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Increased sensitivity of glycogen synthesis to phosphorylase-a and  
impaired expression of the glycogen targeting protein R6  
in hepatocytes from insulin resistant Zucker fa/fa rats

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Abbreviations: DAB, 1,4-dideoxy-1,4-imino-D-arabinitol; G<sub>L</sub>, hepatic glycogen  
targeting subunit of PP1 encoded by the gene PPP1R4(3B); MEM, Minimum

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Essential Medium; MGP, muscle glycogen phosphorylase; PP1C, catalytic sub-unit of protein phosphatase-1; PTG or R5, Protein-Targeting-to-Glycogen, targeting subunit of PP1 encoded by the gene PPP1R5(3C); R6, targeting subunit of PP1 encoded by the gene PPP1R6(3D).

## **ABSTRACT**

Hepatic insulin resistance in the leptin-receptor defective Zucker *fa/fa* rat is associated with impaired glycogen synthesis and increased activity of phosphorylase-a. We investigated the coupling between phosphorylase-a and glycogen synthesis in hepatocytes from *fa/fa* rats by modulating the concentration of phosphorylase-a. Treatment of hepatocytes from *fa/fa* rats and *Fa/?* controls with a selective phosphorylase inhibitor caused depletion of phosphorylase-a, activation of glycogen synthase and stimulation of glycogen synthesis. The flux-control coefficient of phosphorylase on glycogen synthesis was glucose dependent and at 10 mM glucose was higher in *fa/fa* than *Fa/?* hepatocytes. There was an inverse correlation between the activities of glycogen synthase and phosphorylase-a in both *fa/fa* and *Fa/?* hepatocytes. However, *fa/fa* hepatocytes had a higher activity of phosphorylase-a, for a corresponding activity of glycogen synthase. This defect was, in part, normalized by expression of the glycogen-targeting protein, PTG. Hepatocytes from *fa/fa* rats had normal expression of the glycogen-targeting proteins  $G_L$  and PTG but markedly reduced expression of R6. Expression of R6 protein was increased in hepatocytes from Wistar rats after incubation with leptin and insulin. Diminished hepatic R6 expression in the leptin-receptor defective *fa/fa* rat may be a contributing factor to the elevated phosphorylase activity and/or its high control strength on glycogen synthesis.

204 words

## INTRODUCTION

Type 2 diabetes is associated with impaired glucose-induced insulin secretion and insulin resistance in the liver and periphery. Hepatic insulin resistance is attributed to a range of metabolic defects, which include impaired glucose tolerance in the absorptive state and lack of inhibition of hepatic glucose production by hyperglycaemia and hyperinsulinaemia [1,2].

The Zucker fa/fa rat and diabetic db/db mouse, which develop hyperinsulinaemia as a result of mutations in the leptin receptor gene have been widely used as animal models for insulin resistance and type 2 diabetes because they show both hepatic and peripheral insulin resistance [3–7]. The hepatic defect in the fa/fa rat and db/db mouse involves various enzyme abnormalities including elevated activities of glycolytic and lipogenic enzymes [8], phosphorylase [9–12] and glycogen synthase phosphatase [13–15]. It has been proposed that the increased activity of phosphorylase is a contributing factor to impaired hepatic glycogenesis in the fa/fa rat [11]. This hypothesis was supported by the high flux-control coefficient of phosphorylase-a on glycogen synthesis in hepatocytes from Wistar rats under metabolic conditions associated with negligible cycling between glycogen synthesis and degradation [16], and by the finding that in hepatocytes, unlike in muscle, inactivation of phosphorylase rather than inactivation of glycogen synthase kinase-3 is a major component of the mechanism by which insulin stimulates glycogen synthesis [17].

In liver cells there is reciprocal control between the activity of phosphorylase-a and the activation state of glycogen synthase, through allosteric inhibition of glycogen synthase phosphatase by binding of phosphorylase-a (the phosphorylated form of the enzyme) to the C-terminus of the glycogen-targeting protein  $G_L$  [18,19]. However, this mechanism alone cannot account for the high control strength of phosphorylase on glycogen synthesis in hepatocytes from Wistar rats [16,17] or for the impaired glycogen synthesis in hepatocytes from Zucker fa/fa rats, which do not have diminished glycogen synthase activity [7,11,20].  $G_L$  is one of four glycogen-targeting proteins expressed in liver [21–25]. These proteins have binding sites for protein phosphatase-1 (PP1) and for glycogen, and they differ in their relative activities of glycogen synthase phosphatase and phosphorylase phosphatase. They are

designated G<sub>L</sub> or R4, PTG or R5, R6 and R3E [21–25]. The glycogenic effects of G<sub>L</sub> and PTG/R5 in hepatocytes have been demonstrated by adenovirus-mediated enzyme overexpression in hepatocytes [26–28]. However, the contribution of these targeting proteins to the increased activity of glycogen synthase phosphatase in hepatocytes from Zucker fa/fa rats [13–15] has not been explored.

Potent and selective inhibitors of phosphorylase are now available [29,30] which are very powerful experimental tools for selectively modulating either the activity of phosphorylase or the concentration of phosphorylase-a in hepatocytes [31]. They enable investigation into the relative roles of phosphorylase-a, an allosteric ligand of G<sub>L</sub>, as distinct from phosphorylase activity, a determinant of glycogen degradation. In this study we used independent approaches to modulate the activity of phosphorylase or concentration of phosphorylase-a in hepatocytes to determine the mechanism by which phosphorylase contributes to the hepatic defect in the Zucker fa/fa rat.

## RESEARCH DESIGN AND METHODS

**Materials-** CP-91149 [30] was a generous gift from Pfizer Global Research and Development (Groton/New London Laboratories, USA).

**Hepatocyte isolation and culture:** Male, Zucker, 11–13-week-old, genetically obese (fa/fa) or lean (Fa/?) rats (body weight: fa/fa 461 ± 10 g; Fa/? 311 ± 5 g, *n* = 16, *P* < 0.001) were used throughout this study, except for the experiments in Fig. 6 where female (9–11-week-old) Zucker rats were used (body weight: fa/fa 323 ± 12 g; Fa/? 200 ± 5 g, *n* = 4, *P* < 0.001). They were obtained either from AstraZeneca (Alderley Park, UK) or from Harlan Olac (Bicester, UK). All experiments were carried out in accordance with EC Council Directive (86/609/EEC). Hepatocytes were isolated by collagenase perfusion of the liver and suspended in minimal essential medium (MEM) supplemented with 5% (v/v) newborn calf serum and cultured in monolayer [11]. After cell attachment (2–4 h), they were cultured in serum-free MEM containing 10 nM dexamethasone for 18 h.

**Treatment with adenoviruses:** After cell attachment (2 h), the medium was replaced by serum-free MEM containing varying titres of recombinant adenovirus for expression of muscle glycogen phosphorylase [41], glucokinase [42] or PTG [43]. After 2 h, the medium was replaced with serum-free MEM containing 10 nM dexamethasone and the cells were cultured as above.

**Metabolic studies**All metabolic studies were performed after culture of the hepatocytes for 18 h. To determine glycogen synthesis, hepatocyte monolayers were incubated for 3 h in MEM containing [U-<sup>14</sup>C]glucose and 10 mM glucose unless otherwise indicated, without or with inhibitors as indicated. To determine glucokinase, glycogen synthase and phosphorylase, parallel incubations were performed without radiolabel. Glycogen synthesis was determined by ethanol precipitation of the glycogen as described previously [11] and is expressed as nmol of glucose incorporated per 3 h per mg protein.

**Enzyme activity determination:** Glucokinase activity (free and bound) was determined spectrometrically after permeabilization of the hepatocytes with digitonin [32]. To determine phosphorylase and glycogen synthase, cells were snap-frozen in liquid nitrogen [16]. Phosphorylase-a was assayed spectrometrically by coupling to phosphoglucomutase and glucose 6-phosphate dehydrogenase [38]. Total phosphorylase (a + b) was determined radiochemically [44] in the homogenate and 13 000 g supernatant after incubation of the extracts with phosphorylase kinase [11]. The activity of the phosphorylase in cells treated with adenovirus for expression of MGP (Fig. 2A) was determined in the presence 5 mM AMP [16], representing liver phosphorylase-a and muscle a + b. Active or total glycogen synthase were determined without or with glucose 6-phosphate, respectively [45]. The activities of phosphorylase and of active glycogen synthase are expressed as munits/mg protein.

**Metabolic Control Analysis:** Flux-control coefficients of phosphorylase-a on the rate of glycogen synthesis were determined from the initial slope of double log plots of the rate of glycogen synthesis against the activity of phosphorylase-a, as described previously [16,36,37].

**Immunoreactive protein:** Protein expression of the glycogen-targeting proteins:  $G_L$ , PTG and R6 was determined on the hepatocyte suspensions and monolayer cultures using affinity-purified antibodies provided by P.T. Cohen raised in sheep to the GST- $G_L$  protein ( $G_L$ ); peptide GYPNGFQRRNFVNK (R5/PTG) and RPIIQRRSRSLPTSPE (R6). The characterization of these antibodies has been reported previously [22]. Total phosphorylase expression was determined on the monolayer cultures using a commercial mouse antibody (BB Clone 3G1, from Research Diagnostics). Protein of cell lysates (20–30  $\mu\text{g}$ ) were resolved by SDS/PAGE and after electrotransfer of protein to nitrocellulose, membranes were probed with the primary antibody (0.1–0.2  $\mu\text{g}\cdot\text{mL}^{-1}$  affinity purified antibodies or 1 : 1000 for phosphorylase) followed by the appropriate peroxidase conjugated anti-IgG (Jackson Immuno-Research, West Grove, PA) and visualization with an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

**Statistical Analysis:** Results are expressed as means  $\pm$  SE. Statistical analysis was carried out using the Student's *t*-test (either paired or unpaired).

## RESULTS

### High activities of glucokinase and phosphorylase in hepatocytes from fa/fa rats

Hepatocytes from fa/fa rats had a higher total activity of glucokinase (Fa/?  $5 \pm 1$  munits $\cdot\text{mg}^{-1}$ ; fa/fa  $8 \pm 1$  munits $\cdot\text{mg}^{-1}$   $P < 0.01$ ) and a higher proportion of this activity was present in the free (unbound) state (Fa/?  $41 \pm 3\%$ ; fa/fa  $51 \pm 2\%$ ,  $P < 0.05$   $n = 6$ ). The relation between glycogen synthesis and glucokinase activity was determined by overexpression of glucokinase with varying titres of recombinant adenovirus. Although glycogen synthesis increased with titrated glucokinase expression, as expected [32], it was lower in fa/fa hepatocytes for a corresponding glucokinase activity (Fig. 1A). The total activity of phosphorylase (a + b) assayed in the whole homogenate and in the 13 000 *g* supernatant was 24 and 48% higher, respectively, in hepatocytes from fa/fa rats compared with Fa/? controls (Fig. 1B). Immunoreactivity to total phosphorylase determined in the whole homogenate was slightly, but not significantly, higher in fa/fa hepatocytes (Fig. 1C). The total activity

of glycogen synthase was the same in hepatocytes from Fa/? and fa/fa rats ( $1.5 \pm 0.3$  versus  $1.5 \pm 0.3$  munits $\cdot$ mg $^{-1}$ ).

### **Effects of expression of muscle glycogen phosphorylase**

To test whether a higher activity of phosphorylase can account for the lower rate of glycogen synthesis in fa/fa hepatocytes we expressed the muscle isoform of glycogen phosphorylase (MGP), which, unlike the liver isoform, is catalytically active in the dephosphorylated state (phosphorylase b) at physiological AMP concentrations [16]. Titrated MGP expression in hepatocytes causes inactivation of glycogen synthase and inhibition of glycogen synthesis [16]. In this study, expression of MGP was determined from phosphorylase activity assayed in the presence of AMP, which was increased between 1.5- and 5-fold (Fig. 2A). Phosphorylase-a activity, assayed in the absence of AMP, was increased by a lesser extent (1.2 to 1.7-fold, Fig. 2B) because the expressed MGP is only partly phosphorylated [16]. MGP expression was associated with inactivation of glycogen synthase and inhibition of glycogen synthesis. The rate of glycogen synthesis, but not the activity of glycogen synthase, inversely correlated with the activity of phosphorylase-a in hepatocytes overexpressing MGP (Fig. 2C,D), suggesting that the increased activity or concentration of phosphorylase-a is a contributing factor to the glycogenic defect (Fig. 2D) and that there is altered coupling between phosphorylase-a and glycogen synthase in fa/fa compared with Fa/? hepatocytes (Fig. 2C).

### **Effects of activity and concentration of phosphorylase-a on glycogen synthesis**

To test the role of the phosphorylated form of phosphorylase independently of changes in total phosphorylase concentration, we used CP-91149, an indole carboxamide phosphorylase inhibitor [30], which causes conversion of phosphorylase-a to -b with concomitant activation of glycogen synthase and stimulation of glycogen synthesis [16,31]. CP-91149 caused depletion of phosphorylase-a but did not abolish the difference in phosphorylase-a between hepatocytes from fa/fa and Fa/? rats ( $P < 0.03$  at  $10 \mu\text{M}$  CP-91149). When the activation of glycogen synthase and stimulation of glycogen synthesis were plotted against the corresponding activity of phosphorylase-a there was a rightward shift in

both glycogen synthase against phosphorylase-a (Fig. 3B) and glycogen synthesis against phosphorylase-a (Fig. 3C) curves for fa/fa compared with Fa/? hepatocytes.

To test the role of phosphorylase activity, as distinct from the phosphorylation state of the enzyme, we used 1,4-dideoxy-1,4-imino-D-arabinitol (DAB), a potent inhibitor of phosphorylase and of glycogenolysis in hepatocytes with an  $IC_{50} < 2 \mu M$  [33,34], which unlike CP-91149, does not cause conversion of phosphorylase-a to -b [31]. Treatment of hepatocytes from fa/fa rats with DAB (5–20  $\mu M$ ) did not stimulate glycogen synthesis (control,  $9.5 \pm 1.3$ ; 5  $\mu M$  DAB,  $8.7 \pm 1.6$ ; 10  $\mu M$  DAB,  $8.6 \pm 1.6$ ; 20  $\mu M$  DAB,  $5.2 \pm 1.2$  nmol-3 hmg<sup>-1</sup>,  $n = 10$ ). Inhibition at 20  $\mu M$  DAB ( $P < 0.002$ ) was associated with inactivation of glycogen synthase ( $0.42 \pm 0.06$  to  $0.27 \pm 0.07$  munits-mg<sup>-1</sup>,  $P < 0.002$ ) and is explained by conversion of phosphorylase-b to phosphorylase-a [31]. The lack of stimulation of glycogen synthesis by lower DAB concentrations (5–10  $\mu M$ ), which inhibit glycogenolysis [34], is consistent with a lack of cycling between synthesis and degradation [35] confirming that stimulation of glycogen synthesis by CP-91149 is not due to inhibition of glycogen degradation and also the impaired glycogen synthesis in fa/fa hepatocytes is not due to increased glycogen degradation.

### **Effects of overexpression of the glycogen targeting protein PTG**

The rightward shift in the inverse correlation between glycogen synthase against phosphorylase-a in fa/fa and Fa/? hepatocytes (Figs 2C,3B) could be explained by an increased activity of glycogen synthase phosphatase [13–15], because of increased expression of glycogen-targeting proteins [26,27], or by decreased coupling between the glycogen-targeting protein G<sub>L</sub> and its allosteric inhibitor phosphorylase-a, because of altered subcellular distribution of phosphorylase-a or impaired access to G<sub>L</sub>. We determined the effects of expression of the targeting protein, PTG, which causes both dephosphorylation of phosphorylase-a and activation of glycogen synthase [28]. Overexpression of PTG caused inactivation of phosphorylase (Fig. 4A), activation of glycogen synthase and stimulation of glycogen synthesis. Unlike CP-91149, it partially counteracted the rightward shift of the glycogen synthase against phosphorylase-a curve (Fig. 4B). However, it did not abolish the rightward shift of the glycogen synthesis against phosphorylase-a (Fig. 4C). Because PTG mimics the effects of CP-91149 on phosphorylase inactivation, but has a greater effect on

translocation of glycogen synthase and phosphorylase [28], these results suggest that a defect in glycogen-targeting proteins may account for the shift in the glycogen synthase against phosphorylase curves.

### **Higher sensitivity of glycogen synthesis to phosphorylase-a in fa/fa hepatocytes**

To test whether impaired glycogen synthesis in hepatocytes from fa/fa rats can be explained by an altered sensitivity of flux to phosphorylase-a concentration, we used metabolic control analysis [36,37] to determine the flux-control coefficient of phosphorylase-a on glycogen synthesis from the initial slope of the double log plot of glycogen synthesis against phosphorylase-a for the three experimental conditions (incubation with CP-91149 or expression of MGP and PTG) that alter phosphorylase activity (Figs 2–4). The linear plot for the data is shown in Fig. 5A and the corresponding plot for active glycogen synthase against phosphorylase-a is shown in Fig. 5B. PTG expression was more effective than CP-91149 in attenuating the rightward shift for glycogen synthase against phosphorylase-a (Fig. 5B). Flux-control coefficients, which represent the fractional change in flux resulting from a fractional change in phosphorylase-a, were approximately twofold higher in fa/fa hepatocytes (Fig. 5C).

### **Relation between the flux control coefficient and glucose concentration**

In the above experiments the flux-control coefficients of phosphorylase-a on glycogen synthesis were determined from incubations with 10 mM glucose. Because the glycogenic defect in hepatocytes from fa/fa rats is observed at 10 mM, but not 25 mM, glucose [11], we also determined flux-control coefficients for phosphorylase-a on glycogen synthesis at varying glucose concentrations. Flux-control coefficients were highest at 5 mM glucose, and were significantly higher in fa/fa hepatocytes at 5–15 mM glucose with a crossover at 20 mM glucose (Fig. 6). These experiments were performed on hepatocytes from 7–9-week-old female Zucker rats, which have higher rates of glycogen synthesis and lower activities of phosphorylase-a and flux-control coefficients than hepatocytes from 11–13-week-old male rats.

### **Expression of glycogen targeting proteins in hepatocytes from fa/fa rats**

To test whether the defect in hepatocytes from fa/fa rats is associated with altered expression of G<sub>L</sub>, PTG- or R6-targeting proteins, we determined immunoreactivity by western blotting using isoform-specific antibodies [22]. Hepatocytes from fa/fa rats had similar expression of G<sub>L</sub> and PTG as Fa/? controls but markedly decreased expression of R6 (Fig. 7).

### **Effects of leptin and insulin on hepatocytes from Wistar rats**

Because fa/fa rats are homozygous for a mutation in the leptin receptor gene, we tested whether expression of R6 is regulated by leptin in hepatocytes from Wistar rats. The activity of phosphorylase-a was decreased by culture of hepatocytes with leptin and insulin (Fig. 8A) in agreement with previous findings [38]. R6 protein was increased by 75% after combined culture with leptin and insulin (Fig. 8B).

## **DISCUSSION**

The Zucker fa/fa rat is widely used as a model for insulin resistance and type 2 diabetes because it shows impaired glucose tolerance and lack of suppression of hepatic glucose production in response to hyperglycaemia [3–7]. The hepatic enzyme abnormalities include impaired hepatic glycogen synthesis and increased activities of phosphorylase-a [11,12] and glycogen synthase phosphatase [13–15]. However, the total activity of glycogen synthase and the activation state are the same as in control hepatocytes [6,11].

In this study, we used three approaches to modulate the concentration and activity of phosphorylase-a, to determine its role in the glycogenic defect. We applied metabolic control analysis to test whether the glycogenic defect in hepatocytes from fa/fa rats is due to higher phosphorylase activity or to changes in coupling mechanisms between phosphorylase-a and glycogen synthesis. Using three independent methods involving either expression of the muscle isoform of glycogen phosphorylase, or expression of the glycogen-targeting protein PTG or incubation with a selective phosphorylase inhibitor [30] that promotes dephosphorylation of phosphorylase-a [31], we determined the flux-control coefficient of phosphorylase on glycogen synthesis. This is a measure of the sensitivity of flux to small incremental changes in phosphorylase-a concentration or activity [36,37]. It is a property of the

entire metabolic system and depends on the concentrations of other proteins that influence the flux through that pathway.

This study shows that the flux-control coefficient of phosphorylase on glycogen synthesis determined at 10 mM glucose is higher in hepatocytes from fa/fa than Fa/? rats and also that there is a rightward shift in the plots of glycogen synthesis against phosphorylase-a or glycogen synthase against phosphorylase-a in fa/fa compared with Fa/? hepatocytes, which is indicative of a difference in coupling between glycogen synthase and phosphorylase-a.

Flux-control coefficients can be positive or negative, and values greater than unity are rare [37] and indicative of protein–protein interaction and/or downstream mechanisms that act synergistically. Glucokinase has a flux-control coefficient on glycogen synthesis that is greater than unity at low glucose [32], and this is explained by glucokinase binding to an inhibitory regulator protein [39]. Phosphorylase-a, like glucokinase also has a very high flux-control coefficient of glycogen synthesis, particularly at low glucose concentrations. However, unlike in the case of glucokinase, the mechanisms that account for this high control are not fully understood [16]. We can rule out a role for cycling between glycogen synthesis and degradation as a contributory factor to the high control coefficient of phosphorylase on glycogen synthesis because using a potent inhibitor of phosphorylase (DAB) that does not promote conversion of phosphorylase-a to -b [31], it can be shown that there is negligible cycling between glycogen degradation and synthesis [31,35]. Although allosteric inhibition of glycogen synthase phosphatase in association with  $G_L$  is a component of the high control strength of phosphorylase-a [16], several lines of evidence show that this mechanism alone cannot explain the high control strength on glycogen synthesis. One compelling argument is the evidence that inhibitors of glycogen synthase kinase-3 cause marked activation of glycogen synthase but negligible stimulation of glycogen synthesis [17]. This contrasts with the more moderate activation of glycogen synthase by CP-91149 but its greater potency at stimulating glycogen synthesis [17]. Likewise, the potency of PTG overexpression at stimulating glycogen synthesis in hepatocytes when compared with dephosphorylation of phosphorylase-a caused by CP-91149 suggests that translocation of glycogen synthase and phosphorylase is a key contributory factor to

the glycogenic stimulation [28]. We therefore determined the expression of three glycogen-targeting proteins that are known to be expressed in liver.

$G_L$  is thought to be the predominant glycogen-targeting protein in liver [25]. It is the only glycogen-targeting protein that is known to have an allosteric site for phosphorylase-a, which causes inhibition of synthase phosphatase activity [21], accordingly, phosphorylase-a prevents activation of glycogen synthase only in cells expressing  $G_L$ . In agreement with this model, CP-91149 does not cause activation of glycogen synthase in hepatoma cell lines that lack  $G_L$  expression (L. Hampson & L. Agius, unpublished results).  $G_L$  enhances the activity of PP1 on glycogen synthase but suppresses dephosphorylation of phosphorylase-a [21]. It is therefore presumed to function as a synthase phosphatase [21]. Nonetheless, overexpression of  $G_L$  in hepatocytes inactivates phosphorylase, indicating that it does function as a phosphorylase phosphatase [27]. PTG and R6, unlike  $G_L$ , are expressed ubiquitously [22–24]. Expression of PTG in hepatocytes is associated with inactivation of phosphorylase and activation of glycogen synthase and translocation of these proteins [26–28]. The expression of  $G_L$  and PTG, but not R6, in rat liver *in vivo* is insulin-dependent. It declines during insulin deficiency and is restored by insulin treatment [22,40]. Another glycogen-targeting protein expressed in rat liver and designated PPP1RE may also be insulin dependent based on changes in mRNA levels [25]. It is noteworthy that assays of PP1 activity in immunoprecipitates of the glycogen-targeting proteins  $G_L$ , PTG, R6 and PPP1RE have shown in all cases dephosphorylating activity with both glycogen synthase and phosphorylase as substrates. However, whether these activities function as synthase phosphatase (as suggested for  $G_L$ ) or as phosphorylase phosphatase (as suggested for PTG) *in vivo* remains speculative [22,25]. We found no evidence for changes in expression of either  $G_L$  or PTG in hepatocytes from fa/fa rats. However, we demonstrate that expression of R6 protein is markedly decreased in hepatocytes from fa/fa rats. To our knowledge this is the first report of adaptive changes in hepatic R6 protein. The main distinguishing feature of hepatic R6 compared with  $G_L$ , PTG and PPP1RE, in addition to its lack of adaptive change with altered insulin status, is that the protein is recovered mainly from the soluble and microsomal fractions rather than the glycogen fraction of liver extracts [22,40], presumably because of a lower glycogen-binding affinity. This implicates a distinct function from the other targeting proteins.

Based on assays of phosphorylase phosphatase and glycogen synthase phosphatase in both the glycogen fraction and the soluble fraction, R6 appears to have a negligible contribution to phosphatase activity in the glycogen fraction but it can account for as much as 20% of total phosphorylase phosphatase activity in the cell lysate fraction [22]. A key question is whether the markedly reduced expression of R6 in hepatocytes from fa/fa rats could contribute to the elevated phosphorylase-a and the glycolytic defect? Both the activity of phosphorylase-a in hepatocytes and the control strength of phosphorylase on glycogen synthesis are markedly dependent on the age of the rat (S. Aiston & L. Agius, unpublished results). Hepatocytes from 6-week-old rats have a high rate of glycogen synthesis, a low activity of phosphorylase-a and a low flux-control coefficient on glycogen synthesis. With age, glycogen synthesis declines and both the activity of phosphorylase-a and its control coefficient on glycogen synthesis increase markedly. Downregulation of phosphorylase-a activity by leptin is observed in 10-week-old rats but not in 6-week-old rats. A tentative hypothesis to explain a putative link between impaired R6 expression in hepatocytes from fa/fa rats and the elevated activity of phosphorylase-a is that R6 may be involved in the mechanism by which leptin downregulates phosphorylase activity. Our finding that culture of hepatocytes from Wistar rats with leptin and insulin is associated with increased expression of R6 protein with concomitant downregulation of phosphorylase-a activity is consistent with the hypothesis for a putative role for R6 in regulating phosphorylase-a activity and or subcellular location. This hypothesis would be strengthened by use of specific inhibitors of R6, but none are currently available, or by selective downregulation of R6 expression.

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## LEGENDS TO FIGURES

Fig. 1. Impaired glycogen synthesis and elevated phosphorylase activity in fa/fa hepatocytes. A. Glycogen synthesis determined during incubation with 10 mmol/l glucose in hepatocytes from fa/fa (filled symbols) and Fa/? (open symbols) rats with varying degrees of glucokinase overexpression by treatment with 4 titres of recombinant adenovirus. B. Total phosphorylase activity (a + b) determined in the 13,000g supernatant (SN) or in the whole homogenate (HOM) of hepatocytes from fa/fa and Fa/? rats. C. Phosphorylase immunoreactivity (arbitrary densitometry units) and represent immunoblot of 3 fa/fa (■) and 3 Fa/? (□) preparations. Data are means  $\pm$  SE for n=6, (A), 15 (B) and 6, (C), \* P < 0.05 relative to Fa/?.

Fig. 2. Expression of muscle glycogen phosphorylase inhibits glycogen synthesis Hepatocytes from fa/fa (filled symbols) and Fa/? (open symbols) rats were treated with the indicated titres (5-40  $\mu$ l/ml) of adenovirus for expression of MGP. Hepatocytes were incubated for determination of glycogen synthesis and the activities of phosphorylase and glycogen synthase as described in Experimental procedures. A. Phosphorylase activity assayed in the presence of AMP. B. Phosphorylase a activity C. Active glycogen synthase activity versus phosphorylase-a. D. Glycogen synthesis vs phosphorylase-a. Data are the mean  $\pm$  SE for n = 10. \* P < 0.05 relative to Fa/?.

Fig. 3. Effects of CP-91149 on glycogen synthesis and enzyme activities Hepatocytes from fa/fa (filled symbols) and Fa/? (open symbols) rats were incubated for 3 h with the concentrations of CP-91149 indicated for determination of glycogen synthesis and the activities of phosphorylase-a and glycogen synthase. A. phosphorylase-a. B. active glycogen synthase versus phosphorylase-a. C glycogen synthesis versus phosphorylase-a. Data are mean  $\pm$  SE for n = 15.

Fig. 4. Effects of PTG expression on glycogen synthesis and enzyme activities Hepatocytes from fa/fa (filled symbols) and Fa/? (open symbols) rats were treated with varying titres of adenovirus for expression of PTG and cultured for 18 h. A. Hepatocytes were incubated for determination of glycogen synthesis and the activities of phosphorylase and glycogen synthase as in Fig. 2. B. Active glycogen synthase

versus phosphorylase-a. C. Glycogen synthesis versus phosphorylase-a. Data are mean  $\pm$  SE for n = 8.

Fig. 5. Sensitivity of glycogen synthesis to phosphorylase-a during enzyme expression or inactivation. Linear plots of glycogen synthesis against phosphorylase-a (A) and active glycogen synthase against phosphorylase-a (B) for the data in Figs 2-4. C. Flux-control coefficients determined from initial slope of the double log plot of glycogen synthesis against phosphorylase-a.

Fig. 6. Sensitivity of glycogen synthesis to phosphorylase-a as a function of glucose concentration. Glycogen synthesis (A) was determined in hepatocytes from female Zucker fa/fa (filled symbols) and Fa/? (open symbols) rats during incubation with the glucose concentrations indicated without (round symbols) or with (square symbols) 2.5  $\mu$ mol/l CP-91149; B, phosphorylase-a activity. C. Slope of double log plot of glycogen synthesis against phosphorylase-a. Data are mean  $\pm$  SE, n = 4, \* P < 0.05.

Fig. 7. Expression of glycogen targeting proteins in fa/fa and Fa/? hepatocytes. Immunoreactivity to G<sub>L</sub>, PTG/R5 and R6 was determined in the freshly isolated hepatocyte suspensions as described in Experimental procedures and densitometry is expressed as relative arbitrary units (AU). Mean  $\pm$  SE for n = 7; representative blots for three fa/fa and three Fa/? preparations are shown together with the PTG marker. \* P < 0.0001 fa/fa vs Fa/?

Fig. 8. Effects of leptin and insulin on R6-mRNA levels and phosphorylase activity in hepatocytes from Wistar rats.. Hepatocytes were cultured for 18 h without or with 10nmol/l insulin (I) and 500ng/ml leptin (L). Parallel incubations were performed for determination of phosphorylase-a (A) and immunoreactive R6 (B). Data are mean  $\pm$  SE for n=8, \* P < 0.05; \*\*P < 0.005 relative to no additions.

Figure 1

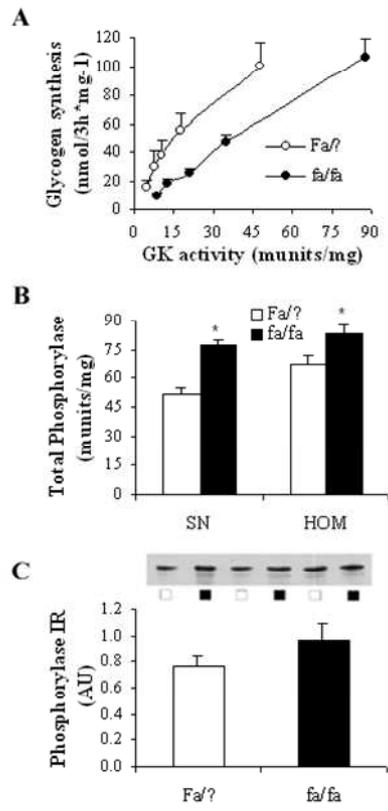


Figure 2

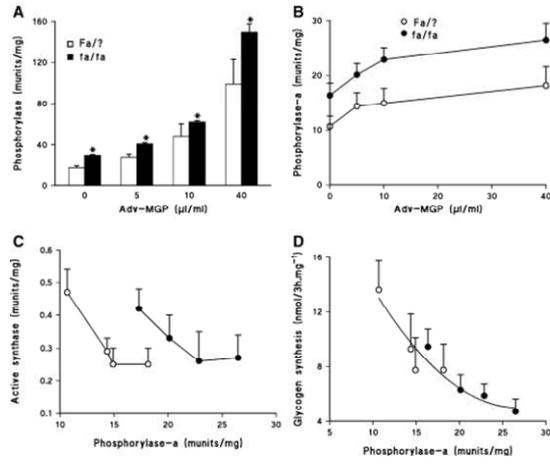


Figure 3

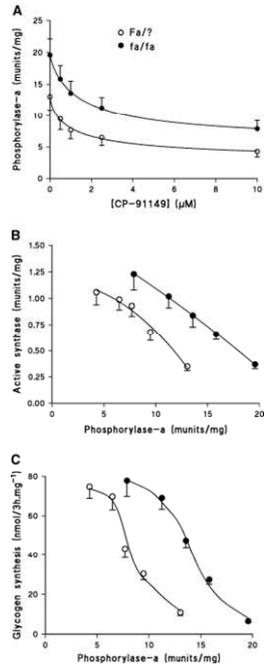


Figure 4

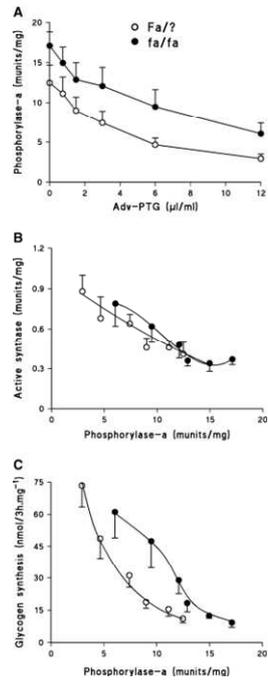


Figure 5

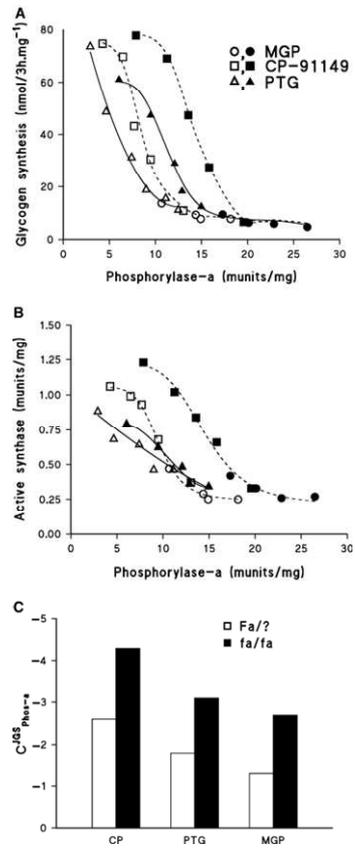


Figure 6

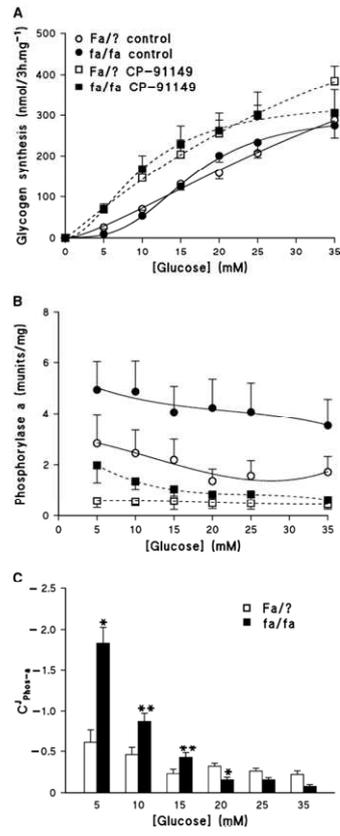


Figure 7

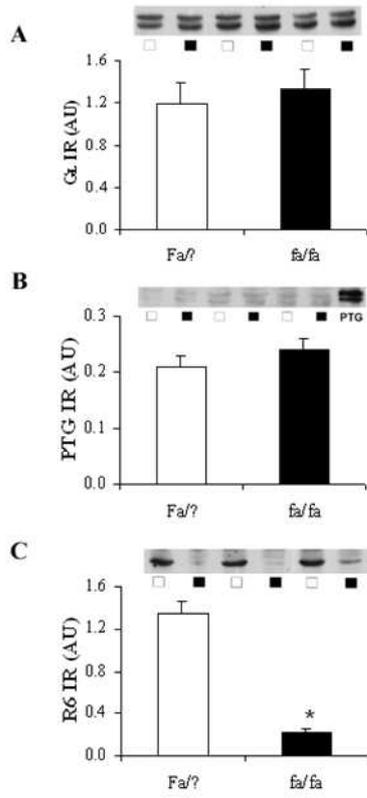


Figure 8

