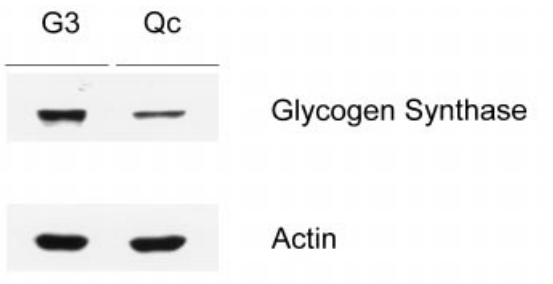

Figure 4 GS protein content is reduced in Qc cells

Representative immunoblots for GS and actin at 120 h from Qc and G3 dishes. Similar results were obtained in three other dishes for each cell line (not shown). SDS/PAGE and immunoblotting were performed as described in the text.

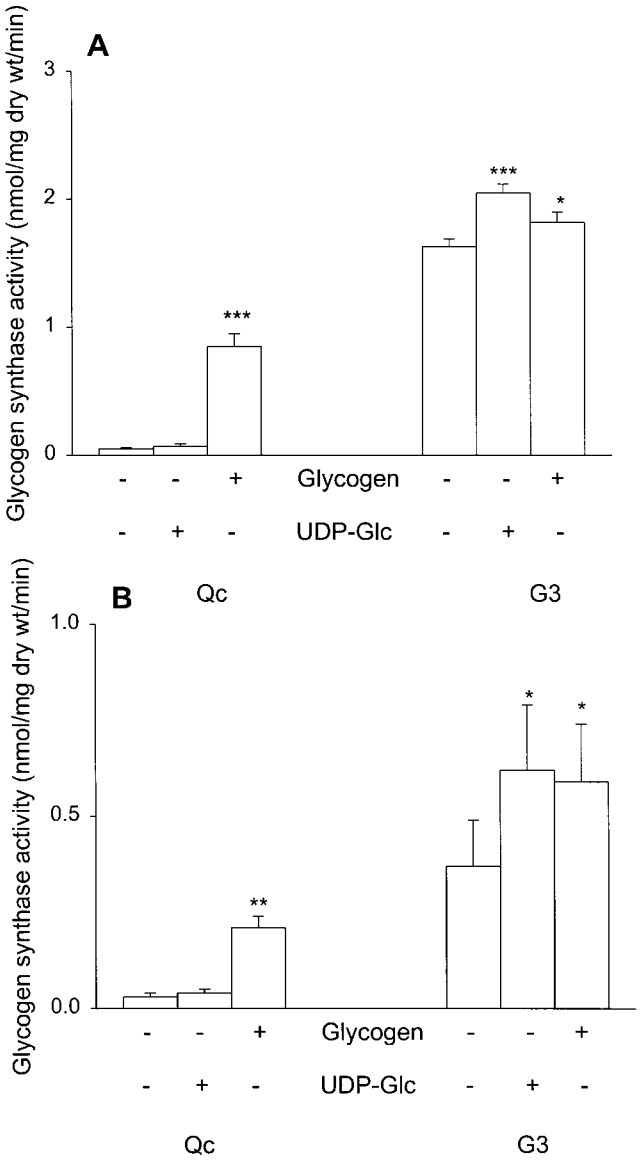

Figure 5 Re-activation of GS *in vitro*

(**A**) Cells were harvested 120 h after seeding, sonicated in ice-cold buffer, centrifuged, and supernatants were incubated in diluting buffer for GS (see the text) for 1 h at 30 °C, followed by 5 h at 4 °C in either the absence (−) or presence (+) of UDP-Glc (0.5 mM) or glycogen (10 mg/ml). Thereafter, samples were diluted and assayed as described in the text. (**B**) Same as (**A**) except that cells were harvested 48 h after seeding. Values are means ± S.E.M. for six (**A**) or four (**B**) dishes. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the absence of UDP-Glc and glycogen.

of G3 cells prior to the GS assay. Addition of Qc extracts to G3 extracts did not appreciably alter the expected activity (expected activity, 0.64 nmol/min per mg of dry weight; measured activity, 0.54 nmol/min per mg). Thus there was no evidence for a soluble inhibitor of GS in the Qc extracts.

Activation of GS *in vitro* was therefore attempted. Thiol compounds have been shown to increase GS activity *in vitro* in the absence and presence of inhibitors [32–34]. Pre-incubation of Qc extracts with 10 mM cysteamine, 50 mM β-mercapthoethanol and both cysteamine and β-mercapthoethanol did not appreciably alter the GS activity from control values of 0.01 ± 0.01 nmol/min per mg of dry weight to 0.01 ± 0.01, 0.01 ± 0.01 and 0.02 ± 0.02 ($n = 3$), respectively. Thus, incubation of Qc extracts with thiol compounds did not restore GS activity. During purification of GS, separation of GS from glycogen results in loss of GS activity, and this can be reversed by addition of exogenous glycogen [35]. Cell extracts prepared from 120 h-old cells were therefore incubated with glycogen for 6 h prior to analysis of GS activity. In G3 cells, prior incubation of extracts with exogenous glycogen resulted in a relatively small ($\approx 10\%$) albeit significant increase in GS activity (Figure 5A). In Qc cells, however, prior incubation of extracts with glycogen increased activity more than 15-fold. Prior incubation with only UDP-Glc had no significant effect on GS activity in Qc extracts, but increased activity in G3 extracts ($\approx 25\%$). Similar results were obtained with extracts from Qc and G3 cells harvested after 48 h (Figure 5B). It is noteworthy that at 48 h neither cell line had detectable amounts of glycogen. Still, GS activity was markedly lower in Qc than in G3 cells (see also Figure 3). Interestingly, at both 48 and 120 h, the activity of GS in Qc extracts after prior incubation with exogenous glycogen was about 50% of that seen in control G3 extracts. Since GS protein content was reduced by

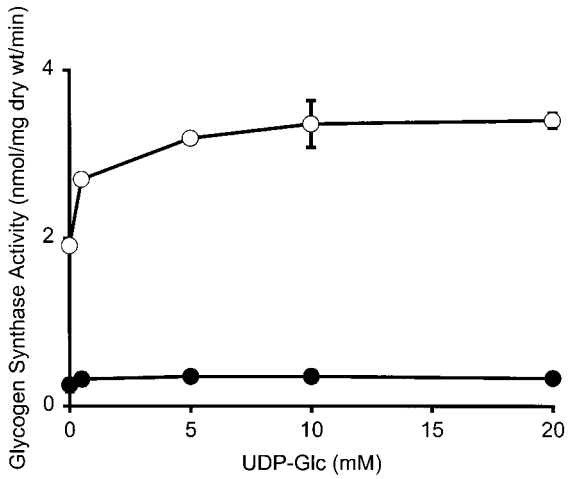

Figure 6 Concentration dependency of UDP-Glc on reactivation of GS *in vitro* in G3 but not Qc extracts

Prior incubation was performed under the conditions described in the legend to Figure 5. The final concentration of UDP-Glc during the assay of GS in this experiment was 2.7 mM. Values are means ± S.E.M. from three dishes cultured for 120 h. ○, G3 cells; ●, Qc cells.

Table 3 Binding of radioactive UDP-[6-^{3}H]Glc to GS in cell extracts

Values are means ± S.E.M. from 3–5 dishes and are given in c.p.m./mg of protein. Cells were cultured for 120 h. Extracts were incubated with or without glycogen (5 mg/ml) and UDP-[6-^{3}H]Glc for 48 h at 4 °C, followed by addition of GS antibody for an additional 24 h. Protein L–agarose beads were then added and 24 h later samples were centrifuged. The pellets were washed and counted. $^*P < 0.05$ versus G3.

	Binding (c.p.m./mg of protein)	
Glycogen	−	+
G3	7711 ± 3832	5020 ± 946
Qc	41 ± 41*	2571 ± 354*

about 50 % in Qc cells, this suggests that we were successful in fully re-activating GS in the Qc cells.

The finding that incubation with exogenous UDP-Glc resulted in significant activation of GS from G3 cells but not in Qc cells raised the possibility that the affinity of GS from Qc cells for UDP-Glc was decreased. If so, then increasing the concentration of UDP-Glc during prior incubation should increase the activity of GS. Such a concentration-dependent activation of GS was observed in G3 extracts (Figure 6). However, no activating effect in Qc extracts was observed, even at 20 mM UDP-Glc. This suggested that GS in Qc cells had lost the ability to bind UDP-Glc.

To test this possibility, cell extracts were incubated with radioactive UDP-Glc, and the amount of radioactivity associated with immunoprecipitated GS was determined. In G3 cells, significant radioactivity was detected in the immunoprecipitate, but none was detected in the Qc immunoprecipitate (Table 3). Since prior incubation with glycogen fully restored GS activity in the Qc cells (Figure 5), we determined whether this was associated with restoration of UDP-Glc binding to GS. Indeed, binding of UDP-Glc to GS in Qc extracts incubated with glycogen was increased to about 50 % of that seen in G3 cells (Table 3). Considering that the amount of GS protein was reduced by 50 % in the Qc cells, this indicates that glycogen fully restored UDP-Glc binding to GS.

DISCUSSION

A major finding in the present study was the virtually complete lack of GS activity in Qc cells. About 50 % of the decreased activity could be accounted for by a decrease in the amount of GS protein. But how UDPG:PP gene inactivation results in inactivation of the remaining protein is not straightforward. One limitation with gene-modification studies is that adaptive changes may occur in the expression of known or unknown proteins (e.g. [36]) that affect the parameter of investigation. One approach to circumvent this limitation is to study the variable of interest under *in vitro* conditions where adaptive responses are minimized or abolished. Inactivation of UDPG:PP resulted in low levels of both UDP-Glc and glycogen, either of which could be responsible for the loss of GS activity. It is well established that glycogen is needed to stabilize GS activity *in vitro* (e.g. [35]) and it might be argued that the inactivation of GS in Qc cells is due to low levels of endogenous glycogen. This possibility can be ruled out for the cells studied 48 h after seeding since neither cell line had detectable amounts of glycogen, and still GS activity in Qc cells was less than 5 % of that observed in G3 cells (Figures 3 and 5). Indeed, others have shown that virtually complete lack of endogenous glycogen can still be associated with substantial GS activity [23,29,37] and even increases in GS activity [38]. It has also been shown that the starvation-induced increase in rat heart glycogen content is associated with a 60 % decrease in total GS activity [39]. Therefore, the low GS activity in the Qc cells cannot be explained solely by lack of endogenous glycogen. More likely is that the lack of adequate UDP-Glc was responsible for the low GS activity in the Qc cells. This explanation is also supported by the finding that depletion of UDP-Glc by incubating G3 cells with pyruvate results in decreased GS activity, and is consistent with the observation that addition of exogenous UDP-Glc to purified preparations of GS prevents loss of enzyme activity during storage [40].

If our explanation is correct, then the question arises of how chronically low UDP-Glc levels in the intact cell result in virtually undetectable GS activity when measured under *in vitro* conditions? Prior incubation of Qc cell extracts with UDP-Glc did not significantly activate GS either at 48 or 120 h, while prior incubation of G3 cell extracts with UDP-Glc significantly increased GS activity at those times. It is unlikely that endogenous glycogen is required for UDP-Glc to exert its stimulatory effect during prolonged prior incubation since at 48 h the G3 cells had no significant amounts of glycogen, but GS activity was still increased by prior incubation with UDP-Glc (Figure 5B). Our findings demonstrate that UDP-Glc binding to GS was lost in the Qc cells. Moreover, the glycogen-mediated restoration of GS activity in the Qc cells could be explained by the restoration of UDP-Glc binding to GS. These data may also provide a mechanism for how glycogen stabilizes GS during storage; namely, glycogen maintains the ability of GS to bind UDP-Glc. Thus in addition to being a substrate for GS and regulating the cellular localization of GS [16], UDP-Glc availability may also dictate the extent of GS activity by a non-substrate-dependent mechanism.

Finally, one could question the relevance of fibroblasts as a model to study glycogen metabolism. However, fibroblasts have previously been used as a diagnostic tool for glycogen storage diseases [41], as well as studying glycogen biogenesis [42] and GS activity [43]. Indeed, the present study demonstrates that, as in skeletal muscle, insulin accelerates both glucose uptake and

glycogen synthesis (G3). Therefore, the results from the present study are likely to be of physiological significance.

In conclusion, our findings indicate that UDP-Glc, in addition to being the glucosyl donor for GS, can regulate glycogen biogenesis via non-substrate-dependent control of GS activity.

This research was supported by grants from the Swedish Cancer Society (no. 3826) and the Lundström Foundation.

REFERENCES

1 Bogardus, C. and Lillioja, S. (1990) Where all the glucose doesn't go in non-insulin-dependent diabetes mellitus. N. Engl. J. Med. **322**, 262–263

2 Smythe, C. and Cohen, P. (1991) The discovery of glycogenin and the priming mechanism for glycogen biogenesis. Eur. J. Biochem. **200**, 625–631

3 Alonso, M. D., Lomako, J., Lomako, W. M. and Whelan, W. J. (1995) A new look at the biogenesis of glycogen. FASEB J. **9**, 1126–1137

4 Roach, P. J. and Skurat, A. V. (1997) Self-glucosylating initiator proteins and their role in glycogen biosynthesis. Progr. Nucleic Acid Res. Mol. Biol. **57**, 289–316

5 Friedman, D. L. and Larner, J. (1963) Studies on UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochemistry **2**, 669–675

6 Roach, P. J., Takeda, Y. and Larner, J. (1976) Rabbit skeletal muscle glycogen synthase. I. Relationship between phosphorylation state and kinetic properties. J. Biol. Chem. **251**, 1913–1919

7 Roach, P. J. (1990) Control of glycogen synthase by hierarchal protein phosphorylation. FASEB J. **4**, 2961–2968

8 Spiro, M. J. and Spiro, R. G. (1971) Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of basement membranes and collagens. J. Biol. Chem. **246**, 4899–4909

9 Larner, J., Takeda, Y. and Hizukuri, S. (1976) The influence of chain size and molecular weight on the kinetic constants for the span glucose to polysaccharide for rabbit muscle glycogen synthase. Mol. Cell. Biochem. **12**, 131–136

10 Alonso, M. D., Lagzdins, E. J., Lomako, J., Lomako, W. M. and Whelan, W. J. (1995) New and specific nucleoside diphosphate glucose substrates for glycogenin. FEBS Lett. **359**, 110–112

11 Just, I., Selzer, J., Wilm, M., Eichel-Streiber, C., Mann, M. and Aktories, K. (1995) Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature (London) **375**, 500–503

12 Sousa, M. and Parodi, A. J. (1995) The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase. EMBO J. **14**, 4196–4203

13 Chambers, J. K., Macdonald, L. E., Sarau, H. M., Ames, R. S., Freeman, K., Foley, J. J., Zhu, Y., McLaughlin, M. M., Murdock, P., McMillan, L. et al. (2000) A G protein-coupled receptor for UDP-glucose. J. Biol. Chem. **275**, 10767–10771

14 Wittenberger, T., Schaller, H. C. and Hellebrand, S. (2001) An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J. Mol. Biol. **307**, 799–813

15 Gustafson, L. A., Jumelle-Laclau, M. N., van Woerkom, G. M., van Kuilenburg, A. B. and Meijer, A. J. (1997) Cell swelling and glycogen metabolism in hepatocytes from fasted rats. Biochim. Biophys. Acta **1318**, 184–190

16 Brady, M. J., Kartha, P. M., Aysola, A. A. and Saltiel, A. R. (1999) The role of glucose metabolites in the activation and translocation of glycogen synthase by insulin in 3T3-L1 adipocytes. J. Biol. Chem. **274**, 27497–27504

17 Chaves-Olarte, E., Florin, I., Boquet, P., Popoff, M., Eichel-Streiber, C. and Thelestam, M. (1996) UDP-glucose deficiency in a mutant cell line protects against glucosyltransferase toxins from *Clostridium difficile* and *Clostridium sordellii*. J. Biol. Chem. **271**, 6925–6932

18 Flores-Diaz, M., Alape-Giron, A., Persson, B., Pollesello, P., Moos, M., Eichel-Streiber, C., Thelestam, M. and Florin, I. (1997) Cellular UDP-glucose deficiency caused by a single point mutation in the UDP-glucose pyrophosphorylase gene. J. Biol. Chem. **272**, 23784–23791

19 Flores-Diaz, M., Alape-Giron, A., Titball, R. W., Moos, M., Guillouard, I., Cole, S., Howells, A. M., Eichel-Streiber, C., Florin, I. and Thelestam, M. (1998) UDP-glucose deficiency causes hypersensitivity to the cytotoxic effect of *Clostridium perfringens* phospholipase C. J. Biol. Chem. **273**, 24433–24438

20 Thomas, J. A., Schlender, K. K. and Larner, J. (1968) A rapid filter paper assay for UDPglucose-glycogen glucosyltransferase, including an improved biosynthesis of UDP-^{14}C-glucose. Anal. Biochem. **25**, 486–499

21 Guinovart, J. J., Salavert, A., Massague, J., Ciudad, C. J., Salsas, E. and Itarte, E. (1979) Glycogen synthase: a new activity ratio assay expressing a high sensitivity to the phosphorylation state. FEBS Lett. **106**, 284–288

22 Tan, A. W. and Nuttall, F. Q. (1975) Characteristics of the dephosphorylated form of phosphorylase purified from rat liver and measurement of its activity in crude liver preparations. Biochim. Biophys. Acta **410**, 45–60

23 Jiao, Y., Shashkina, E., Shashkin, P., Hansson, A. and Katz, A. (1999) Manganese sulfate-dependent glycosylation of endogenous glycoproteins in human skeletal muscle is catalyzed by a nonglucose 6-P-dependent glycogen synthase and not glycogenin. Biochim. Biophys. Acta **1427**, 1–12

24 Harris, R. C., Hultman, E. and Nordesjo, L. O. (1974) Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. Scand. J. Clin. Lab. Invest. **33**, 109–120

25 Lowry, O. H. and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York

26 Katz, A. and Raz, I. (1995) Rapid activation of glycogen synthase and protein phosphatase in human skeletal muscle after isometric contraction requires an intact circulation. Pflugers Arch. **431**, 259–265

27 Shashkin, P. N., Koshkin, A., Langley, D. R., Ren, J.-M., Westerblad, H. and Katz, A. (1995) Effects of CGS 9343B (a putative calmodulin antagonist) on isolated skeletal muscle: dissociation of signaling pathways for insulin-mediated activation of glycogen synthase and hexose transport. J. Biol. Chem. **270**, 25613–25618

28 Piras, R. and Staneloni, R. (1969) *In vivo* regulation of rat muscle glycogen synthetase activity. Biochemistry **8**, 2153–2160

29 Yan, Z., Spencer, M. K. and Katz, A. (1992) Effect of low glycogen on glycogen synthase in human muscle during and after exercise. Acta Physiol. Scand. **145**, 345–352

30 Larner, J., Villar-Palasi, C. and Richman, D. J. (1960) Insulin-stimulated glycogen formation in rat diaphragm. Levels of tissue intermediates in short-term experiments. Arch. Biochem. Biophys. **86**, 56–60

31 Lawrence, Jr, J. C. and Larner, J. (1978) Activation of glycogen synthase in rat adipocytes by insulin and glucose involves increased glucose transport and phosphorylation. J. Biol. Chem. **253**, 2104–2113

32 Rosell-Perez, M., Villar-Palasi, C. and Larner, J. (1962) Studies on UDPG-glycogen transglucosylase. I. Preparation and differentiation of two activities of UDPG-glycogen transglucosylase from rat skeletal muscle. Biochemistry **1**, 763–768

33 Rosell-Perez, M. and Larner, J. (1962) Studies on UDPG-glycogen transglucosylase. II. Species variation of glucose-6-phosphate sensitivity of UDPG-glycogen transglucosylase. Biochemistry **1**, 769–772

34 Tagaya, M., Nakano, K. and Fukui, T. (1985) A new affinity labeling reagent for the active site of glycogen synthase. Uridine diphosphopyridoxal. J. Biol. Chem. **260**, 6670–6676

35 Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) The purification and properties of rabbit skeletal muscle glycogen synthase. Eur. J. Biochem. **68**, 21–30

36 Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, III, B., Johnson, R. S. and Kahn, C. R. (1994) Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature (London) **372**, 186–190

37 Nur, T., Sela, I., Webster, N. J. and Madar, Z. (1995) Starvation and refeeding regulate glycogen synthase gene expression in rat liver at the posttranscriptional level. J. Nutr. **125**, 2457–2462

38 Cheng, C., Mu, J., Farkas, I., Huang, D., Goebl, M. G. and Roach, P. J. (1995) Requirement of the self-glucosylating initiator proteins Glg1p and Glg2p for glycogen accumulation in *Saccharomyces cerevisiae*. Mol. Cell Biol. **15**, 6632–6640

39 Gannon, M. C. and Nuttall, F. Q. (1984) Effect of prolonged starvation on glycogen synthase and glycogen synthase phosphatase activity in rat heart. J. Nutr. **114**, 2147–2154

40 Miller, R. E., Miller, E. A., Fredholm, B., Yellin, J. B., Eichner, R. D., Mayer, S. E. and Steinberg, D. (1975) Enzymes regulating glycogen metabolism in swine subcutaneous adipose tissue. II. Glycogen synthase. Biochemistry **14**, 2481–2488

41 Ausems, M. G., Lochman, P., van Diggelen, O. P., Ploos van Amstel, H. K., Reuser, A. J. and Wokke, J. H. (1999) A diagnostic protocol for adult-onset glycogen storage disease type II. Neurology **52**, 851–853

42 Skurat, A. V., Lim, S. S. and Roach, P. J. (1997) Glycogen biogenesis in rat 1 fibroblasts expressing rabbit muscle glycogenin. Eur. J. Biochem. **245**, 147–155

43 Skurat, A. V., Dietrich, A. D. and Roach, P. J. (2000) Glycogen synthase sensitivity to insulin and glucose-6-phosphate is mediated by both NH_2- and COOH-terminal phosphorylation sites. Diabetes **49**, 1096–1100

Received 21 August 2002/27 November 2002; accepted 2 December 2002
Published as BJ Immediate Publication 2 December 2002, DOI 10.1042/BJ20021320