Glucose 6-phosphate causes translocation of phosphorylase in hepatocytes and inactivates the enzyme synergistically with glucose

Susan AISTON, Andrew GREEN, Mohammed MUKHTAR and Loranne AGIUS[1]

Department of Diabetes, School of Clinical Medical Sciences, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

The role of glucose 6-P (glucose 6-phosphate) in regulating the activation state of glycogen synthase and its translocation is well documented. In the present study, we investigated the effects of glucose 6-P on the activation state and compartmentation of phosphorylase in hepatocytes. Glucose 6-P levels were modulated in hepatocytes by glucokinase overexpression or inhibition with 5-thioglucose and the effects of AMP were tested using AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), which is metabolized to an AMP analogue. Inhibition of glucokinase partially counteracted the effect of glucose both on the inactivation of phosphorylase and on the translocation of phosphorylase *a* from a soluble to a particulate fraction. The increase in glucose 6-P caused by glucokinase overexpression caused translocation of phosphorylase *a* to the pellet and had additive effects with glucose on inactivation of phosphorylase. It decreased the glucose concentration that caused half-maximal inactivation from 20 to 11 mM, indicating that it acts synergistically with glucose. AICAR activated phosphorylase and counteracted the effect of glucose 6-P on phosphorylase inactivation. However, it did not counteract translocation of phosphorylase by glucose 6-P. Glucose 6-P and AICAR had opposite effects on the activation state of glycogen synthase, but they had additive effects on translocation of the enzyme to the pellet. There was a direct correlation between the translocation of phosphorylase *a* and of glycogen synthase to the pellet, suggesting that these enzymes translocate in tandem. In conclusion, glucose 6-P causes both translocation of phosphorylase and inactivation, indicating a more complex role in the regulation of glycogen metabolism than can be explained from regulation of glycogen synthase alone.

Key words: glucose 6-phosphate, glycogen synthase, hepatocyte, phosphorylase, translocation.

INTRODUCTION

Hepatic glycogen metabolism is regulated by allosteric control of glycogen synthase and phosphorylase and by changes in the phosphorylation state of these proteins [1]. Glycogen synthase is inactivated by multi-site phosphorylation, whereas phosphorylase is activated by phosphorylation of a single serine residue close to the N-terminus. The phosphorylated and unphosphorylated forms of phosphorylase are designated *a* and *b* respectively [1]. Glycogen synthase is also regulated by changes in subcellular compartmentation [2]. Glucose causes translocation of the enzyme from a soluble to a particulate fraction [3] and also from a diffuse location in the cytoplasm to the cell periphery [4,5]. Changes in the subcellular compartmentation of phosphorylase have not been reported.

Glucose 6-P (glucose 6-phosphate) is the main metabolic intermediate involved in the regulation of glycogen synthase [6]. It is an allosteric activator of the enzyme and also determines its phosphorylation state by making the enzyme a better substrate for synthase phosphatase. In addition, glucose 6-P regulates the subcellular compartmentation of glycogen synthase by causing translocation from a soluble to a particulate fraction [7,8]. Since the activation state of glycogen synthase correlates with the cellular content of glucose 6-P in hepatocytes [9,10], glycogen synthase is often described as the glucose 6-P sensor of the hepatocyte [6].

Glucose, rather than glucose 6-P, is considered to be the main small molecule regulator of phosphorylase in liver, and phosphorylase is often regarded as the glucose sensor of hepatocytes [1]. Phosphorylase exists in either a relaxed (R) or a tense (T) conformation [11]. Glucose binds to phosphorylase *a* and promotes the T state, which is less active and also a better substrate for dephosphorylation by the phosphatase. Accordingly, glucose causes dephosphorylation of phosphorylase *a* in hepatocytes [12,13]. AMP is an allosteric activator of phosphorylase and favours the R state [11]. It is a potent activator of the muscle isoform of phosphorylase *b*, but it stimulates the liver isoform [14] to a lesser extent. The activation of muscle phosphorylase *b* by AMP is competitively inhibited by glucose 6-P, which binds to the same site [15]. The liver isoform of phosphorylase *b* is virtually catalytically inactive under physiological conditions [16] and accordingly glucose 6-P has a negligible role as an allosteric inhibitor in liver. However, glucose 6-P mimics glucose in stimulating the dephosphorylation of phosphorylase *a* by the protein phosphatase *in vitro* [17–19] and it also inhibits phosphorylation of phosphorylase *b* by phosphorylase kinase by a substrate-directed mechanism [20,21]. We have shown recently that glucose 6-P also has a physiological role in regulating the activation state of phosphorylase in hepatocytes [22]. Given the complexity of the mechanism(s) by which glucose 6-P regulates glycogen synthase [2], the question is raised whether glucose 6-P also regulates phosphorylase by diverse mechanisms. The present study had three aims: (i) to determine the extent by which glucose 6-P is involved in the glucose-induced inactivation of phosphorylase; (ii) to determine whether the regulation of the activation state of phosphorylase by glucose 6-P is competitive with respect to AMP; and (iii) to explore whether glucose 6-P regulates the subcellular compartmentation of phosphorylase and if so whether this might share a similar mechanism as for glycogen synthase.

Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; glucose 6-P, glucose 6-phosphate.

[1] To whom correspondence should be addressed (e-mail Loranne.Agius@ncl.ac.uk).

MATERIALS AND METHODS

Materials

AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), 5-thioglucose, glucagon, substrates and cofactors were from Sigma–Aldrich (St. Louis, MO, U.S.A.), UDP-[6-^{3}H]glucose, [U-^{14}C]glucose 1-P and [^{32}P]ATP were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Antibody to phosphorylase was from Research Diagnostics (Flanders, NJ, U.S.A.). The recombinant adenovirus for expression of rat liver glucokinase [23] was a gift from Dr C. B. Newgard (Duke University Medical Center, Durham, NC, U.S.A.). SAMS peptide (the synthetic peptide HMRSAMSGLHLVKRR) [24] was synthesized by Dr G. Bloomberg (University of Bristol, U.K.). Sources of other reagents were as described previously [22].

Hepatocyte isolation and treatment

Hepatocytes were isolated by collagenase perfusion of the liver from *ad lib*.-fed male Wistar rats obtained from Bantin and Kingman (Hull, U.K.). The hepatocytes were suspended in minimum essential medium containing 7 % (v/v) newborn calf serum and plated in multi-well dishes [25]. Cell attachment was allowed for 4 h, except in experiments involving glucokinase overexpression using recombinant adenovirus [22], where the medium was changed 2 h after plating with serum-free medium containing adenovirus. The medium was replaced 4 h after plating with serum-free minimum essential medium containing 5 mM glucose and 10 nM dexamethasone, and the cells were cultured for 16 h [25]. Incubations for determining the effects of substrates or other additions were performed after 16 h culture.

Enzyme assays

For the determination of phosphorylase *a* and glycogen synthase, incubations were terminated by snap-freezing in liquid nitrogen. For the determination of phosphorylase *a*, cells were extracted in 25 mM Hepes, 150 mM potassium fluoride, 0.5 mM EDTA, 1 mM PMSF and 1 mM benzamidine (pH 7.5), and for glycogen synthase they were extracted in 10 mM Tris, 150 mM potassium fluoride, 15 mM EDTA, 600 mM sucrose, 50 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine and 3.5 μg/ml leupeptin (pH 7.5) [26]. The cells were scraped in extraction buffer at a protein concentration of approx. 1 mg/ml and the extracts were sonicated. Assays were performed either on the whole homogenate or on the supernatant and pellet fractions after centrifuging at 13 000 ***g*** (10 min, 4 °C). Activities were expressed as m-units/mg of protein in the whole homogenate. Unless otherwise indicated, phosphorylase *a* was assayed spectrometrically in the whole homogenate and the supernatant fractions in the glycogenolytic direction by the phosphoglucomutase/glucose 6-P dehydrogenase-coupled assay [27]. This assay underestimates activity in the pellet and to a lesser extent in the homogenate relative to the supernatant because of interference from NADPH oxidase and/or degradation of glucose 6-P by glucose 6-phosphatase. The distribution of phosphorylase *a* between the supernatant and particulate fraction was determined radiochemically from the incorporation of [U-^{14}C]glucose 1-P into glycogen in the presence of 1 mM caffeine [28]. The concentration of glucose that causes half-maximal effect ($S_{0.5}$) on inactivation of phosphorylase in the homogenate or supernatant by the spectrometric assay was determined from Hill plots using the Fig. P. Biosoft program. Glycogen synthase (active and total) was assayed radiochemically from the incorporation of UDP-[1-^{3}H]-glucose into glycogen in the absence or presence of 6.7 mM glucose 6-P respectively [29]. The active form of the enzyme is expressed as the activity ratio (−/+ glucose 6-P), whereas the total glycogen synthase (+ glucose 6-P) is expressed as m-units/mg of cell protein: 1 m-unit is defined as the amount that converts 1 nmol/min at 30 °C.

Glucokinase activity in untreated cells and cells treated with recombinant adenovirus encoding rat liver glucokinase (AdCMV-GK) was determined by the digitonin-release assay [30]. Glucokinase overexpression was 8–10-fold higher than endogenous activity.

For the determination of the activity of AMPK (AMP-activated protein kinase), cells were permeabilized with digitonin (0.2 mg/ml for 20 min) as described in [31]. The digitonin eluate was then sedimented with ammonium sulphate and the precipitate from the phosphorylation of SAMS peptide with [^{32}P]ATP was assayed as described in [31]. Activity is expressed as pmol · min^{-1} · (mg of protein)$^{-1}$.

Metabolite determination

For determination of glucose 6-P, the incubations were terminated by snap-freezing in liquid nitrogen. They were extracted in $HClO_4$ and glucose 6-P was assayed fluorimetrically [22].

Immunoblotting

The distribution of phosphorylase and glycogen synthase between the supernatant and pellet fractions was determined by immunoblotting using a mouse monoclonal antibody to human phosphorylase *b* isoform (Clone 3G1) from Research Diagnostics or a rabbit antibody raised against the sequence I^{690}PKGKKKL-HGEYKN^{703} [5] of rat liver glycogen synthase (Sigma-GenoSys, Cambridge, U.K.). Cells were extracted as for the respective enzymic assays and protein was fractionated by SDS/PAGE and transferred to nitrocellulose. Membranes were probed with primary antibody (1:2000 dilution for phosphorylase and 1:500 for glycogen synthase) followed by incubation with peroxidase-conjugated antibodies against mouse or rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.). Immunoreactive bands were visualized using an ECL® kit (Amersham Biosciences) and quantified by densitometry.

RESULTS

Relative effects of glucose and glucose 6-P on phosphorylase determined using a glucokinase inhibitor

To determine the extent to which glucose 6-P contributes to the inactivation of phosphorylase caused by high glucose concentration, we used 5-thioglucose, a potent competitive inhibitor of glucokinase [32]. 5-Thioglucose (3 mM) counteracted the increase in glucose 6-P caused by 10–35 mM glucose (Figure 1A). Glucose (20–35 mM) decreased the activity of phosphorylase *a* in the whole homogenate by 34–63 % relative to the activity at 5 mM glucose, and this effect of glucose was partially counteracted by 5-thioglucose, which lowered the inhibition to 24–38 % (Figure 1B). This indicates that 32–43 % of the inactivation of phosphorylase at high glucose concentration is due to glucose 6-P. In the same experiments, the activity of phosphorylase *a* in the high-speed supernatant was decreased by 26–65 % at 15–35 mM glucose and 5-thioglucose partially reversed this decrease (17–38 %; Figure 1C). The concentration of glucose that caused $S_{0.5}$ in decreasing phosphorylase *a* activity was lower in the supernatant when compared with the homogenate and 5-thioglucose increased $S_{0.5}$ for glucose in the supernatant (Table 1). Together these results suggest that glucose 6-P acts synergistically with glucose by increasing the affinity for glucose. The greater potency of glucose at decreasing phosphorylase *a* in the supernatant relative to the

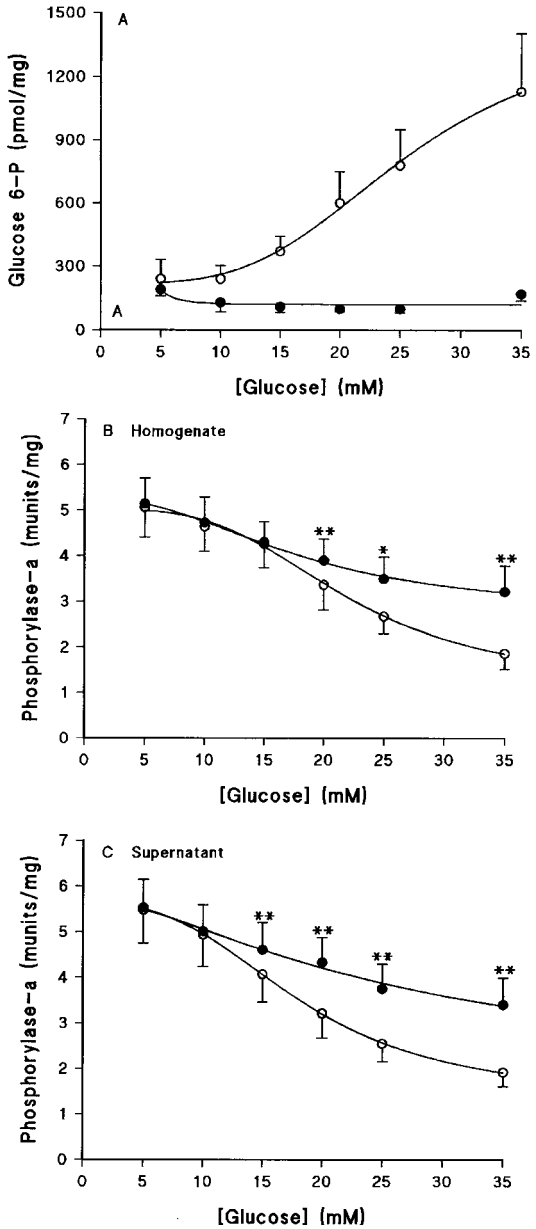

Figure 1 Increase in glucose 6-P and inactivation of phosphorylase by glucose counteracted completely and partially by 5-thioglucose respectively

Hepatocytes were incubated for 1 h with the glucose concentrations indicated in the absence (○) or presence (●) of 3 mM 5-thioglucose. (**A**) Glucose 6-P (pmol/mg of protein), (**B**, **C**) activity of phosphorylase *a* (m-units/mg of cell protein) determined spectrometrically in the whole homogenate (**B**) or supernatant (**C**). Means ± S.E.M. from four (**A**) or six (**B**, **C**) experiments. $^{*}P < 0.05$, $^{**}P < 0.005$, presence versus absence of 5-thioglucose.

homogenate suggests a role for either glucose or glucose 6-P in causing translocation of enzyme from the supernatant to the pellet. To test this possibility, the activity of phosphorylase *a* was determined radiochemically in the supernatant and pellet fractions. At 25 mM glucose, there was an increase in the activity of phosphorylase *a* associated with the pellet both in absolute terms (3-fold; Figure 2A) and when expressed as a percentage of total phosphorylase *a* activity in the supernatant and pellet (3.4-fold; Figure 2B), relative to values at 5 mM glucose. 5-Thioglucose partially counteracted (75%) this effect, indicating that glucose

Table 1 Glucose 6-P increases the affinity for glucose

The concentration of glucose that causes half-maximal effect in decreasing the activity of phosphorylase *a* in the experiments in Figures 1 and 3 was determined from Hill plots. $^{*}P < 0.05$, $^{**}P < 0.005$ relative to respective control; $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ relative to corresponding homogenate.

	Glucose $S_{0.5}$ (mM)	
	Homogenate	Supernatant
Figure 1		
Control	19.6 ± 0.6	15.9 ± 0.8$^{\#}$
5-Thioglucose	18.4 ± 1.3	22.8 ± 1.2**
Figure 3		
Control	20.4 ± 0.8	17.4 ± 1.3$^{\#\#}$
Glucokinase overexpression	10.7 ± 1.2**	12.6 ± 0.8*

6-P causes translocation of phosphorylase *a* from the supernatant to the pellet, in addition to an overall decrease in phosphorylase *a* in the whole homogenate. When the increase in activity of phosphorylase *a* caused by 25 mM glucose in the pellet was plotted against the respective decrease in phosphorylase *a* in the whole homogenate, there was an inverse correlation (Figure 2C). This suggests either that inactivation of phosphorylase in the whole homogenate is a sequential event to translocation of phosphorylase *a* from the supernatant to the pellet fraction or that glucose 6-P causes either translocation of phosphorylase *a* to the pellet or dephosphorylation in the supernatant.

Relative effects of glucose and glucose 6-P determined by glucokinase overexpression

To determine the effects of glucose on the activation state of phosphorylase at saturating concentrations of glucose 6-P, cells overexpressing glucokinase by 6–8-fold above endogenous levels by treatment with recombinant adenovirus were used. At 5 mM glucose, cells overexpressing glucokinase had an increased glucose 6-P content (4-fold), in agreement with previous findings [22,33] and similar to the glucose 6-P content of untreated cells incubated with 25 mM glucose (Figure 3A). Glucokinase overexpression significantly decreased the activity of phosphorylase *a* in the whole homogenate at all glucose concentrations tested, indicating that glucose 6-P has an additive effect with glucose in causing inactivation of phosphorylase (Figure 3B). At 5–10 mM glucose, glucokinase overexpression caused a greater decrease in the activity of phosphorylase *a* in the supernatant (53–62%) when compared in the homogenate (26–45%), indicating that glucose 6-P causes translocation as well as inactivation. However, at higher glucose concentrations (20–35 mM), the decrease was similar in the homogenate (53–66%) and supernatant (49–59%), suggesting inactivation after translocation to the pellet (Figures 3B and 3C) or preferential inactivation in the supernatant. Glucokinase overexpression decreased the concentration of glucose that caused half-maximal inactivation of phosphorylase from 20 to 11 mM in the whole homogenate and from 17 to 13 mM in the supernatant, indicating that glucose 6-P acts synergistically with glucose.

AICAR activates phosphorylase and counteracts inactivation by glucose 6-P

Since glucose 6-P binds to phosphorylase at the AMP-binding site [11], we used an AMP analogue AICAR, which is metabolized

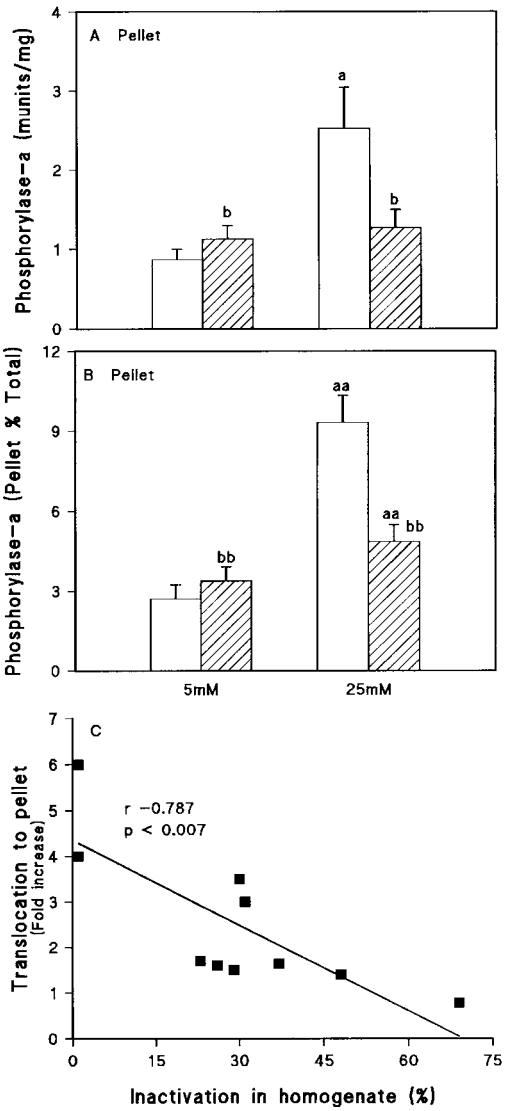

Figure 2 Glucose-induced translocation of phosphorylase *a* to the pellet counteracted by 5-thioglucose

Hepatocytes were incubated for 1 h with 5 or 25 mM glucose in the absence (open bars) or presence (hatched bars) of 3 mM 5-thioglucose. Phosphorylase *a* activity was determined radiochemically in the homogenate, supernatant and pellet. (**A**) Phosphorylase *a* activity in the pellet expressed as m-units/mg of whole cell protein. (**B**) Phosphorylase *a* activity in the pellet expressed as percentage of the activity in the supernatant plus pellet. (**C**) Fold increase in phosphorylase *a* activity in the pellet at 25 mM glucose relative to 5 mM glucose versus fractional decrease in phosphorylase *a* in the homogenate at 25 mM glucose relative to 5 mM. (**A**, **B**) Means ± S.E.M. from five experiments; (**C**) individual data from ten experiments. $^{a}P < 0.05$, $^{aa}P < 0.005$, relative to the corresponding 5 mM glucose; $^{b}P < 0.05$, $^{bb}P < 0.005$, relative to no 5-thioglucose.

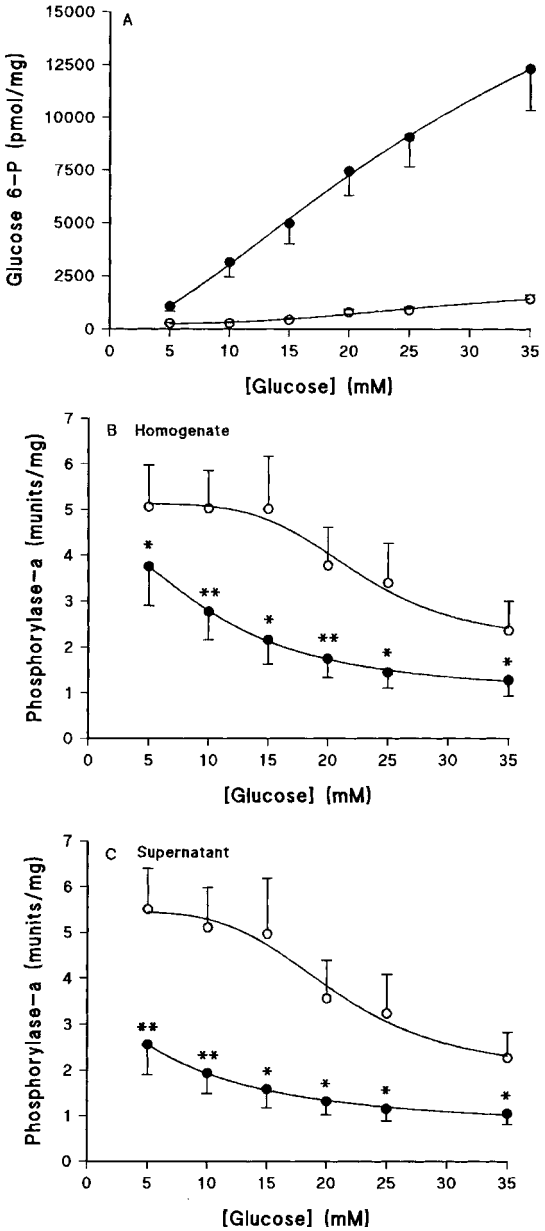

Figure 3 Additive effect of glucokinase overexpression with glucose on inactivation of phosphorylase

Hepatocytes were either untreated (○) or treated with recombinant adenovirus (●) for overexpression of glucokinase by 8–10-fold above endogenous activity. After 16 h culture they were incubated for 1 h with the glucose concentrations indicated. (**A**) Glucose 6-P (pmol/mg of protein). (**B**, **C**) Activity of phosphorylase *a* (m-units/mg of cell protein) determined spectrometrically in the homogenate (**B**) or supernatant (**C**). Means ± S.E.M. from six (**A**) or seven (**B**, **C**) experiments. $^{*}P < 0.05$; $^{**}P < 0.005$, glucokinase overexpression versus respective controls.

by hepatocytes to 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide [34], to test whether the effects of glucose 6-P can be explained by competition with AMP. Since AICAR also causes activation of AMPK, we compared the effects of AICAR with 0.5 mM cyanide, which also activates AMPK (Figure 4A) by depletion of ATP (results not shown). This concentration of cyanide, unlike AICAR, did not activate phosphorylase, despite causing a similar activation of AMPK. This indicates that the activation of phosphorylase by AICAR cannot be explained by activation of AMPK. When the combined effects of AICAR and glucokinase overexpression were tested at 10 mM glucose, AICAR counteracted the effects of glucokinase overexpression on phosphorylase *a* activity in the homogenate but not in the supernatant, suggesting counteraction of inactivation but not translocation (Figure 4B versus 4C).

AICAR (200–500 μM) increased the activity of phosphorylase *a* ($P < 0.001$) at all glucose concentrations tested (Figure 5A) and it also lowered ($P < 0.05$) the hepatocyte content of glucose 6-P by 50 % at glucose concentrations higher than 15 mM (Figure 5B) in agreement with previous findings by Vincent et al. [35]. The

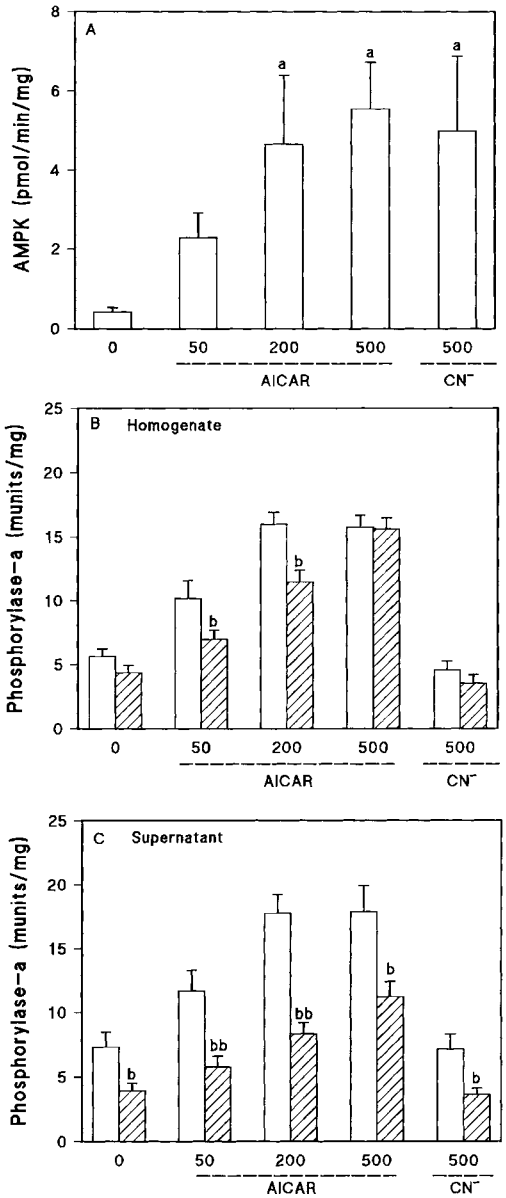

Figure 4 Effects of AICAR and cyanide on AMPK and phosphorylase activity

Hepatocytes were either untreated (open bars) or treated with recombinant adenovirus for expression of glucokinase (hatched bars). After 16 h culture, they were incubated for 1 h with the concentrations (μM) of AICAR or cyanide indicated at either 5 mM (**A**) or 10 mM glucose (**B**, **C**). (**A**) AMPK activity. (**B**, **C**) Activity of phosphorylase *a* determined spectrometrically in the homogenate (**B**) and supernatant (**C**) fractions. Means $\pm$ S.E.M. from four experiments. $^{a}P < 0.05$, relative to no additions (**A**); $^{b}P < 0.05$, $^{bb}P < 0.005$, relative to no glucokinase overexpression (**B**, **C**).

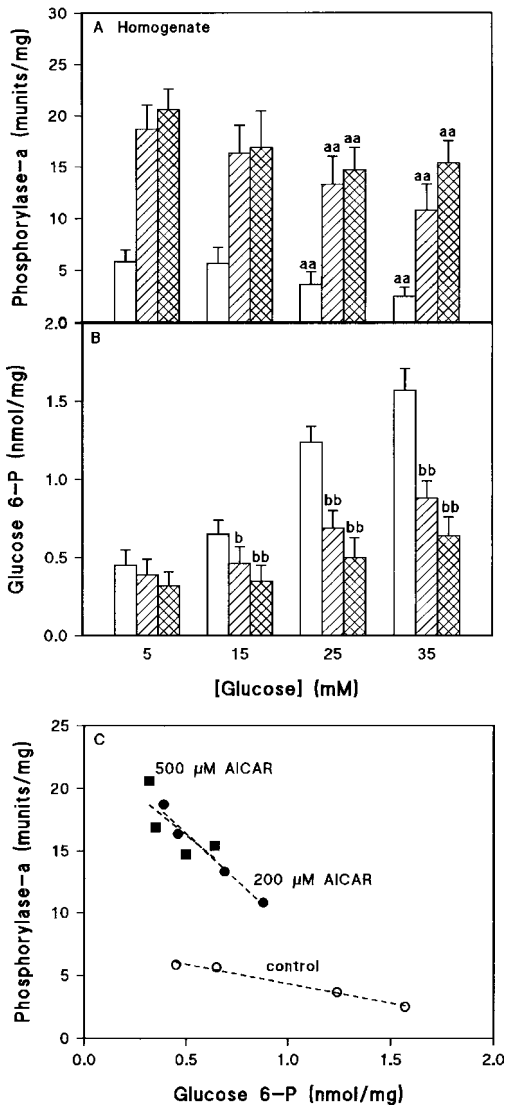

Figure 5 Greater activation of phosphorylase by AICAR than predicted by suppression of glucose 6-P

Hepatocytes were incubated for 1 h with the glucose concentrations indicated in either the absence (open bars) or presence of 200 μM (hatched bars) or 500 μM (crossed bars) AICAR. (**A**) Phosphorylase *a* activity in the whole homogenate. (**B**) Cell content of glucose 6-P. (**C**) Plot of phosphorylase *a* activity against glucose 6-P. Means $\pm$ S.E.M. from seven experiments. $^{aa}P < 0.005$, relative to 5 mM glucose (**A**); $^{b}P < 0.05$; $^{bb}P < 0.005$, AICAR relative to respective control (**B**).

activation of phosphorylase by AICAR was more than the value obtained from the suppression of glucose 6-P (Figure 5C), consistent with the activation of phosphorylase by the AMP analogue, additional to the lowering of glucose 6-P.

AICAR and glucokinase overexpression have additive effects on translocation

The interactions between AICAR and glucose 6-P on translocation were tested using the radiochemical assay for phosphorylase *a* activity in the supernatant and pellet (Figure 6) and by immunoblotting for total phosphorylase protein (Figure 7). AICAR increased the activity of phosphorylase *a* by 2-fold in the homogenate and supernatant (Figures 6A and 6B) and by 3–4-fold in the pellet (Figure 6C). Glucokinase overexpression increased the activity of phosphorylase *a* in the pellet (Figure 6C) despite an overall decrease in phosphorylase *a* in the whole homogenate (Figure 6A). AICAR and glucokinase overexpression had additive effects on the activity of phosphorylase *a* in the pellet (Figure 6C) and on the fractional distribution of phosphorylase *a* in the pellet (Figure 6D), despite having opposite effects on the activation state of phosphorylase *a* in the whole homogenate. To test whether the additive effects of AICAR and glucokinase overexpression on phosphorylase *a* activity in the pellet were due to a specific effect of the AMP analogue, cells overexpressing glucokinase were

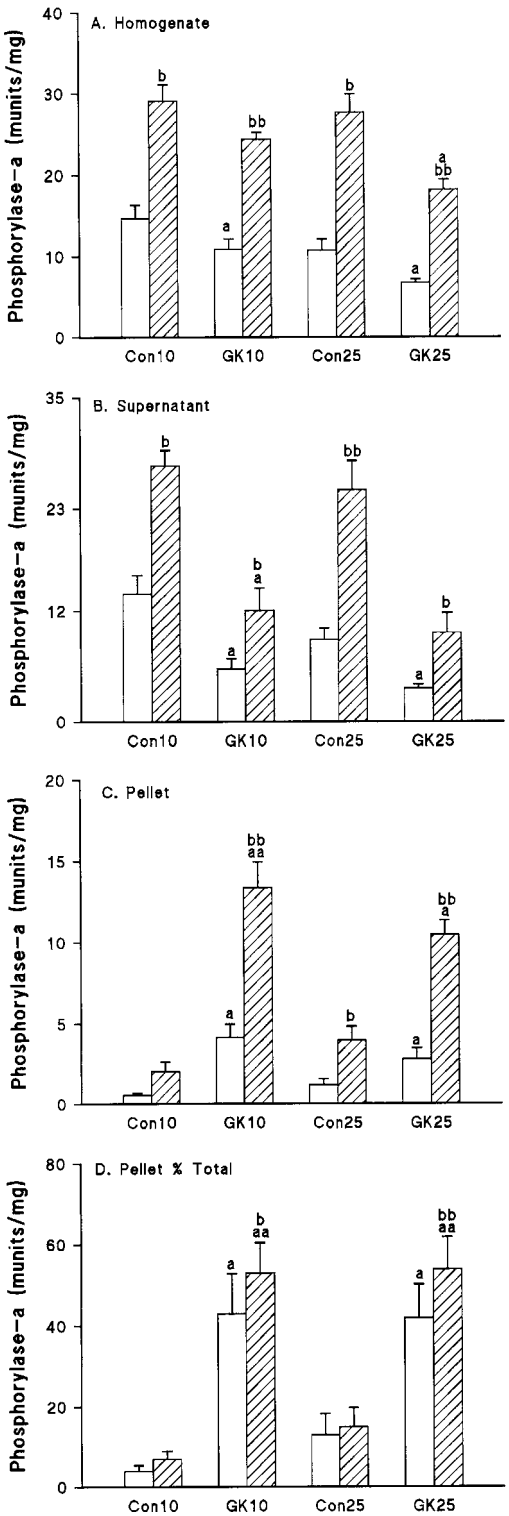

Figure 6 Combined effects of AICAR and glucokinase overexpression on phosphorylase *a* activity and translocation

Hepatocytes were either untreated (Con) or treated with recombinant adenovirus for expression of glucokinase (GK) as in Figure 3. They were then incubated for 1 h with 10 or 25 mM glucose as indicated in the absence (open bars) or presence (hatched bars) of 500 μM AICAR. Phosphorylase *a* activity was determined radiochemically in the homogenate (**A**), supernatant (**B**) or pellet (**C**) fractions (m-units/mg). (**D**) Activity in the pellet fraction is expressed as percentage of supernatant plus pellet activity. Means $\pm$ S.E.M. from four experiments. $^{a}P < 0.05$, $^{aa}P < 0.005$, glucokinase overexpression versus respective controls; $^{b}P < 0.05$, $^{bb}P < 0.005$, AICAR relative to respective control.

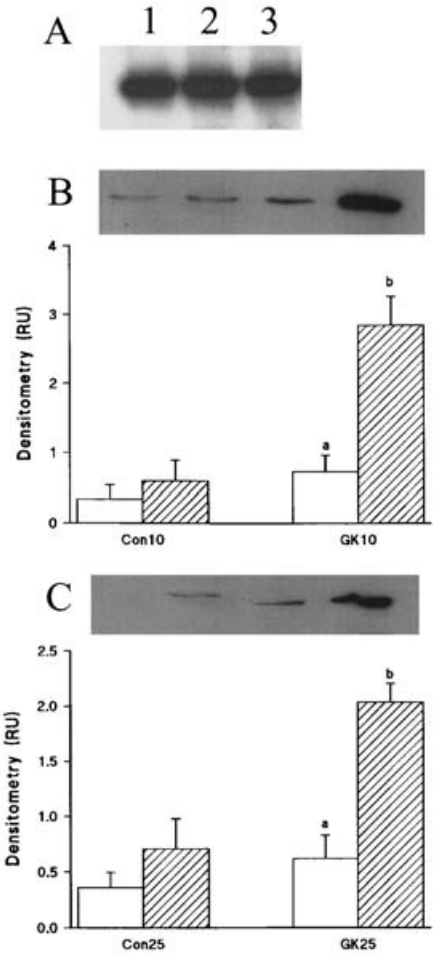

Figure 7 Translocation of phosphorylase protein to the pellet determined by immunoblotting

(**A**) Immunoblot showing immunoreactivity to phosphorylase in cells that were either untreated (lane 1) or incubated with CP-91149 (lane 2) of glucagon (lane 3), showing lack of selectivity with an antibody to phosphorylase *a* and *b*. (**B**, **C**) Experimental conditions were as in Figure 6. Cells were either untreated (Con) or overexpressing glucokinase (GK) and were incubated in the absence (open bars) or presence of 500 μM AICAR at 10 mM (**B**) or 25 mM (**C**) glucose. The protein in the supernatant and pellet was fractionated by SDS/PAGE and immunoblotted with the phosphorylase antibody. RU, Relative densitometric units. Means $\pm$ S.E.M. from four experiments; $^{a}P < 0.05$, GK relative to control; $^{b}P < 0.05$, AICAR relative to no AICAR.

incubated with glucagon (100 nM), which, similar to AICAR, causes activation of phosphorylase in the homogenate. Glucagon-like AICAR had additive effects on phosphorylase *a* in the pellet (results not shown), suggesting that the additive effect of AICAR and glucokinase overexpression of phosphorylase *a* in the pellet is due to the increase in the activation state of phosphorylase in the supernatant or inhibition of dephosphorylation of phosphorylase *a* in the pellet, rather than a specific effect of the AMP analogue.

These findings suggest that glucokinase overexpression causes translocation of phosphorylase *a* from the soluble to the particulate fraction and that AICAR has an additive effect on phosphorylase *a* activity in the pellet. To confirm that the increased activity of phosphorylase *a* in the pellet under these conditions is due to translocation of the protein from the soluble to the particulate fraction, in contrast with the selective activation of pre-existing phosphorylase *b* in the pellet, we determined the

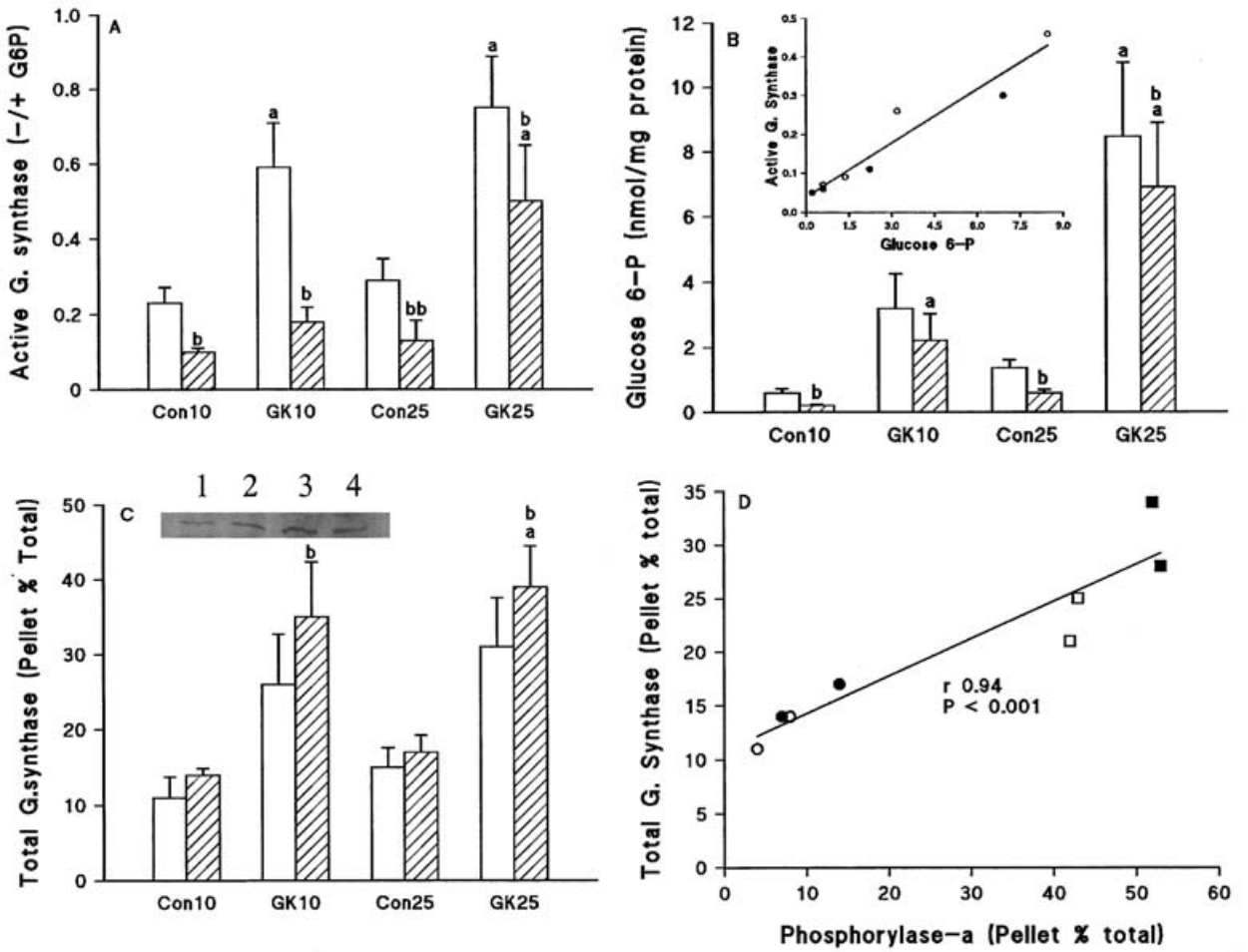

Figure 8 Translocation of glycogen synthase correlates with translocation of phosphorylase

Experimental conditions were as in Figure 6. (**A**) The activity ratio of glycogen synthase (−/+ glucose 6-P) in the whole homogenate. (**B**) Glucose 6-P (nmol/mg of protein); (**B**) inset: active glycogen synthase versus glucose 6-P. (**C**) The activity of total glycogen synthase (+ glucose 6-P) in the pellet fraction as percentage of supernatant + pellet. The immunoblot shows glycogen synthase immunoreactivity in the pellet at 10 mM glucose: lane 1, control; lane 2, AICAR; lane 3, GK10 overexpression; lane 4, GK10 overexpression + AICAR. (**D**) Correlation between the distribution of total glycogen synthase (**C**) and of phosphorylase *a* (Figure 6D) in the pellet fraction. $^{a}P < 0.05$, glucokinase overexpression versus respective controls; $^{b}P < 0.05$, $^{bb}P < 0.005$, AICAR relative to respective control.

distribution of total phosphorylase by immunoblotting using an antibody against the human brain isoform. We confirmed using recombinant human liver and brain phosphorylase isoforms and the rabbit muscle isoform that this antibody is not isoform-specific (A. Green, unpublished work). This antibody is not selective for either the phosphorylated or unphosphorylated forms of the enzyme, because pretreatment of cells with CP-91149 that causes dephosphorylation [36], or with glucagon that causes phosphorylation, had no effect on immunoreactivity in the whole homogenate (Figure 7A). Overexpression of glucokinase and treatment with AICAR had additive effects on phosphorylase immunoreactivity in the pellet at both 10 and 25 mM glucose (Figures 7B and 7C), and similar trends were observed when the immunoreactivity in the pellet was expressed as a percentage of the value in the whole homogenate (control, 10 ± 6; AICAR, 20 ± 11; glucokinase overexpression, 21 ± 8; glucokinase overexpression + AICAR, $61 \pm 10\%$; means ± S.E.M., $n = 4$). This confirms that there is a net translocation of phosphorylase protein to the pellet that is greatest in cells overexpressing glucokinase and treated with AICAR.

Translocation of phosphorylase correlates with translocation of glycogen synthase

Glucose 6-P causes translocation of glycogen synthase from the supernatant to the particulate fraction of hepatocytes [7,8]. However, the effects of AICAR or AMP on translocation of glycogen synthase have not been reported. To test whether glycogen synthase and phosphorylase translocate in parallel in response to glucokinase overexpression and AICAR, we determined the distribution of glycogen synthase (assayed in the presence of glucose 6-P) in the supernatant and pellet fractions as well as the activity ratio of the enzyme in the whole homogenate. Glycogen synthase was activated by glucokinase overexpression, as expected [33], and inactivated by AICAR (Figure 8A). The latter can be explained by the suppression of glucose 6-P (Figure 8B) as shown by the correlation (Figure 8B, inset). The relative distribution of glycogen synthase in the pellet (Figure 8C) showed similar trends as with phosphorylase *a* activity (Figure 6), and immunoreactive protein (Figure 7) and was increased in the pellet additively by glucokinase overexpression and AICAR. Translocation

of glycogen synthase was also confirmed by immunoblotting (Figure 8C, inset). There was a positive correlation between translocation of phosphorylase *a* and glycogen synthase to the pellet (Figure 8D). It is noteworthy that whereas glucokinase overexpression stimulates glycogen synthesis [33], AICAR inhibits glycogen synthesis [25 mM glucose, 92 ± 8; $+500\ \mu$M AICAR, 17 ± 2; nmol $\cdot$ (3 h)$^{-1}$ $\cdot$ mg^{-1}, means $\pm$ S.E.M., $n = 3$], consistent with inactivation of glycogen synthase and activation of phosphorylase. Thus translocation of glycogen synthase does not correlate with glycogen synthesis.

DISCUSSION

The role of glucose 6-P in regulating the activation state of glycogen synthase [9,10] and its subcellular compartmentation [7,8,37] is well established. The activation state of glycogen synthase in hepatocytes correlates with the cell content of glucose 6-P irrespective of whether this is derived from the glucose or from gluconeogenic precursors. This is explained by a conformational change in glycogen synthase on binding glucose 6-P, which makes the enzyme a better substrate for dephosphorylation by the synthase phosphatase [6]. In the present study, we confirmed a direct correlation between the activation state of glycogen synthase and the cell content of glucose 6-P in cells overexpressing glucokinase and incubated with or without AICAR, which lowers glucose 6-P [34]. Although the role of glucose in regulating the activation state of phosphorylase is well documented [12,13], the physiological role of glucose 6-P in regulating the activation state of phosphorylase in hepatocytes has only been reported recently [22].

The aim of the present study was to determine the relative roles of glucose and glucose 6-P in regulating the activation state of phosphorylase and to investigate whether the actions of glucose 6-P can be explained by competition with AMP. Several lines of evidence from this study suggest that glucose 6-P has a dual role in regulating both the activation state of phosphorylase and its subcellular location analogous to its effects on glycogen synthase and that the action of glucose 6-P on the activation state of phosphorylase is synergistic with glucose. Thus inhibition of glucose phosphorylation counteracts by 40% the decrease in phosphorylase *a* activity caused by glucose, whereas glucokinase overexpression has an additive effect with glucose. Moreover, glucose 6-P also markedly increases the affinity for glucose in causing inactivation of phosphorylase. The significance of these findings is that the sensitivity of phosphorylase inactivation to glucose is modulated by the cellular content of glucose 6-P to the extent that metabolic conditions that lower the cell content of glucose 6-P such as suppression of glucokinase activity or an increase in glucose 6-phosphatase activity would be expected to decrease the affinity for glucose. Thus the inability of hyperglycaemia to suppress hepatic glucose production in Type II diabetes could be explained by a low activity of glucokinase [38] and the improved suppression of hepatic glucose production by hyperglycaemia during a fructose infusion [39] could be explained by the increase in glucose 6-P, which results during the stimulation of glucokinase translocation [40].

AMP and glucose 6-P bind to phosphorylase at the same site and favour the R and T conformation respectively [11]. The activation of phosphorylase by AICAR, which is metabolized to an AMP analogue (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide), cannot be explained by activation of AMPK, since low concentrations of cyanide that activate AMPK did not mimic the effect of AICAR on phosphorylase. This agrees with previous findings that AMPK does not affect the activity of phosphorylase kinase [19]. Stimulation of glycogenolysis and activation of phosphorylase by AICAR have also been reported in skeletal and cardiac muscles [41,42]. In muscle cells, unlike in hepatocytes, AICAR stimulates glucose transport and this accounts for the lack of inhibition of glycogen synthesis, despite activation of phosphorylase [43]. The inhibition of glycogen synthesis by AICAR in hepatocytes is explained at least in part by the lowering of glucose 6-P [35], which causes inactivation of glycogen synthase. The counteraction by AICAR of the inactivation of phosphorylase by glucokinase overexpression is consistent with binding of glucose 6-P to the AMP site [11]. The lack of complete counteraction of the inactivation of phosphorylase by AICAR at 25 mM glucose suggests that AICAR is more effective at counteracting the effects of glucose 6-P than glucose.

We show in the present study that, whereas glucose causes a decrease in the activity of phosphorylase *a* in both the whole homogenate and the high-speed supernatant, it also causes an increase in phosphorylase *a* activity in the pellet. This effect of glucose is in part counteracted by 5-thioglucose, indicating that it is mediated by both glucose and glucose 6-P. The role of the latter metabolite in causing translocation was confirmed by glucokinase overexpression, which increased the activity of phosphorylase *a* in the pellet. Since dihydroxyacetone, which causes a large increase in glucose 6-P in hepatocytes, also increases the activity of phosphorylase *a* in the pellet [22], this supports a role for glucose 6-P as distinct from glucokinase itself in causing translocation. We can rule out the possibility that the increase in phosphorylase *a* in the pellet results from the activation of phosphorylase *b* within this fraction (in the absence of translocation) because of the increase in phosphorylase protein associated with the pellet determined by immunoblotting with an antibody that is not selective for the phosphorylation state of phosphorylase. The additive effects of AICAR and glucokinase overexpression on phosphorylase *a* activity or immunoreactive protein in the pellet can be explained by the combined effects of glucose 6-P in causing translocation and the effect of the AMP analogue favouring phosphorylation of phosphorylase *b* in the supernatant or inhibiting dephosphorylation of phosphorylase *a* in the pellet fraction.

A tentative hypothesis based on the inverse correlation between glucose-induced translocation of phosphorylase *a* to the pellet and the net inactivation of phosphorylase *a* in the homogenate is that the rate of dephosphorylation of phosphorylase *a* in the pellet may be greater than that in the soluble fraction and that the increase in phosphorylase *a* in the pellet with glucose or glucokinase overexpression reflects a greater rate of translocation to the pellet relative to dephosphorylation within this fraction (Figure 9).

Previous work has shown that glucose induces translocation of glycogen synthase from a soluble to a particulate fraction [3,7] and from a diffuse location in the cytoplasm to the cell periphery [4,5] and that this effect is not mimicked by activation of the enzyme with a glycogen synthase kinase-3 inhibitor, indicating that activation of glycogen synthase is not sufficient to cause translocation [5]. A key finding from the present study was the correlation between translocation of phosphorylase *a* (determined either radiochemically or as immunoreactivity) and glycogen synthase from the soluble to the pellet fraction, in cells overexpressing glucokinase and incubated with or without AICAR. Since AICAR lowered glucose 6-P levels and caused inactivation of glycogen synthase, translocation of glycogen synthase cannot be explained by either the activation state of the enzyme, in agreement with previous findings [5], or with the cell content of glucose 6-P alone (present study). This suggests that translocation of glycogen synthase is controlled co-ordinately with translocation of phosphorylase *a*, which in turn depends both

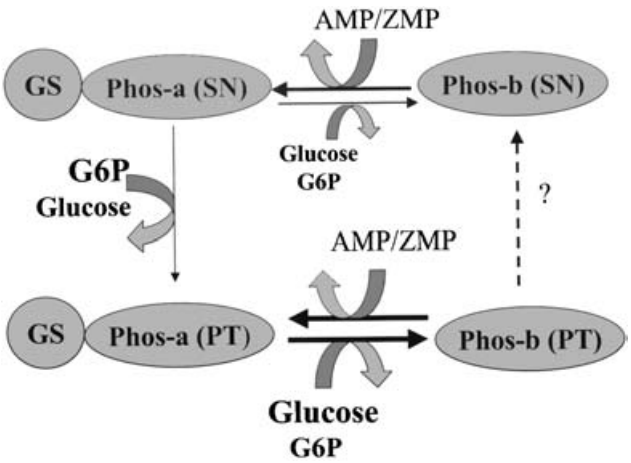

Figure 9 Effects of glucose 6-P and AICAR on translocation and inactivation of phosphorylase

Proposed model to account for the effects of glucose, glucose 6-P and AMP on the activation state of phosphorylase (Phos *a* and Phos *b*) and on the distribution of phosphorylase and glycogen synthase (GS) between the supernatant (SN) and pellet (PT) fractions. ZMP, 5-aminoimidazole-4-carboxamide ribotide.

on the cell content of glucose 6-P and on the activation state of phosphorylase.

In conclusion, glucose 6-P has a more complex role in regulating glycogen metabolism than can be accounted for from its effects on the activation state and translocation of glycogen synthase alone. Glucose 6-P acts co-ordinately with glucose in regulating both the translocation of phosphorylase *a* to the particulate fraction and the dephosphorylation of the enzyme. Glucose 6-P acts synergistically with glucose and decreases the concentration of glucose that causes half-maximal inactivation. Thus the hepatocyte content of glucose 6-P is a major determinant not only of the rate of the glycogen synthesis but also of the rate of glycogenolysis, despite the fact that glucose 6-P is not a physiological allosteric inhibitor of phosphorylase *b* in liver. The question could be raised as to the physiological significance of the co-ordinate translocation of glycogen synthase and phosphorylase *a*, which catalyse synthesis and degradation of glycogen respectively. Since phosphorylase *a* is a potent allosteric inhibitor of glycogen synthase phosphatase [1], and the latter co-localizes with glycogen synthase through the glycogen targeting proteins [44], co-ordinate translocation of phosphorylase *a* and glycogen synthase may be important for maximizing allosteric control of synthase phosphatase by phosphorylase *a*.

We thank Dr Chris Newman for generously providing the recombinant adenovirus, and Diabetes, U.K., for the project grant and equipment grant support.

REFERENCES

1 Bollen, M., Keppens, S. and Stalmans, W. (1998) Specific features of glycogen metabolism in liver. Biochem. J. **336**, 19–31

2 Guinovart, J. J., Gomez-Foix, A. M., Seoane, J., Fernandez-Novell, J. M., Bellido, D. and Vilaro, S. (1997) Bridging the gap between glucose phosphorylation and glycogen synthesis in the liver. Biochem. Soc. Trans. **25**, 157–160

3 Fernandez-Novell, J. M., Arino, J., Vilaro, S. and Guinovart, J. J. (1992) Glucose induces the translocation and the aggregation of glycogen synthase in rat hepatocytes. Biochem. J. **281**, 443–448

4 Fernandez-Novell, J. M., Bellido, D., Vilaro, S. and Guinovart, J. J. (1997) Glucose induces the translocation of glycogen synthase to the cell cortex in rat hepatocytes. Biochem. J. **321**, 227–231

5 Garcia-Rocha, M., Roca, A., De La Iglesia, N., Baba, O., Fernandez-Novell, J. M., Ferrer, J. C. and Guinovart, J. J. (2001) Intracellular distribution of glycogen synthase and glycogen in primary cultured rat hepatocytes. Biochem. J. **357**, 17–24

6 Villar-Palasi, C. and Guinovart, J. J. (1997) The role of glucose 6-phosphate in the control of glycogen synthase. FASEB J. **11**, 544–558

7 Fernandez-Novell, J. M., Arino, J., Vilaro, S., Bellido, D. and Guinovart, J. J. (1992) Role of glucose 6-phosphate in the translocation of glycogen synthase in rat hepatocytes. Biochem. J. **288**, 497–501

8 Fernandez-Novell, J. M., Roca, A., Bellido, D., Vilaro, S. and Guinovart, J. J. (1996) Translocation and aggregation of hepatic glycogen synthase during the fasted-to-refed transition in rats. Eur. J. Biochem. **238**, 570–575

9 Ciudad, C. J., Carabaza, A. and Guinovart, J. J. (1986) Glucose 6-phosphate plays a central role in the activation of glycogen synthase by glucose in hepatocytes. Biochem. Biophys. Res. Commun. **141**, 1195–1200

10 Gomis, R. R., Favre, C., Garcia-Rocha, M., Fernandez-Novell, J. M., Ferrer, J. C. and Guinovart, J. J. (2003) Glucose 6-phosphate produced by gluconeogenesis and by glucokinase is equally effective in activating hepatic glycogen synthase. J. Biol. Chem. **278**, 9740–9746

11 Newgard, C. B., Huang, P. K. and Fletterick, R. J. (1989) The family of glycogen phosphorylases: structure and function. Crit. Rev. Biochem. Mol. Biol. **24**, 69–99

12 Hue, L., Bontemps, F. and Hers, H. G. (1975) The effect of glucose and of potassium ions on the interconversion of the two forms of glycogen phosphorylase and of glycogen synthase in isolated rat liver preparations. Biochem. J. **152**, 105–114

13 Carabaza, A., Ciudad, C. J., Baque, S. and Guinovart, J. J. (1992) Glucose has to be phosphorylated to activate glycogen synthase, but not to inactivate glycogen phosphorylase in hepatocytes. FEBS Lett. **296**, 211–214

14 Coats, W. S., Browner, M. F., Fletterick, R. J. and Newgard, C. B. (1991) An engineered liver glycogen phosphorylase with AMP allosteric activation. J. Biol. Chem. **266**, 16113–16119

15 Johnson, L. N., Snape, P., Martin, J. L., Acharya, K. R., Barford, D. and Oikonomakos, N. G. (1993) Crystallographic binding studies on the allosteric inhibitor glucose-6-phosphate to T state glycogen phosphorylase *b*. J. Mol. Biol. **232**, 253–267

16 Stalmans, W. and Gevers, G. (1981) The catalytic activity of phosphorylase *b* in the liver. With a note on the assay in the glycogenolytic direction. Biochem. J. **200**, 327–336

17 Hurd, S. S., Teller, D. and Fischer, E. H. (1966) Probable formation of partially phosphorylated intermediates in the interconversions of phosphorylase *a* and *b*. Biochem. Biophys. Res. Commun. **24**, 79–84

18 Martensen, T. M., Brotherton, J. E. and Graves, D. J. (1973) Kinetic studies of the activation of muscle phosphorylase phosphatase. J. Biol. Chem. **248**, 8329–8336

19 Cadefau, J., Bollen, M. and Stalmans, W. (1997) Glucose-induced glycogenesis in the liver involves the glucose-6-phosphate-dependent dephosphorylation of glycogen synthase. Biochem. J. **322**, 745–750

20 Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. and Fischer, E. H. (1964) Purification and properties of rabbit skeletal muscle phosphorylase *b* kinase. Biochemistry **3**, 1022–1033

21 Tu, J.-U. and Graves, D. J. (1973) Inhibition of the phosphorylase kinase catalysed reaction by glucose-6-P. Biochem. Biophys. Res. Commun. **53**, 59–65

22 Aiston, S., Andersen, B. and Agius, L. (2003) Glucose 6-phosphate regulates hepatic glycogenolysis through inactivation of phosphorylase. Diabetes **52**, 1340–1346

23 Becker, T. C., Noel, R. J., Johnson, J. H., Lynch, R. M., Hirose, H., Tokuyama, Y., Bell, G. I. and Newgard, C. B. (1996) Differential effects of overexpressed glucokinase and hexokinase I in isolated islets. Evidence for functional segregation of the high and low K_m enzymes. J. Biol. Chem. **271**, 390–394

24 Hardie, D. G. and Carling, D. (1997) The AMP-activated protein kinase. Fuel gauge of the mammalian cell? Eur. J. Biochem. **246**, 259–273

25 Agius, L., Peak, M. and Alberti, K. G. M. M. (1990) Regulation of glycogen synthesis from glucose and gluconeogenic precursors by insulin in periportal and perivenous rat hepatocytes. Biochem. J. **266**, 91–102

26 Aiston, S., Hampson, L., Gomez-Foix, A. M., Guinovart, J. J. and Agius, L. (2001) Hepatic glycogen synthesis is highly sensitive to phosphorylase activity: evidence from metabolic control analysis. J. Biol. Chem. **276**, 23856–23866

27 Aiston, S. and Agius, L. (1999) Leptin enhances glycogen storage in hepatocytes by inhibition of phosphorylase and exerts an additive effect with insulin. Diabetes **48**, 15–20

28 Gilboe, D. P., Larson, K. L. and Nuttall, F. Q. (1972) Radioactive method for the assay of glycogen phosphorylases. Anal. Biochem. **47**, 20–27

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

30 Agius, L., Peak, M., Newgard, C. B., Gomez-Foix, A. M. and Guinovart, J. J. (1996) Evidence for a role of glucose-induced translocation of glucokinase in the control of hepatic glycogen synthesis. J. Biol. Chem. **271**, 30479–30486

31 Witters, L. A. and Kemp, B. E. (1992) Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5′-AMP-activated protein kinase. J. Biol. Chem. **267**, 2864–2867
32 Agius, L. and Stubbs, M. (2000) Investigation of the mechanism by which glucose analogues cause translocation of glucokinase in hepatocytes: evidence for two glucose binding sites. Biochem. J. **346**, 413–421
33 Seoane, J., Gomez-Foix, A. M., O'Doherty, R. M., Gomez-Ara, C., Newgard, C. B. and Guinovart, J. J. (1996) Glucose 6-phosphate produced by glucokinase, but not hexokinase I, promotes the activation of hepatic glycogen synthase. J. Biol. Chem. **271**, 23756–23760
34 Vincent, M. F., Marangos, P. J., Gruber, H. E. and Van den Berghe, G. (1991) Inhibition by AICA riboside of gluconeogenesis in isolated rat hepatocytes. Diabetes **40**, 1259–1266
35 Vincent, M. F., Bontemps, F. and Van den Berghe, G. (1992) Inhibition of glycolysis by 5-amino-4-imidazolecarboxamide riboside in isolated rat hepatocytes. Biochem. J. **281**, 267–272
36 Latsis, T., Andersen, B. and Agius, L. (2002) Diverse effects of two allosteric inhibitors on the phosphorylation state of glycogen phosphorylase in hepatocytes. Biochem. J. **368**, 309–316
37 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
38 Caro, J. F., Triester, S., Patel, V. K., Tapscott, E. B., Frazier, N. L. and Dohm, G. L. (1995) Liver glucokinase: decreased activity in patients with Type II diabetes. Horm. Metab. Res. **27**, 19–22
39 Hawkins, M., Gabriely, I., Wozniak, R., Vilcu, C., Shamoon, H. and Rossetti, L. (2002) Fructose improves the ability of hyperglycemia *per se* to regulate glucose production in Type II diabetes. Diabetes **51**, 606–614
40 Agius, L. (1997) Involvement of glucokinase translocation in the mechanism by which resorcinol inhibits glycolysis in hepatocytes. Biochem. J. **325**, 667–673
41 Young, M. E., Radda, G. K. and Leighton, B. (1996) Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR – an activator of AMP-activated protein kinase. FEBS Lett. **382**, 43–47
42 Longnus, S. L., Wambolt, R. B., Parsons, H. L., Brownsey, R. W. and Allard, M. F. (2003) 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) stimulates myocardial glycogenolysis by allosteric mechanisms. Am. J. Physiol. **284**, R936–R944
43 Halse, R., Fryer, L. G., McCormack, J. G., Carling, D. and Yeaman, S. J. (2003) Regulation of glycogen synthase by glucose and glycogen: a possible role for AMP-activated protein kinase. Diabetes **52**, 9–15
44 Newgard, C. B., Brady, M. J., O'Doherty, R. M. and Saltiel, A. R. (2000) Organizing glucose disposal: emerging roles of the glycogen targeting subunits of protein phosphatase-1. Diabetes **49**, 1967–1977

Received 6 August 2003; accepted 18 September 2003
Published as BJ Immediate Publication 18 September 2003, DOI 10.1042/BJ20031191