Expression and glycogenic effect of glycogen-targeting protein phosphatase 1 regulatory subunit G_L in cultured human muscle

Marta MONTORI-GRAU*, Maria GUITART*, Carles LERIN*¹, Antonio L. ANDREU†, Christopher B. NEWGARD‡, Cèlia GARCÍA-MARTÍNEZ* and Anna M. GÓMEZ-FOIX*²

*Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, 08028-Barcelona, Spain, †Centre d'Investigació en Bioquímica i Biologia Molecular (A.L.A.), University Hospital Vall d'Hebron, 08035-Barcelona, Spain, and ‡Sarah W. Stedman Nutrition and Metabolism Center and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27704, U.S.A.

Glycogen-targeting PP1 (protein phosphatase 1) subunit G_L (coded for by the *PPP1R3B* gene) is expressed in human, but not rodent, skeletal muscle. Its effects on muscle glycogen metabolism are unknown. We show that G_L mRNA levels in primary cultured human myotubes are similar to those in freshly excised muscle, unlike subunits G_M (gene *PPP1R3A*) or PTG (protein targeting to glycogen; gene *PPP1R3C*), which decrease strikingly. In cultured myotubes, expression of the genes coding for G_L, G_M and PTG is not regulated by glucose or insulin. Overexpression of G_L activates myotube GS (glycogen synthase), glycogenesis in glucose-replete and -depleted cells and glycogen accumulation. Compared with overexpressed G_M, G_L has a more potent activating effect on glycogenesis, while marked enhancement of their combined action is only observed in glucose-replete cells. G_L does not affect GP (glycogen phosphorylase) activity, while co-overexpression with muscle GP impairs G_L activation of GS

in glucose-replete cells. G_L enhances long-term glycogenesis additively to glucose depletion and insulin, although G_L does not change the phosphorylation of GSK3 (GS kinase 3) on Ser⁹ or its upstream regulator kinase Akt/protein kinase B on Ser⁴⁷³, nor its response to insulin. In conclusion, in cultured human myotubes, the G_L gene is expressed as in muscle tissue and is unresponsive to glucose or insulin, as are G_M and PTG genes. G_L activates GS regardless of glucose, does not regulate GP and stimulates glycogenesis in combination with insulin and glucose depletion.

Key words: protein phosphatase 1 subunit G_L (*PPP1R3B* gene product), protein phosphatase 1 subunit G_M (*PPP1R3A* gene product), protein phosphatase 1 subunit PTG (*PPP1R3C* gene product), human myotube, glycogen synthase, glycogen phosphorylase.

INTRODUCTION

Muscle glycogen synthesis is considered to be enhanced by recruitment/activation of phosphatases, primarily PP1 (protein phosphatase 1), and by phosphorylation-inactivation of kinases, mainly GSK3 (glycogen synthase kinase 3) [1,2], acting on the rate-limiting enzyme GS (glycogen synthase). PP1, which activates GS and inactivates GP (glycogen phosphorylase), is recruited into glycogen particles and regulated by GRSs (glycogen-targeting regulatory subunits) [1]. In muscle tissue, several PP1 GRSs are expressed and their specific regulatory roles are undergoing investigation. In the human muscle these are: the muscle-specific subunit G_M (gene *PPP1R3A*), which is largely expressed in both rodent and human tissues [3,4]; PTG (protein targeting to glycogen; gene *PPP1R3C*), which is robustly expressed in skeletal and cardiac muscle and liver in humans [5,6]; and the G_L subunit (gene *PPP1R3B*), which is expressed in muscle and liver in humans at comparable levels [4], whereas in rodents and rabbits it is not present in skeletal muscle [4,7]; the ubiquitous isoform R6 (*PPP1R3D*) [6] that is also found in muscle; and R3E (*PPP1R3E*) [8], which is expressed in liver and heart in rodents, but is most abundant in skeletal muscle in humans.

G_L has been shown to modulate the activity of PP1 on glycogen-metabolizing enzymes, either purified or in the context of hepatic

cells. Moreover, the human *PPP1R3B* gene has been examined as a candidate gene for the Type 2 diabetes and MODY (maturity onset diabetes of the young) loci on chromosome 8p23 [9]. Purified G_L enhances the muscle GS phosphatase activity of PP1, whereas it decreases muscle GP phosphatase activity [7]. Effects of G_L on PP1 GS phosphatase activity are inhibited by addition to the assay of GP *a* (phosphorylated active form), and the GP counteraction is suppressed by AMP [7]. The G_L regulatory binding site for GP *a* comprises the 16 amino acids at the C-terminus [10]. This mechanism is effective in liver [11], and it has been proposed to contribute to the stimulation of glycogen synthesis by insulin and high glucose [12]. G_L regulatory effects in liver glycogen metabolism have been described as occurring when the gene is delivered to primary cultured rat hepatocytes [13,14] or rat liver [15]. When overexpressed in hepatocytes, G_L was found to have the highest glycogenic and GS-activating capacity when compared with PTG or G_M. Likewise, GP was strongly inactivated by G_L expression [13], and glycogenolysis in response to glucose depletion was prevented [14]. Delivery of G_L to the liver in rats fed on a high-fat diet increased glycogen content in the fed and fasted condition and reduced plasma triacylglycerols in fed animals as well as reducing lactate after fasting [15].

The metabolic-control role of G_L in muscle has not been examined. Only the glycogenic effectiveness of G_M and PTG in

Abbreviations used: Ad-, adenovirus; Ad-GFP, a non-replicating adenovirus encoding GFP (green fluorescent protein); C_t, threshold cycle; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GP, glycogen phosphorylase; GRS, glycogen targeting regulatory subunit; GS, glycogen synthase; GSK3, glycogen synthase kinase 3; MGP, muscle GP; PDK1, 3-phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B (Akt); PP1, protein phosphatase 1; *PPP1R3B*, *PPP1R3A*, *PPP1R3C*, *PPP1R3D* and *PPP1R3E*, genes coding for PP1 subunits G_L, G_M, PTG (protein targeting to glycogen), R6 and R3E respectively; SR, sarcoplasmic reticulum.

¹ Present address: Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02115, U.S.A.

² To whom correspondence should be addressed (email agomezfoix@ub.edu).

muscle has been previously explored. Overexpressed G_M [16] activates GS in glycogen-depleted cultured human myotubes more than in glycogen-replete cells and has an equivalent reverse action on GP. Consequently, overexpressed G_M shows a modest glycogen-accumulating effect and impairs glycogenolysis. Specific overexpression of G_M in muscle of transgenic mice [17] is also characterized by an enhanced GS activity ratio and a moderate increase in glycogen content. However, disruption of the *PPP1R3A* gene in mice lowers GS activity and glycogen in muscle and causes muscle insulin resistance with aging [17,18]. Overexpression of PTG in cultured human myotubes [19] markedly activates GS in both glucose-deprived and glucose-treated cells and causes a huge accumulation of glycogen, whereas it does not significantly modify GP activity. Mice with a heterozygous deletion of the PTG gene [20] show reduced accumulation of glycogen in muscle, as well as in liver and adipose tissue, and muscle insulin resistance with aging.

In summary, the regulation of the human G_L gene and its metabolic control role in muscle cells is unknown. To address these issues, we studied the expression of the G_L gene in primary cultured human myotubes and its response to glucose and insulin. Moreover, we overexpressed G_L , by means of adenovirus, in these cultured cells, and studied its effects on glycogen-metabolizing enzymes and glycogen metabolism. We also sought to study whether GP modulated the regulatory action of G_L on GS within the muscle cell and the combined effects of G_L with G_M , glucose or insulin stimulation.

EXPERIMENTAL

Muscle samples and culture

Biopsies, from gastrocnemius and peroneus muscles, were obtained during surgery from four donors devoid of muscle disease. The biopsies were obtained with the approval of the Ethics Committee of the Hospital Vall d'Hebrón, Barcelona, Spain, and with the informed consent of the subjects concerned. Myoblast cell populations were isolated from the muscle biopsies by the explant culture technique [21]. Myoblasts were grown in DMEM (Dulbecco's modified Eagle's medium)/M-199 medium (3:1, v/v), with 10% (v/v) FBS (fetal bovine serum), 10 μ g/ml insulin, 4 mM glutamine, 25 ng/ml fibroblast growth factor and 10 ng/ml epidermal growth factor. Immediately after myoblast fusion, the medium was replaced by DMEM/M-199 devoid of growth factors and glutamine and with 10% FBS.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from tissue and cell samples following the instructions of the RNeasy Minikit (Qiagen, Valencia, CA, U.S.A.). Extracts were homogenized with a Polytron (Kynematica Polytron, Westbury, NY, U.S.A.). A 0.5 μ g portion of total RNA was retrotranscribed with TaqMan reverse-transcription reagents from Applied Biosystems (Branchburg, NJ, U.S.A.) using random hexamers. Real-time PCR was performed in the ABI PRISM 7700 sequence detection system with the TaqMan universal PCR master mix and probes for human *PPP1R3A*, *PPP1R3B* and *PPP1R3C* genes and *PPP1R3B* rat and *PPP1R3A* rabbit genes from Applied Biosystems. β_2 -Microglobulin was used as the endogenous control to normalize the threshold cycle (C_t) for each probe assay as stated. Relative gene expression was estimated as $2^{-\Delta C_t}$ and gene fold change was estimated by the $2^{-\Delta\Delta C_t}$ method [22].

Transduction with recombinant adenoviruses

10-day-old myotubes were transduced with adenoviruses at an MOI (multiplicity of infection) of 20 for 4 h. Under these conditions, transduction efficiency was about 90% (results not shown). At 48 h before the metabolic experiments, cells were depleted of insulin and, 24 h before the metabolic experiments, depleted of FBS. An adenovirus expressing the EGFP (enhanced green fluorescent protein) under the control of the CMV (cytomegalovirus) promoter Ad-GFP [a non-replicating adenovirus encoding GFP (green fluorescent protein)] was used as a control. Construction of Ad- G_L , encoding rat G_L , and Ad- G_M , encoding rabbit G_M , are described in [13]; Ad-MGP encoding rabbit MGP (muscle GP) is described in [23].

Enzyme activity and metabolite assays

To measure GS and GP activities, 100 μ l of homogenization buffer consisting of 10 mM Tris/HCl, pH 7.0, 150 mM KF, 15 mM EDTA, 600 mM sucrose, 15 mM 2-mercaptoethanol, 17 μ g/l leupeptin, 1 mM benzamidine and 1 mM PMSF was used in order to scrape the cell monolayers off the frozen plates prior to sonication. The resulting homogenates were used for the determination of enzyme activities. GP activity was determined by the incorporation of [U - 14 C]glucose 1-phosphate into glycogen in the absence or presence of the allosteric activator AMP (1 mM) [24]. GS activity was measured in the absence or presence of 10 mM glucose 6-phosphate as described in [25].

To assess glycogen synthesis, cells were incubated with 10 mM [U - 14 C]glucose (0.05 μ Ci/ μ mol). To measure glycogen, cell monolayers were scraped into 100 μ l of 30% (w/v) KOH and homogenates boiled for 15 min. An aliquot of the homogenates was used for the measurement of protein concentration. Homogenates were spotted on to Whatman 3MM paper, and glycogen was precipitated by immersing the papers in ice-cold 66% (v/v) ethanol. Radioactivity in dried papers was counted in a β -radiation counter or papers were incubated in 0.4 M acetate buffer, pH 4.8, with 25 units/ml α -amylglucosidase (Sigma) for 120 min at 37°C. Glucose released was measured enzymatically with a glucose kit from Biosystems (Barcelona, Spain). Glucose transport was assessed by 2-deoxy-D- 3 H]glucose uptake in cells grown in 24-well plates as described previously [26].

Western blotting

Extracts were prepared by scraping cell monolayers from 6-cm-diameter dishes into 120 μ l of homogenization buffer consisting of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4 , 1 mM NaF, 2 μ g/ μ l leupeptin, 2 μ g/ml aprotinin, 1% (v/v) Nonidet P40 and 1 mM dithiothreitol. Lysates were then rocked gently for 60 min at 4°C. The resulting homogenates were centrifuged at 13 000 g for 5 min at 4°C and the supernatants were collected. An aliquot of the cell extracts was used for the measurement of protein concentration. A 20 μ g portion of protein was resolved by SDS/10%-(w/v)-PAGE, and immunoblotting was performed with antibodies against the Ser⁴⁷³ phosphorylation site on Akt1 and equivalent sites in Akt2/3, protein Akt1, Akt2 and Akt3 (Cell Signaling), actin (Sigma), α -actinin (Chemicon) or GSK3- α and - β (Upstate) and GSK- α and - β in which Ser²¹ and Ser⁹ are respectively phosphorylated (Cell Signaling). Protein bands were revealed and quantified with a LAS-3000 luminescent image analyser (FujiFilm). Results are the ratios, in arbitrary units, between the Akt and actin signals or between GSK3 and the α -actinin signal and are expressed as

Table 1 Expression of G_L, PTG and G_M genes in human muscle biopsies and cultured human myotubes

The relative expression of G_L (*PPP1R3B*), PTG (*PPP1R3C*) and G_M (*PPP1R3A*) genes was analysed in muscle biopsies and cultured myotubes from four subjects by reverse-transcription PCR. The ΔC_t values for the reference probe β_2 -microglobulin were subtracted from each probe C_t (ΔC_t). Expression relative to that of β_2 -microglobulin was calculated using the ΔC_t method. Mean values of $2^{-\Delta C_t}$ or $1/2^{-\Delta C_t} \pm$ S.E.M. are shown. The former are shown by '+' and the latter by '-'. To determine changes due to culture handling, the ΔC_t values for muscle were subtracted from the ΔC_t values of cells cultured with glucose. To determine regulation of gene expression by glucose and insulin, cultured cells were incubated for 18 h with or without 25 mM glucose and then further incubated with 100 nM insulin for 4 h where stated. The ΔC_t values for cells treated without glucose and without insulin were subtracted from each treatment. Data are expressed as mean values of $2^{-\Delta \Delta C_t}$ for up-regulated genes and $1/2^{-\Delta \Delta C_t}$ for down-regulated ones. The former are shown by '+' and the latter by '-'. The significance of the difference between tissue and cultured muscle, as assessed with REST was * $P < 0.01$.

Gene	Relative expression		Effect of treatment			
	Muscle biopsy	Cultured myotubes	Biopsy/myotubes	Cultured myotubes		
				Glucose	Insulin	Glucose + insulin
PPP1R3B	-117 $\pm$ 47	-87 $\pm$ 10	+1.15	-1.19	-1.63	-1.10
PPP1R3C	+1.92 $\pm$ 0.47	-8.70 $\pm$ 0.72	-14.7*	+1.14	+1.10	+1.20
PPP1R3A	-2.67 $\pm$ 0.31	-12612 $\pm$ 4325	-3408*	-1.32	-1.27	+1.63

percentages with respect to Ad-GFP-treated cells incubated with glucose and without insulin.

Statistical analysis

All data are presented as means $\pm$ S.E.M., and the significance of the difference was analysed by Student's *t* test. Values were considered significant at $P < 0.05$. Results for gene expression (fold change from the real-time PCR analysis) were examined using REST (Relative Expression Software Tool) [27].

RESULTS AND DISCUSSION

Relative expression of glycogen-targeting PP1 regulatory subunits, G_L, G_M and PTG genes in human muscle: effect of glucose and insulin

Since G_L is only expressed in human muscle, mRNA levels of *PPP1R3B* (coding for G_L), in comparison with those of *PPP1R3A* (coding for G_M) and *PPP1R3C* (coding for PTG), and potential regulation of these genes by glucose and insulin, was analysed in primary cultured human myotubes.

In muscle biopsies, relative *PPP1R3C* and *PPP1R3A* mRNA levels were both comparable with those of the reference gene coding for β_2 -microglobulin, whereas expression of *PPP1R3B* was about 100-fold lower than the reference gene (Table 1). In primary cultured myotubes, relative levels of *PPP1R3B* expression were steady compared with the muscle biopsies. *PPP1R3C* expression was about 14-fold lower, whereas *PPP1R3A* was reduced by about 3400-fold in cultured myotubes compared with muscle tissue. The relative abundances of the targeting subunit isoforms changed from *PPP1R3A* = *PPP1R3C* >> *PPP1R3B* to *PPP1R3C* > *PPP1R3B* >> *PPP1R3A*. Therefore these data suggest weaker expression of the G_L PP1 regulatory subunit compared with G_M or PTG in human muscle tissue. However, G_L (*PPP1R3B*) expression in cultured myotubes is preserved, unlike the muscle-specific PP1 GRS, G_M (*PPP1R3A*), which plunges after culture, and the non-muscle-specific PTG (*PPP1R3C*) subunit, which is decreased.

In cultured muscle cells [28,29] and muscle *in vivo* [30,31], glucose depletion or insulin are positive signals for the GS activity ratio [32]. There are several hypotheses about the mechanisms of these adaptations, which are still not completely elucidated [2,32]. We reasoned that one possible contributing factor could be the up-regulation of a PP1 GRS subunit. Thus we examined

whether glucose or insulin regulated the expression of *PPP1R3B* (G_L), *PPP1R3A* (G_M) and *PPP1R3C* (PTG) genes in cultured myotubes. We found no effects of glucose or insulin alone or of glucose plus insulin on their respective mRNA levels (Table 1). Previous studies showed that G_M expression is not modified in the muscle [33] of streptozotocin-type 1 diabetic rats. On the other hand, in liver extracts of insulin-dependent diabetic and adrenalectomized starved rats, G_L expression is reduced, an effect which is reverted by insulin administration [34]. Nevertheless, unlike the human gene, the rat G_L gene is not expressed in muscle tissue.

Effect of G_L overexpression on GS and GP activities in cultured human myotubes (combined effect with G_M; response to glucose, muscle GP and insulin)

We studied the impact of rat G_L overexpression on glycogen-metabolizing enzymes in cultured muscle cells alone or combined with delivered G_M, since, as shown above, this muscle-specific subunit is highly down-regulated in these cells. Rat G_L protein is 89% identical with human G_L [4]. Moreover, the 16-amino-acid region in the C-terminal region that binds GP *in vitro* is identical in the human and rat proteins [10].

To overexpress G_L, cultured human myotubes were treated with an adenovirus (Ad-G_L) containing the full-length rat G_L cDNA. Cells were harvested 24 or 48 h after transduction, and the expression of the rat G_L mRNA was analysed by reverse-transcription PCR relative to β_2 -microglobulin. Cells exposed to Ad-G_L showed levels of G_L (expressed as $1/2^{-\Delta C_t} \pm$ S.E.M.) of -3.68 ± 0.03 at 24 h and -1.50 ± 0.08 at 48 h, whereas, in cells treated with the control virus (Ad-GFP), values of -2424 ± 571 were assessed. Under equivalent conditions, cells were exposed to Ad-G_M, leading to a relative mRNA level of rabbit G_M of -18.0 ± 1.9 at 24 h; the value in Ad-GFP cells was -10615 ± 2503 (minus signs indicate that expression was lower than that of the control gene).

Delivery of G_L increased (between 2- and 3-fold) the activity ratio of GS, irrespective of whether cells had been incubated with or without glucose (Table 2) during the period of overexpression. Total GS activity was similar in controls (glucose-replete, 11.3 ± 1.1 munits/mg of protein; glucose-depleted, 15.3 ± 2.1 munits/mg of protein) and G_L overexpressers (glucose-replete, 12.8 ± 0.9 munits/mg of protein; glucose-depleted, 13.2 ± 0.7 munits/mg of protein). As shown previously [16], G_M activated GS more in glucose-depleted (85%) than in glucose-replete

Table 2 Effect of G_L overexpression on GS and GP activity ratios in glucose-replete and -depleted cells

Muscle cells were transduced with Ad-GFP, Ad- G_L and/or Ad-MGP and incubated without or with 25 mM glucose for 18 h. Then, where indicated, 100 nM insulin was added for 30 min. GS and GP activity ratios were measured in cell extracts. Data are means $\pm$ S.E.M. for four independent experiments performed in duplicate. Significances of differences are: * P < 0.01 and ** P < 0.001 versus controls (Ad-GFP) and † P < 0.05 and ‡ P < 0.005 versus Ad- G_L , under the same incubation conditions; ¶ P < 0.05 no glucose versus glucose without insulin in controls; and § P < 0.05 insulin versus no insulin in G_L overexpressers without glucose. ND, means not determined.

Treatment	GS				GP			
	Glucose ...							
	+	–	–	+	+	–	–	+
Insulin ...	–	+	–	+	–	+	–	+
Ad-GFP	0.050 $\pm$ 0.006	0.057 $\pm$ 0.004	0.081 $\pm$ 0.016¶	0.058 $\pm$ 0.003	0.65 $\pm$ 0.08	0.78 $\pm$ 0.03	0.74 $\pm$ 0.03	0.88 $\pm$ 0.07
Ad- G_L	0.170 $\pm$ 0.020**	0.154 $\pm$ 0.025**	0.196 $\pm$ 0.001**	0.126 $\pm$ 0.013**§	0.55 $\pm$ 0.03	0.65 $\pm$ 0.05	0.66 $\pm$ 0.10	0.79 $\pm$ 0.04
Ad-MGP	0.055 $\pm$ 0.005	ND	0.111 $\pm$ 0.007*	ND	0.25 $\pm$ 0.07**	ND	0.37 $\pm$ 0.02*	ND
Ad- G_L + Ad-MGP	0.127 $\pm$ 0.008†	ND	0.193 $\pm$ 0.008	ND	0.30 $\pm$ 0.04‡	ND	0.35 $\pm$ 0.02‡	ND

Figure 1 Combined effect of G_L and G_M on GS (A) and GP (B) activity ratios and glycogen synthesis (C)

Muscle cells were transduced with Ad-GFP, Ad- G_L or Ad- G_M and incubated with (white columns) or without (black columns) 25 mM glucose for 18 h. (A) GS and (B) GP activity ratios were measured in cell extracts. (C) Cells were further incubated with 10 mM [14 C]glucose for 8 h. Data are means $\pm$ S.E.M. from three (A, B) and two (C) experiments performed in triplicate. Significance of differences: versus cells treated with Ad-GFP under the same incubation conditions, * P < 0.05 and ** P < 0.001; cells without glucose versus with glucose for any viral treatment, † P < 0.05 and ‡ P < 0.001; and (C) versus cells treated with Ad- G_L (§ P < 0.005) or Ad- G_M (¶ P < 0.005) under the same incubation conditions.

(35%) cells (Figure 1A), whereas G_M did not modify total GS activity levels (results not shown). Indeed, high glucose/glycogen exerts a negative effect on GS (Table 2) and G_M [16], through mechanisms that are not yet clearly established [28–31]. Therefore the GS-activating effect of G_L is comparable with that of G_M in glucose-depleted cells and higher in glucose-replete cells [16], but weaker than that shown for PTG [19] in muscle cells, unlike in liver cells, where G_L shows the highest GS-activating capacity

compared with PTG or G_M [13,14]. When overexpressed together in myotubes, G_L and G_M did not significantly further activate GS compared with G_L alone (Figure 1A).

G_L overexpression caused no statistically significant change in the GP activity ratio, and glucose had no further effect on these values (Table 2). By contrast, G_M inactivated GP in cells incubated with glucose (Figure 1B) [16], irrespective of G_L overexpression. A possible explanation for this lack of effect in muscle cells is that GP is located preferentially in glycogen particles that accumulate close to the SR (sarcoplasmic reticulum) in a protein complex including phosphorylase kinase [35] and, presumably, G_M . Neither G_L nor PTG [19], which are not targeted to the SR, regulate this enzyme. By contrast, G_M , which includes an SR-binding motif, does inactivate GP, whereas a truncated G_M protein lacking the regulatory-subunit-binding motif does not [16].

Since G_L effects on PP1 GS phosphatase activity are inhibited by GP α (dephosphorylated, active form) *in vitro* [7], we examined the joint effect of G_L and muscle GP overexpression on GS activation. Transduction with the Ad-MGP virus increased total GP activity (456 $\pm$ 97 and 311 $\pm$ 38 munits/mg of protein in glucose-replete and -depleted cells respectively) by more than 3-fold compared with controls (100 $\pm$ 8 and 86.4 $\pm$ 8.0 munits/mg of protein in glucose-replete and -depleted cells respectively). The levels of the active form of GP (GP α) were increased by about 2-fold (114 $\pm$ 14 and 113 $\pm$ 12 munits/mg of protein in GP overexpressers with or without glucose compared with 64.2 $\pm$ 9.0 and 64.8 $\pm$ 7.8 in glucose-replete and -depleted control cells respectively). Higher levels of GP did not modify the GS activity ratio in cells incubated with glucose, whereas they did cause an additional increase when cells were deprived of glucose (Table 2), as we have previously shown [29]. Concomitant overexpression of G_L and GP did not modify GP activity ratio (Table 2) compared with GP overexpression alone, irrespective of whether glucose was present or not. GP inhibited the effect of G_L on GS activation by 25% in glucose-replete cells, whereas the inhibition was not significant in glucose-depleted cells. These results support the concept that GP α is an allosteric inactivator of G_L in muscle cells, as has been shown in liver cells [11] and *in vitro* [7]. The lack of inhibition in glucose-depleted cells may be due to the suppression of GP action on G_L by AMP [7] or other inhibitory metabolites. On the other hand, it has to be taken into account that, in glucose-depleted cells, muscle GP has a stimulatory effect by itself, which opposes the allosteric inhibition of G_L .

Since insulin is a key physiological regulator of glycogen metabolism, we examined the effects of G_L under insulin stimulation. Activation of muscle GS may be mediated, besides enhancement

Figure 2 Time-dependent synthesis of glycogen in glucose-replete (A) and -depleted (B) cells

Muscle cells were transduced with Ad-GFP (□) or Ad- G_L (■) and incubated with 25 mM glucose (A), or switched to glucose-depleted medium (B), for 18 h. Then, cells were incubated with 10 mM [U - ^{14}C]glucose for the times indicated. Data are means $\pm$ S.E.M. for three experiments performed in triplicate. Significance of differences versus cells treated with Ad-GFP: * $P < 0.01$ and ** $P < 0.001$.

of phosphatases, by inactivation of kinases phosphorylating GS, mainly GSK3, which is a target of insulin [36,32]. Insulin metabolic effects in muscle are triggered by the phosphorylation of the insulin receptor substrate 1 and subsequent activation of PI3K (phosphatidylinositol 3-kinase) [37,38]. Activated PI3K produces $Ptd(3,4,5)P_3$, which recruits proteins with a pleckstrin homology domain, such as PDK1 (3-phosphoinositide-dependent protein kinase) and Akt/PKB (protein kinase B) [38]. The activated phospho-Akt then inactivates GSK3 by phosphorylation of N-terminal Ser²¹ on GSK3- α and Ser⁹ on GSK3- β , which is involved in GS regulation [32,36]. We found that insulin had no significant effect on GS activity ratio (Table 2) in control cells or G_L overexpressers incubated with glucose, whereas GS activity ratios showed a tendency to decrease in cells depleted of glucose and treated with insulin, which reached statistical significance in G_L overexpressers. Insulin did not change GS total activity under any studied conditions (results not shown). The lack of activation of GS by insulin is a consistent observation in cultured myotubes [29], in comparison with the observations by others in cultured human myoblasts, where insulin doubles the GS activity ratio [39]. However, we found that, in myotubes cultured with glucose, short-term treatment with insulin stimulates Akt phosphorylation on Ser⁴⁷³ and GSK3- β phosphorylation on Ser⁹, as expected. Treatment of Ad-GFP-transduced cells incubated with glucose with 100 nM insulin for 10 min stimulated Akt/PKB phosphorylation on Ser⁴⁷³ compared with Ad-GFP cells ($136 \pm 9\%$, $P < 0.005$) and slightly increased total Akt protein ($120 \pm 9\%$; $P < 0.05$). By contrast, in G_L overexpressers, no significant changes were observed in Akt phosphorylation ($104 \pm 8\%$) or protein content ($108 \pm 13\%$) compared with Ad-GFP cells. Insulin had equivalent stimulatory effects on Akt phosphorylation ($141 \pm 19\%$; $P < 0.05$) and did not change total Akt levels ($94 \pm 10\%$) in G_L overexpressers relative to Ad-GFP. Regarding GSK3, the major phosphorylated band was GSK3- β (results not shown). Phosphorylation of GSK3- β on Ser⁹ was increased by insulin ($148 \pm 19\%$; $P < 0.05$) relative to Ad-GFP glucose-replete cells, while expression of the GSK3 β -isoform was not modified by insulin ($102 \pm 15\%$). In cells overexpressing G_L , GSK3- β phosphorylation ($93 \pm 13\%$) exhibited no changes compared with Ad-GFP cells, nor was there change in the total protein content ($105 \pm 9\%$). Insulin enhancement of GSK3- β phosphorylation in G_L overexpressers ($150 \pm 27\%$, $P < 0.05$) was still observed as in controls, while no differences were found in total GSK3- β expression levels ($112 \pm 26\%$). A possible reason

for the discrepancy between the insulin effect on transducing kinases and lack of GS activation in myotubes is the regulation of different intracellular pools of kinases. For instance, it has been suggested that there are at least two distinct pools of GSK3- β and that the one responsive to cyclic AMP is not directly involved in the phosphorylation-inactivation of GS in isolated soleus muscles [40]. It is also possible that, besides inactivation of GSK3, insulin exerts other effects in human myotubes, which counteract the activation of GS. Regarding G_L , the rat and human proteins have a theoretical PDK1-binding motif (Ser¹⁹² and Ser¹⁹³ respectively) according to [41]; however, our data indicate that no interaction between PDK1 and G_L takes place that affects regulation of the phosphorylation of Akt or GSK3 or its response to insulin.

Effect of G_L overexpression on glycogen synthesis in cultured human myotubes: response to insulin and glucose

The ability of G_L to stimulate glycogen synthesis was examined. First, a time-course study was performed in cells incubated with or without glucose (Figure 2). In glucose-replete cells (Figure 2A), G_L stimulated glucose incorporation into glycogen at all time points tested between 1 and 48 h. At the early time points, increments were about 2.5-fold and a maximal 5-fold stimulation was observed at 8 h. In glucose-depleted cells (Figure 2B), where glucose incorporation was higher compared with that in cells preincubated with glucose, no stimulatory effect of G_L on early glycogen resynthesis was observed, findings similar to those we obtained with G_M [16], whereas persistent increments of about 3-fold were detected between 8 and 48 h. Next, we examined the effect of G_L on glycogen synthesis as a function of glucose concentration (Figure 3A). In cells preincubated without glucose, G_L overexpression enhanced glycogen synthesis at every glucose concentration tested from 0.5 to 10 mM, indicating that stimulation is not a function of glucose concentration in the range tested. We also assessed (Figure 1C) the combined effects of G_L and G_M , which had a fainter (about 3-fold lower) glycogenic capacity than did G_L . Glycogen synthesis was further enhanced (about 2-fold) by the co-overexpression of both PP1 GRSs in glucose-replete cells, whereas in glucose-depleted cells no extra stimulation was accomplished. Next the combined effect on glycogen synthesis of G_L and insulin was examined (Figure 3B). Insulin exerted a stimulatory effect on glycogen synthesis, of about 2-fold in cells preincubated with glucose and of 1.4-fold in cells preincubated without glucose. In G_L overexpressers, incubated with or without

Figure 3 Glycogen synthesis: (A) glucose-concentration-dependence and (B) combined effect of G_L and insulin

Muscle cells were transduced with Ad-GFP (A, □) or Ad- G_L (A, ■) and (A) incubated without glucose or (B) with or without 25 mM glucose as stated, for 18 h. Then, cells were incubated with [^{14}C]glucose at (A) the indicated concentrations or at (B) 10 mM with or without 100 nM insulin, for 8 h. Data are means $\pm$ S.E.M. for three experiments performed in triplicate. Significance of differences: (A) versus cells treated with Ad-GFP: * P < 0.01 and ** P < 0.001 at equivalent glucose concentration; (B) in every group of treatments; * P < 0.01 and ** P < 0.001 in cells incubated with insulin versus without insulin, within controls and G_L overexpressers; † P < 0.001 in cells incubated without glucose and without insulin versus with glucose and without insulin; and ‡ P < 0.001 in G_L overexpressers versus controls incubated with glucose and without insulin.

glucose, insulin had a further stimulatory effect, with values 1.4- and 1.6-fold higher respectively. Thus, despite the fact that insulin has no activating effect on GS, it stimulates glycogen synthesis and combines its effects with G_L and glucose depletion. Moreover, glucose depletion and G_L , both of which activate GS, have an additive stimulatory effect on glycogen synthesis.

Finally the effects of overexpression of G_L in glycogen accumulation were assessed (Figure 4). In cells transduced with the control virus, glycogen content was steady over the time studied, whereas in cells transduced with Ad- G_L , glycogen levels were 2-fold higher 18 h after G_L delivery and progressively increased throughout the ensuing 48 h. Then, G_L causes a time-dependent increase in glycogen content.

In summary, G_L gene expression in human muscle tissue is relatively lower than that of PTG or G_M ; moreover, it is preserved in cultured human myotubes, where, like the expression of G_M or PTG genes, it is not regulated by insulin or glucose. In cultured myotubes, overexpression of G_L activates GS irrespective of glucose, without affecting the phosphorylation of the regulatory kinase GSK3 and does not modulate GP. G_L exerts a greater glycogenic effect than G_M , although weaker than that shown in

Figure 4 Time-course of glycogen accumulation

Muscle cells were transduced with Ad-GFP (□) or Ad- G_L (■) and incubated with 25 mM glucose for 18 h (time zero) or further incubated for the indicated times and then harvested to assess glycogen content. Data are means $\pm$ S.E.M. for three experiments performed in triplicate. Significance of differences versus cells treated with Ad-GFP: * P < 0.05, ** P < 0.005; and versus G_L overexpressers at time zero: † P < 0.05 and ‡ P < 0.01.

liver cells, where G_L powerfully activates GS and inactivates GP. G_L stimulation of glycogenesis in myotubes combines additively to the glycogenic effects of insulin and glucose depletion.

C. G. M. was a recipient of a Ramon y Cajal fellowship from the Ministerio de Educación y Ciencia, Spain. M. M. G. was a recipient of a fellowship from the Ministerio de Educación y Ciencia. This work was supported by grants SAF-2003-04223 and SAF-2006-07228 from the MCyT (Ministerio de Ciencia y Tecnología) and a grant [REDIMET (Red de Diabetes y Enfermedades Metabólicas Asociadas) RD06/0015/0018] from the Instituto de Salud Carlos III, Spain. We thank Ms Ana Orozco for technical assistance.

REFERENCES

- 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
- Nielsen, J. N. and Wojtaszewski, J. F. (2004) Regulation of glycogen synthase activity and phosphorylation by exercise. *Proc. Nutr. Soc.* **63**, 233–237
- Tang, P. M., Bondor, J. A., Swiderek, K. M. and DePaoli-Roach, A. A. (1991) Molecular cloning and expression of the regulatory (RGL) subunit of glycogen-associated protein phosphatase. *J. Biol. Chem.* **266**, 15782–15789
- Munro, S., Cuthbertson, D. J., Cunningham, J., Sales, M. and Cohen, P. T. (2002) Human skeletal muscle expresses a glycogen targeting subunit of PP1 that is identical to the insulin sensitive glycogen targeting subunit GL of liver. *Diabetes* **51**, 591–598
- Printen, J. A., Brady, M. J. and Saltiel, A. R. (1997) PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. *Science* **275**, 1475–1478
- Armstrong, C. G., Browne, G. J., Cohen, P. and Cohen, P. T. (1997) PPP1R6, a novel member of the family of glycogen-targeting subunits of protein phosphatase 1. *FEBS Lett.* **418**, 210–214
- Doherty, M. J., Moorhead, G., Morrice, N., Cohen, P. and Cohen, P. T. (1995) Amino acid sequence and expression of the hepatic glycogen-binding (GL)-subunit of protein phosphatase-1. *FEBS Lett.* **375**, 294–298
- Munro, S., Ceulemans, H., Bollen, M., Dilexio, J. and Cohen, P. T. (2005) A novel glycogen-targeting subunit of protein phosphatase 1 that is regulated by insulin and shows differential tissue distribution in humans and rodents. *FEBS J.* **272**, 1478–1489
- Dunn, J. S., Mlynarski, W. M., Pezzolesi, M. G., Borowiec, M., Powers, C., Krolewski, A. S. and Doria, A. (2006) Examination of PPP1R3B as a candidate gene for the type 2 diabetes and MODY loci on chromosome 8p23. *Ann. Hum. Genet.* **70**, 587–593
- Armstrong, C. G., Doherty, M. J. and Cohen, P. T. (1998) Identification of the separate domains in the hepatic glycogen-targeting subunit of protein phosphatase 1 that interact with phosphorylase a, glycogen and protein phosphatase 1. *Biochem. J.* **336**, 699–704
- Aiston, S., Hampson, L., Gomez-Fox, 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**, 23858–23866
- Bollen, M. and Stalmans, W. (1992) The structure, role, and regulation of type 1 protein phosphatases. *Crit. Rev. Biochem. Mol. Biol.* **27**, 227–281

- 13 Gasa, R., Jensen, P. B., Berman, H. K., Brady, M. J., DePaoli-Roach, A. A. and Newgard, C. B. (2000) Distinctive regulatory and metabolic properties of glycogen-targeting subunits of protein phosphatase-1 (PTG, GL, GM/RGL) expressed in hepatocytes. *J. Biol. Chem.* **275**, 26396–26403
- 14 Yang, R., Cao, L., Gasa, R., Brady, M. J., Sherry, A. D. and Newgard, C. B. (2002) Glycogen-targeting subunits and glucokinase differently affect pathways of glycogen metabolism and their regulation in hepatocytes. *J. Biol. Chem.* **277**, 1514–1523
- 15 Gasa, R., Clark, C., Yang, R., DePaoli-Roach, A. A. and Newgard, C. B. (2002) Reversal of diet-induced glucose intolerance by hepatic expression of a variant glycogen-targeting subunit of protein phosphatase-1. *J. Biol. Chem.* **277**, 1524–1530
- 16 Lerin, C., Montell, E., Nolasco, T., Clark, C., Brady, M. J., Newgard, C. B. and Gomez-Foix, A. M. (2003) Regulation and function of the muscle glycogen-targeting subunit of protein phosphatase 1 (GM) in human muscle cells depends on the COOH-terminal region and glycogen content. *Diabetes* **52**, 2221–2226
- 17 Aschenbach, W. G., Suzuki, Y., Breeden, K., Prats, C., Hirshman, M. F., Dufresne, S. D., Sakamoto, K., Vilardo, P. G., Steele, M., Kim, J. H. et al. (2001) The muscle-specific protein phosphatase PP1G/RGL (GM) is essential for activation of glycogen synthase by exercise. *J. Biol. Chem.* **276**, 39959–39967
- 18 Delibegovic, M., Armstrong, C. G., Dobbie, L., Watt, P. W., Smith, A. J. and Cohen, P. T. (2003) Disruption of the striated muscle glycogen targeting subunit PPP1R3A of protein phosphatase 1 leads to increased weight gain, fat deposition, and development of insulin resistance. *Diabetes* **52**, 596–604
- 19 Lerin, C., Montell, E., Berman, H. K., Newgard, C. B. and Gomez-Foix, A. M. (2000) Overexpression of protein targeting to glycogen in cultured human muscle cells stimulates glycogen synthesis independent of glycogen and glucose 6-phosphate levels. *J. Biol. Chem.* **275**, 39991–39995
- 20 Crosson, S. M., Khan, A., Printen, J., Pessin, J. E. and Saltiel, A. R. (2003) PTG gene deletion causes impaired glycogen synthesis and developmental insulin resistance. *J. Clin. Invest.* **111**, 1423–1432
- 21 Askanas, V. and Gallez-Hawkins, G. (1985) Synergistic influence of polypeptide growth factors on cultured human muscle. *Arch. Neurol.* **42**, 749–752
- 22 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−ΔΔC_T} method. *Methods* **25**, 402–408
- 23 Gomez-Foix, A. M., Coats, W. S., Baque, S., Alam, T., Gerard, R. D. and Newgard, C. B. (1992) Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. *J. Biol. Chem.* **267**, 25129–25134
- 24 Gilboe, D. P., Larson, K. L. and Nuttall, F. Q. (1972) Radioactive method for the assay of glycogen phosphorylases. *Anal. Biochem.* **47**, 20–27
- 25 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-¹⁴C-glucose. *Anal. Biochem.* **25**, 486–499
- 26 Montell, E., Turini, M., Marotta, M., Roberts, M., Noe, V., Ciudad, C. J., Mace, K. and Gomez-Foix, A. M. (2001) DAG accumulation from saturated fatty acids desensitizes insulin stimulation of glucose uptake in muscle cells. *Am. J. Physiol. Endocrinol. Metab.* **280**, E229–E237
- 27 Pfaffl, M. W., Horgan, G. W. and Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36
- 28 Halse, R., Bonavaud, S. M., Armstrong, J. L., McCormack, J. G. and Yeaman, S. J. (2001) Control of glycogen synthesis by glucose, glycogen, and insulin in cultured human muscle cells. *Diabetes* **50**, 720–726
- 29 Montell, E., Arias, A. and Gómez-Foix, A. M. (1999) Glycogen depletion rather than glucose 6-P increments controls early glycogen recovery in human cultured muscle. *Am. J. Physiol.* **45**, R1489–R1495
- 30 Bloch, G., Chase, J. R., Meyer, D. B., Avison, M. J., Shulman, G. I. and Shulman, R. G. (1994) *In vivo* regulation of rat muscle glycogen resynthesis after an intense exercise. *Am. J. Physiol.* **266**, E85–E91
- 31 Danforth, W. H. (1965) Glycogen synthetase activity in skeletal muscle interconversion of two forms and control of glycogen synthesis. *J. Biol. Chem.* **240**, 588–593
- 32 Yeaman, S. J., Armstrong, J. L., Bonavaud, S. M., Poinasamy, D., Pickersgill, L. and Halse, R. (2001) Regulation of glycogen synthesis in human muscle cells. *Biochem. Soc. Trans.* **29**, 537–541
- 33 Lanner, C., Suzuki, Y., Bi, C., Zhang, H., Cooper, L. D., Bowker-Kinley, M. M. and DePaoli-Roach, A. A. (2001) Gene structure and expression of the targeting subunit, RGL, of the muscle-specific glycogen-associated type 1 protein phosphatase, PP1G. *Arch. Biochem. Biophys.* **388**, 135–145
- 34 Doherty, M. J., Cadefau, J., Stalmans, W., Bollen, M. and Cohen, P. T. (1998) Loss of the hepatic glycogen-binding subunit (GL) of protein phosphatase 1 underlies deficient glycogen synthesis in insulin-dependent diabetic rats and in adrenalectomized starved rats. *Biochem. J.* **333**, 253–257
- 35 Cuenda, A., Nogues, M., Henao, F. and Gutierrez-Merino, C. (1995) Interaction between glycogen phosphorylase and sarcoplasmic reticulum membranes and its functional implications. *J. Biol. Chem.* **270**, 11998–12004
- 36 McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, R. and Alessi, D. R. (2005) Role that phosphorylation of GSK3 plays in insulin and Wnt signalling defined by knockin analysis. *EMBO J.* **24**, 1571–1583
- 37 Bjornholm, M. and Zierath, J. R. (2005) Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. *Biochem. Soc. Trans.* **33**, 354–357
- 38 Dong, L. Q. and Liu, F. (2005) PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. *Am. J. Physiol. Endocrinol. Metab.* **289**, E187–E196
- 39 Halse, R., Rochford, J. J., McCormack, J. G., Vandenhede, J. R., Hemmings, B. A. and Yeaman, S. J. (1999) Control of glycogen synthesis in cultured human muscle cells. *J. Biol. Chem.* **274**, 776–780
- 40 Jensen, J., Brennesvik, E. O., Lai, Y. C. and Shepherd, P. R. (2007) GSK-3 β regulation in skeletal muscles by adrenaline and insulin: evidence that PKA and PKB regulate different pools of GSK-3. *Cell. Signalling* **19**, 204–210
- 41 Obenaus, J. C., Cantley, L. C. and Yaffe, M. B. (2003) Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* **31**, 3635–3641

Received 18 October 2006/9 February 2007; accepted 27 February 2007

Published on the Internet 13 June 2007, doi:10.1042/BJ20061572