

# Regulation and Function of the Muscle Glycogen-Targeting Subunit of Protein Phosphatase 1 ( $G_M$ ) in Human Muscle Cells Depends on the COOH-Terminal Region and Glycogen Content

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$G_M$ , the muscle-specific glycogen-targeting subunit of protein phosphatase 1 (PP1) targeted to the sarcoplasmic reticulum, was proposed to regulate recovery of glycogen in exercised muscle, whereas mutation truncation of its COOH-terminal domain is known to be associated with type 2 diabetes. Here, we demonstrate differential effects of  $G_M$  overexpression in human muscle cells according to glycogen concentration. Adenovirus-mediated delivery of  $G_M$  slightly activated glycogen synthase (GS) and inactivated glycogen phosphorylase (GP) in glycogen-replete cells, causing an overaccumulation of glycogen and impairment of glycogenolysis after glucose deprivation. Differently, in glycogen-depleted cells,  $G_M$  strongly increased GS activation with no further enhancement of early glycogen resynthesis and without affecting GP. Effects of  $G_M$  on GS and GP were abrogated by treatment with dibutyryl cyclic AMP. Expression of a COOH-terminal deleted-mutant ( $G_{M\Delta C}$ ), lacking the membrane binding sequence to sarcoplasmic reticulum, failed to activate GS in glycogen-depleted cells, while behaving similar to native  $G_M$  in glycogen-replete cells. This is explained by loss of stability of the  $G_{M\Delta C}$  protein following glycogen-depletion. In summary,  $G_M$  promotes glycogen storage and inversely regulates GS and GP activities, while, specifically, synthase phosphatase activity of  $G_M$ -PP1 is inhibited by glycogen. The conditional loss of function of the COOH-terminal deleted  $G_M$  construct may help to explain the reported association of truncation mutation of  $G_M$  with insulin resistance in human subjects. *Diabetes* 52:2221–2226, 2003

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DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; GP, glycogen phosphorylase; GS, glycogen synthase; PKA, cyclic AMP-dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; PP1, protein phosphatase 1; PTG, protein targeting glycogen; SR, sarcoplasmic reticulum.

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Glycogen metabolism is regulated by the rate-limiting enzymes of its synthesis and breakdown, glycogen synthase (GS) and glycogen phosphorylase (GP). Both enzymes are regulated by allosteric and phosphorylation-dephosphorylation mechanisms. Dephosphorylation, which causes the activation of GS and inactivation of GP, is mainly catalyzed by protein phosphatase 1 (PP1) (1). PP1 activity is regulated by binding to regulatory subunits known collectively as glycogen targeting subunits of PP1. Four targeting subunits have been identified in mammals:  $G_M$ , specifically expressed in skeletal muscle (2);  $G_L$ , expressed mostly in liver (3); protein targeting glycogen (PTG), expressed mainly in insulin-sensitive tissues, such as muscle and fat (4); and PPP1R6, abundant in muscle (5). These targeting subunits localize enzymes of glycogen metabolism to the glycogen particle and differentially affect their catalytic and regulatory properties (6).

$G_M$  shares the  $NH_2$ -terminal glycogen (amino acids 150–159 in  $G_M$ ) (7) and PP1 (64–69 in  $G_M$ ) (8) binding sequences with the rest of glycogen targeting subunits. Although muscle cell synthase phosphatase activity is inhibited by glycogen (9–12), no direct evidence of glycogen effect on  $G_M$ -PP1 activity within the muscle cell has been reported. In vitro,  $G_M$  binds directly to GS through domains localized to amino acids 77–118 and 219–240 in its primary sequence (13), independently of glycogen content (7,13), while there is no evidence of direct binding to GP. Nevertheless, in  $G_M$  knockout mice both GS and GP activity ratios were affected (in opposite directions), while overexpression in skeletal muscle of transgenic mice activated GS only (14). Furthermore, the major finding in  $G_M$  knockout mice was the lack of increase in GS activity after muscle contraction and hence glycogen recovery (14).

On the other hand,  $G_M$  has distinguishable structural features in which the impact on muscle glycogen metabolism has only been partly elucidated.  $G_M$  has an elongated COOH-terminal domain that contains a sarcoplasmic reticulum (SR) binding sequence (1064–1094). Nevertheless, in skeletal muscle,  $G_M$  can be localized in two intracellular compartments, the SR and cytosol (15), bound to the corresponding specific fractions of glycogen (16). Impor-

tantly, truncation mutation of the G<sub>M</sub> gene has been recently associated with insulin resistance in human subjects (17). Unlike wild-type G<sub>M</sub>, the mutant lacking the COOH-terminal SR-binding domain was localized almost exclusively in the cytosol. This mutation was presumed to derange muscle metabolism, but no experimental support was provided of the functional consequences of protein truncation. Much more is known about the role of the two NH<sub>2</sub>-terminal phosphorylation sites, Ser48 (site 1) and Ser67 (site 2), which are hormonally regulated. Phosphorylation of site 1 is induced by insulin and leads to activation of GS in vitro (18). However, G<sub>M</sub> is not required for insulin activation of GS in vivo, as the hormone effect was not impaired in G<sub>M</sub> knockout mice (19). Phosphorylation of sites 1 and 2 is stimulated by adrenaline, via cyclic AMP-dependent protein kinase (PKA) (15,20), in both the cytosolic and SR-localized fractions of G<sub>M</sub>. In in vitro studies, phosphorylation of site 2, which is located in the PP1 binding sequence, causes the dissociation of the catalytic subunit from G<sub>M</sub>. By virtue of this disruption, site 2 phosphorylation dominates the impact of site 1 phosphorylation. Based on this observation, PKA-mediated regulation of the G<sub>M</sub>-PP1 complex was proposed to play a role in the adrenergic control of glycogen synthesis (15).

Therefore, there is limited understanding of how structural features that are unique to G<sub>M</sub> may contribute to regulation/deregulation of glycogen metabolism. In this study, we analyzed the impact of G<sub>M</sub> overexpression in human muscle cells under various cell glycogen concentrations, and more importantly we dissected the importance of the extended COOH-terminal domain in G<sub>M</sub> function.

## RESEARCH DESIGN AND METHODS

**Human muscle primary cultures.** Human muscle primary cultures were initiated from satellite cells of muscle biopsies obtained with informed consent and approval of the Human Use Committee of the Hospital Vall d'Hebrón (Barcelona). Aneurular muscle cultures were established in a monolayer according to the technique described by Askanas and Engel (21). Cultures were grown in Dulbecco's modified Eagle's medium (DMEM)/M-199 medium 3:1, supplemented with 10% FBS, 10 μg/ml insulin (Sigma), 2 mmol/l glutamine (Sigma), 25 ng/ml fibroblast growth factor (FGF), and 10 ng/ml epidermal growth factor (EGF). Immediately after myoblast fusion, the medium was replaced by a medium devoid of FGF, EGF, and glutamine, which was maintained until experiments were performed. All cultures were kept at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. All experiments were performed with biopsies from at least two different patients.

**Transduction with adenovirus.** Recombinant adenovirus containing wild-type rabbit muscle G<sub>M</sub>/R<sub>G1</sub> (AdCMV-G<sub>M</sub>/R<sub>G1</sub>) (22), a truncated version of rabbit G<sub>M</sub>/R<sub>G1</sub> (1.1-kb fragment of the G<sub>M</sub>/R<sub>G1</sub> cDNA encoding amino acids 1–375 and lacking the 735 COOH-terminal amino acids) (AdCMV-G<sub>M</sub>ΔC) (23), and the entire protein coding sequence of mouse protein targeting to glycogen (AdCMV-PTG) (24) were used. In addition, AdCMV-β-GAL, a virus containing the bacterial β-galactosidase gene was used as a control in the metabolic studies (25). Recombinant viruses were amplified in 293 cells and viral stocks of 1 × 10<sup>9</sup> plaque-forming units (pfu)/ml were prepared in 10% FBS-DMEM by standard techniques. Gene delivery to muscle cultures was achieved by exposing 12-day-old fibers to the virus for 2 h at a multiplicity of infection (moi) of 10.

**Western blot analysis.** Cell monolayers were scraped into 100 μl homogenization buffer consisting of 10 mmol/l Tris-HCl (pH 7.0), 150 mmol/l KF, 15 mmol/l EDTA, 600 mmol/l sucrose, 10 μg/ml leupeptin, 1 mmol/l benzamide, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) and then sonicated. Protein concentration was measured using the Bio-Rad protein assay reagent. Immunoblot analysis of whole cell extracts was performed using specific antibodies. Polyclonal antibody raised against rabbit G<sub>M</sub>/R<sub>G1</sub> protein (14) was kindly provided by Dr. R. Cussó (Hospital Clínic de Barcelona). Polyclonal antibody raised against GS was kindly provided by Dr. J.J. Guinovart (Parc científic de Barcelona). PP1 antibody (sc-7482) was purchased from Santa-

cruz Biotechnologies, and β-actin antibody (A2066) was purchased from Sigma. Detection of the secondary antibody was accomplished using the ECL Plus kit (Roche).

**Enzyme activity assays.** To measure GS and GP activities, 100 μl homogenization buffer consisting of 10 mmol/l Tris-HCl (pH 7.0), 150 mmol/l KF, 15 mmol/l EDTA, 600 mmol/l sucrose, 15 mmol/l 2-mercaptoethanol, 10 μg/ml leupeptin, 1 mmol/l benzamide, and 1 mmol/l PMSF was used to scrape frozen plates containing the cell monolayers before sonication. The resulting homogenates were used for the determination of enzyme activities. Protein concentration was measured as described above. GP activity was determined by the incorporation of [U-<sup>14</sup>C]glucose 1-phosphate into glycogen in the absence or presence of the allosteric activator AMP (5 mmol/l) (26). GS activity was determined by the incorporation of [U-<sup>14</sup>C]UDP-glucose into glycogen in the absence or presence of 10 mmol/l glucose 6-P as described (27).

**Determination of glycogen content.** To measure glycogen content, cell monolayers were scraped into 100 μl 30% KOH and the homogenates were boiled for 15 min. An aliquot of the homogenates was used for the measurement of protein concentration as described above. Homogenates were spotted onto Whatman 31ET paper, and glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. Dried papers containing precipitated glycogen were incubated in 0.4 mol/l acetate buffer (pH 4.8) with 25 units/ml α-amyloglucosidase (Sigma) for 90 min at 37°C. Glucose released from glycogen was measured enzymatically in a Cobas Fara II autoanalyzer with a GlucoQuant (Roche) kit.

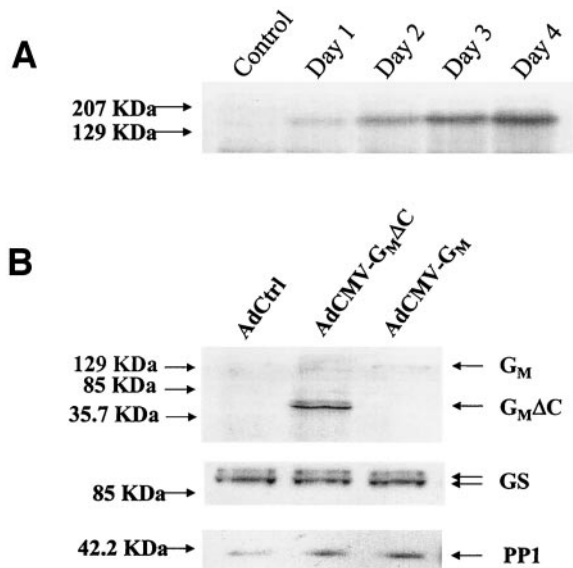
**Determination of [U-<sup>14</sup>C]glucose incorporation into glycogen.** After adenoviral infection and glucose deprivation, cells were incubated in the presence of 10 mmol/l glucose and 0.5 μCi/μl [U-<sup>14</sup>C]glucose for the indicated times. To measure <sup>14</sup>C-glucose incorporation into glycogen, cell monolayers were scraped into 100 μl 30% KOH and the homogenates boiled for 15 min. Homogenates were then spotted onto Whatman 31ET paper, and glycogen was precipitated by immersing the papers in ice-cold 66% ethanol. After two washes with 66% ethanol, papers containing precipitated glycogen were dried and radioactivity measured in a β-counter.

**Statistical analysis.** Data were analyzed for statistical significance by the Student's *t* test.

## RESULTS

**Effect of G<sub>M</sub> and G<sub>M</sub>ΔC delivery in glycogen-replete cells.** Overexpression of G<sub>M</sub> was achieved by exposing cells to adenovirus containing the full-length rabbit G<sub>M</sub> cDNA (AdCMV-G<sub>M</sub>). To analyze the role of SR targeting in G<sub>M</sub> regulatory action, cells were transduced with another adenovirus, AdCMV-G<sub>M</sub>ΔC, containing a G<sub>M</sub> mutant (G<sub>M</sub>ΔC), which lacks the 735 COOH-terminal amino acids. Western blot analysis with an antibody raised against the NH<sub>2</sub>-terminal region of rabbit G<sub>M</sub> revealed transgene expression within 24 h of AdCMV-G<sub>M</sub> treatment, with a progressive increase with time up to 4 days (Fig. 1A), whereas endogenous protein could not be detected. In cells exposed to AdCMV-G<sub>M</sub>ΔC, an immunoreactive band corresponding to the molecular weight of the truncated protein was detected (Fig. 1B). Protein levels of the glycogen synthetic complex were analyzed. Delivery of either G<sub>M</sub> or G<sub>M</sub>ΔC did not modify muscle GS content, but an increase in PP1 was revealed. Because glycogen synthesis is limited by the rate of glucose uptake, this parameter was ascertained in G<sub>M</sub> overexpressers. No difference in 2-deoxyglucose uptake was observed between control (176 ± 7 pmol/min per well) and G<sub>M</sub> (173 ± 5 pmol/min per well) cells.

To study the effects of G<sub>M</sub> overexpression on GS and GP activity ratio in the glycogen-replete condition, cells were incubated at a high glucose concentration for 3 days after viral treatment. After this treatment, G<sub>M</sub>-overexpressing cells showed a slightly higher GS activity ratio compared with controls (Table 1), whereas GP activity ratio was slightly decreased. Total activities of both GS and GP were unchanged by G<sub>M</sub> overexpression, indicating that protein



**FIG. 1.** Immunoblot analysis of  $G_M$  and  $G_M\Delta C$  expression. **A:** Primary human muscle cells exposed to AdCtrl or AdCMV- $G_M$  were incubated in the presence of 25 mmol/l glucose and collected to perform the immunoblot analysis at the indicated times. Whole cell extracts (30  $\mu$ g protein) were separated on a 6% gel, and  $G_M$  protein was detected by immunoblotting. A representative autoradiogram of three independent experiments is shown. **B:** Cells were infected with AdCtrl, AdCMV- $G_M\Delta C$ , or AdCMV- $G_M$  and then incubated with 25 mmol/l glucose for 36 h.  $G_M$ ,  $G_M\Delta C$ , PPI, and GS proteins were detected by immunoblotting in whole cell extracts. A representative autoradiogram of three independent experiments is shown.

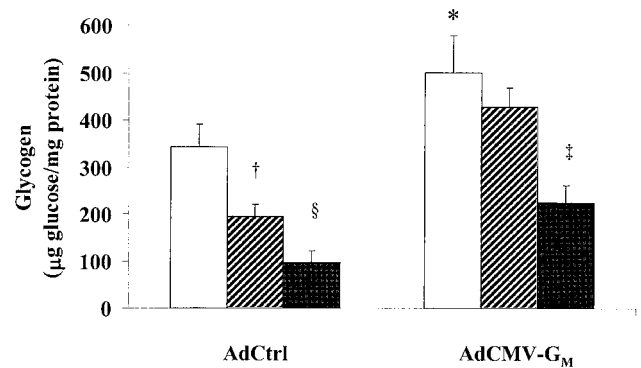
levels were not altered. Glycogen content was increased by 50% in cells overexpressing  $G_M$  compared with untreated control cells. In glycogen-replete cells overexpressing  $G_M\Delta C$ , GS activity ratio and glycogen content were similar to those in cells overexpressing  $G_M$  (Table 1). Unlike  $G_M$ , overexpression of  $G_M\Delta C$  did not modify GP activity ratio in glycogen-replete cells.

**Effect of  $G_M$  overexpression on basal and cyclic AMP-stimulated glycogen hydrolysis.** Because  $G_M$  overexpression slightly inactivated GP, we next studied whether glycogen breakdown was impaired. To this end, cells were treated with AdCMV- $G_M$  or a control virus, AdCtrl, and then incubated in the absence of glucose for 4 h to activate glycogenolysis. After this manipulation,  $G_M$ -overexpressing cells had degraded significantly less glycogen than control cells (15 vs. 45%) (Fig. 2). Since phosphorylation of  $G_M$  by PKA was reported to dissociate the  $G_M$ -PPI complex, we tested whether this mechanism could abrogate  $G_M$ -mediated effects on glycogenolysis.

**TABLE 1**  
Effect of  $G_M$  and  $G_M\Delta C$  delivery on GS and GP activities and glycogen levels

	AdCtrl	AdCMV- $G_M$	AdCMV- $G_M\Delta C$
GS activity ratio (-G6P/+G6P)	0.07 $\pm$ 0.01	0.10 $\pm$ 0.01*	0.09 $\pm$ 0.01†
GS total activity (mU/mg protein)	10.5 $\pm$ 0.8	10.1 $\pm$ 1.3	10.9 $\pm$ 0.3
GP activity ratio (-AMP/+AMP)	0.73 $\pm$ 0.04	0.61 $\pm$ 0.03†	0.71 $\pm$ 0.02
GP total activity (mU/mg protein)	52.7 $\pm$ 2.1	51.9 $\pm$ 3.1	47.2 $\pm$ 1.5
Glycogen ( $\mu$ g glucose/mg protein)	344 $\pm$ 48	501 $\pm$ 78‡	521 $\pm$ 56§

Data are means  $\pm$  SD of at least four independent experiments performed in duplicate (GS and GP activities) or triplicate (glycogen levels). Muscle cells were infected with AdCtrl, AdCMV- $G_M$ , or AdCMV- $G_M\Delta C$  and incubated with 25 mmol/l glucose for 3 days. Metabolic parameters were assessed at the end of this period. \* $P$  < 0.01, † $P$  < 0.001, ‡ $P$  < 0.0005, and § $P$  < 0.00005 for control cells vs.  $G_M$  or  $G_M\Delta C$ -overexpressing cells.



**FIG. 2.** Effect of  $G_M$  overexpression on the glycogenolytic response. Primary human muscle cells infected with AdCtrl or AdCMV- $G_M$  were maintained with 25 mmol/l glucose for 3 days ( $\square$ ) and then deprived of glucose in the absence ( $\square$ ) or presence ( $\blacksquare$ ) of 2 mmol/l dibutyryl cyclic AMP. Glycogen levels were measured before and after 4 h of treatment. \* $P$  < 0.0005 for control cells vs.  $G_M$  overexpressing cells. † $P$  < 0.005, ‡ $P$  < 0.0005, and § $P$  < 0.00005, respectively, for differences versus glucose-incubated cells. Data are means  $\pm$  SD of four independent experiments performed in triplicate.

Cells were treated with dibutyryl cyclic AMP, which activates PKA, concomitantly with glucose deprivation. The glycogenolytic rate was markedly increased by this treatment in  $G_M$  and control cells. The response, assessed by the ratio between the decay in glycogen caused by cyclic AMP or glucose depletion alone, was even higher in  $G_M$  (3.6-fold) than control (1.6-fold) cells, indicating that  $G_M$  inhibition of glycogenolysis was overcome by elevation of cellular cyclic AMP.

**Effect of  $G_M$  on GS and GP activities in glycogen-depleted cells.** The data summarized to this point indicate that  $G_M$  effects on GS were modest. A possible explanation for these weak effects could be that high glycogen levels prevented  $G_M$ -PPI activation of GS. To test this hypothesis, myotubes were treated with viruses and then incubated without glucose for 18 h to allow glycogen depletion. At this time, glycogen levels were decreased to  $43.9 \pm 4.3$  and  $45.5 \pm 4.1$   $\mu$ g glucose/mg protein in control and  $G_M$ -overexpressing cells, respectively. In glycogen-depleted control cells, GS activation state was double that in glycogen-replete cells, confirming previous findings (28), whereas  $G_M$  overexpression caused a twofold additional activation of GS (Fig. 3A). Glycogen resynthesis was monitored after 48 h. Strikingly, no increment was observed during the first 4 h, while 50% increment was observed at 24 h (Fig. 4). To test whether cyclic AMP abrogated  $G_M$  effects on GS in glycogen-depleted cells,

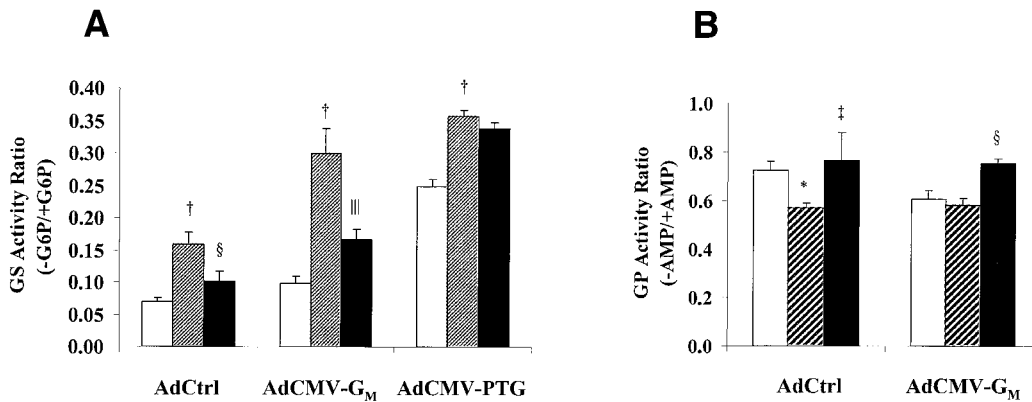


FIG. 3. Effect of dibutyryl cyclic AMP on GS and GP activity ratios in glycogen-depleted cells. Primary human muscle cells were infected with AdCtrl, AdCMV-G<sub>M</sub>, or AdCMV-PTG and incubated with 25 mmol/l glucose for 3 days (□) or concomitantly switched to glucose-depleted medium for 18 h. Then, cells were treated with (■) or without (▨) 2 mmol/l dibutyryl cyclic AMP for 30 min. GS (A) and GP (B) activity ratios were measured as described in RESEARCH DESIGN AND METHODS. \**P* < 0.001 and †*P* < 0.00001, respectively, versus glucose-incubated cells. ‡*P* < 0.01, §*P* < 0.005, and ||*P* < 0.001, respectively, versus cells incubated without dibutyryl cyclic AMP. Data are means ± SD of at least four independent experiments performed in duplicate.

cells were treated with dibutyryl cyclic AMP for 30 min after the glycogen depletion regimen. As shown in Fig. 3A, activation of PKA caused GS activity ratio to decrease dramatically in control and G<sub>M</sub>-overexpressing cells, such that GS activity ratio reached similar values in both cell types.

To test whether the foregoing effects of G<sub>M</sub> overexpression were specific to that particular targeting subunit isoform, we compared the results with those obtained in response to overexpression of PTG. Consistent with our previous findings (29), PTG activated GS irrespective of cell glycogen content, although again higher activity ratios were achieved in glycogen-depleted cells. Treatment of glycogen-depleted PTG-overexpressing cells with dibutyryl cyclic AMP did not inactivate GS. Thus, in glycogen-depleted cells PTG overexpression prevented the phosphorylation of GS by PKA, while overexpression of G<sub>M</sub> enhanced this signaling.

The GP activity ratio decreased in control glycogen-depleted cells (Fig. 3B), whereas G<sub>M</sub> overexpression did not additionally inactivate GP. To test whether cyclic AMP could activate GP in these conditions, cells were treated with dibutyryl cyclic AMP for 30 min. This incubation caused similar activation of GP in controls and G<sub>M</sub>-overexpressing cells, indicating that regulation of GP by cyclic AMP-mediated mechanisms was dominant relative to glucose deprivation or G<sub>M</sub> effects.

**Effects of G<sub>M</sub>ΔC expression in glycogen-depleted cells.** We next considered whether the putative SR local-

ization domain in the COOH-terminus of G<sub>M</sub> was involved in the effects described previously. To this end, we overexpressed a truncated form of G<sub>M</sub> (G<sub>M</sub>ΔC) in glycogen-depleted cells. Strikingly, G<sub>M</sub>ΔC was unable to increase GS activity ratio relative to AdCtrl-treated controls in the manner previously described for native G<sub>M</sub> (Table 2). GP activity was also unaffected by G<sub>M</sub>ΔC expression in glycogen-depleted cells. We examined G<sub>M</sub>ΔC expression by immunoblot analysis. In glycogen-replete cells, a band of the size expected was consistently and easily detectable (Fig. 5A). In contrast, G<sub>M</sub>ΔC protein was not detectable in glycogen-depleted cells again in multiple experiments (Fig. 5A). These results suggested that the COOH-terminus of G<sub>M</sub> plays an important role in stabilization of the protein in muscle cells with low glycogen content. To confirm such an assumption, glycogen-replete cells expressing G<sub>M</sub> or G<sub>M</sub>ΔC cells were switched to a medium devoid of glucose (Fig. 5B). Transduction with the adenovirus encoding native G<sub>M</sub> resulted in immunodetectable expression of a protein of expected size irrespective of glucose presence. In contrast, a time-dependent decline in G<sub>M</sub>ΔC protein level was observed in G<sub>M</sub>ΔC-transduced cells after glucose depletion.

## DISCUSSION

In this study, we demonstrate that the regulatory effects of the glycogen targeting subunit G<sub>M</sub> are strongly influenced by glycogen content and require the presence of an intact

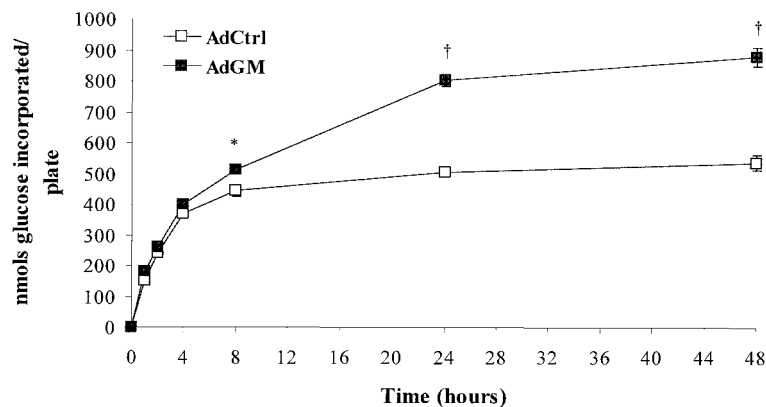


FIG. 4. Effect of G<sub>M</sub> overexpression on glycogen resynthesis. Cells were infected with AdCtrl or AdCMV-G<sub>M</sub> and concomitantly incubated in the absence of glucose for 18 h. Cells were then incubated with [U-<sup>14</sup>C]glucose for the indicated times. Incorporation of [U-<sup>14</sup>C]glucose into glycogen was determined. \**P* < 0.05 and †*P* < 0.0001, respectively, versus control. Data are means ± SD of four independent assays.

TABLE 2  
Impact of  $G_M\Delta C$  expression on GS and GP activities in glycogen-depleted cells

	AdCtrl	AdCMV- $G_M\Delta C$
GS activity ratio (-G6P/+G6P)	0.16 ± 0.01	0.16 ± 0.01
GS total activity (mU/mg protein)	9.9 ± 0.9	10.1 ± 1.0
GP activity ratio (-AMP/+AMP)	0.58 ± 0.02	0.62 ± 0.03
GP total activity (mU/mg protein)	42.6 ± 1.3	42.0 ± 0.8

Data are means ± SD of three independent experiments performed in duplicate. Muscle cells were infected with AdCtrl or AdCMV- $G_M\Delta C$  and incubated with no glucose for 18 h. GS and GP activities were determined.

COOH-terminal domain in the  $G_M$  protein.  $G_M$  has little activating effect on GS when cells are glycogen-replete, which is associated with a rise in PP1 content, as observed in transgenic mice (14), without alteration of GS protein or cell glucose uptake. In contrast, after acute depletion of glycogen stores,  $G_M$  markedly activates GS. In vitro experiments have demonstrated that the two proteins  $G_M$  and GS associate directly through specific sequences in  $G_M$  (mapped to aminoacids 77–118 and 219–240) (13) and do not require glycogen, consistent with our data, but they have also shown that glycogen does not impair  $G_M$ -GS binding (7,13). In contrast, our data suggests that cell glycogen inhibits the synthase phosphatase activity of the  $G_M$ -PP1 complex. Despite all of this, early glycogen resynthesis is not further activated by  $G_M$ , while glycogen accumulation is enhanced in a saturable manner. This  $G_M$  regulatory mechanism is strikingly different from that of PTG, as GS achieves higher activity ratios and glycogen resynthesis/accumulation are regulated independent of glycogen levels, in muscle cells with overexpression of the latter targeting isoform (29). Thus, even though our data are compatible with the proposal that  $G_M$  is essential to activate GS in glycogen-depleted exercised muscle (14), they do not entirely support that  $G_M$  is the target for

glycogen autoregulation on glycogen recovery (9,11,12). A possible explanation for the lack of correlation between  $G_M$ -mediated GS activation and glycogen synthesis is the targeting of this protein to the glycogen-SR versus the glycogen-cytoplasmic localization of PTG. Thereby, a plausible deduction is that glycogen synthesis does not initiate in the SR but rather in the cytoplasm.

The phosphatase activity of the  $G_M$ -PP1 complex on GP appears to be less potent but is not inhibited by glycogen. To the contrary,  $G_M$  mildly promotes the inactivation of GP in glycogen-replete cells, with no further inactivation of GP in glycogen-depleted cells. These data may explain why in transgenic mice overexpressing  $G_M$  no alteration was detected in GP activity ratio, whereas in  $G_M$  knockout mice GP activity ratio was clearly elevated (14). Remarkably, we show that this inactivation of GP is encompassed with a substantial impairment of the glycogenolytic response to glucose deprivation.

We also demonstrate that the effects of  $G_M$  on GS and GP were abrogated by raising cellular cyclic AMP levels, as consistently demonstrated (15,20). The reported mechanism involves PKA phosphorylation of  $G_M$  and subsequent dissociation of PP1. In acute contrast, dibutyryl cyclic AMP did not impair PTG-mediated activation of GS. PKA phosphorylates GS, rendering the enzyme more susceptible to inactivation via phosphorylation by other kinases (20,30). Thus, our data reveals that whereas  $G_M$  cannot prevent or undo PKA action on GS, PTG does, suggesting that PTG complexes either dephosphorylate or hide specific sites on GS targeted by PKA.

Finally, to evaluate the importance of  $G_M$  binding to the SR membrane, we tested the metabolic impact of a deletion-mutant (truncated at aminoacid 375) lacking the COOH-terminal region ( $G_M\Delta C$ ) in which the specific binding site is located (15). When this mutant was overexpressed in hepatocytes, it displayed a higher stimulatory effect on glycogen synthesis than the wild-type  $G_M$  (23). Interestingly, a spontaneous mutation of the  $G_M$  gene leading to a truncated protein (premature stop 668) has recently been described in human subjects and shown to be associated with insulin resistance, although corresponding metabolic data were not provided (17). Here, we demonstrate that expressed  $G_M\Delta C$  had similar effects on GS activation as the intact  $G_M$  construct in glycogen-replete cells, whereas this effect of the truncated construct was lost in glycogen-depleted cells. Moreover, the COOH-terminal truncated form failed to induce GP inactivation. These differential effects of intact and truncated versions of  $G_M$  in glycogen-depleted muscle cells are likely explained by the apparent loss of stability of the  $G_M\Delta C$  protein under these conditions. A time-dependent decay in protein level was observed after glucose depletion. One interpretation of our findings is that binding of intact  $G_M$  to a cellular structure (e.g., SR) serves as a means of preventing degradation of the protein in glycogen-depleted muscle cells. In contrast,  $G_M\Delta C$ , which lacks the COOH-terminal binding motif, may not be protected in this fashion. If correct, this model may explain why the human truncation mutant (17) predisposes to insulin resistance. Thus, in conditions of glycogen depletion (e.g., fasting and exercise), this protein may be susceptible to degradation, leading to an overall lowering of targeting subunit expres-

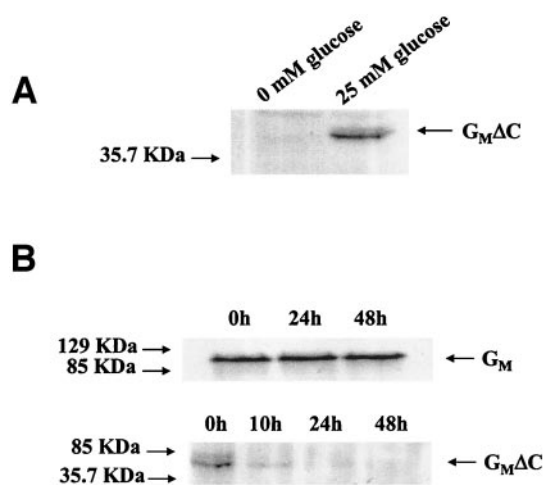


FIG. 5.  $G_M\Delta C$  protein levels in glucose deprived cells. **A:** Cells were infected with AdCMV- $G_M\Delta C$  and incubated in the absence or presence of 25 mmol/l glucose for 36 h. **B:** Cells were infected with AdCMV- $G_M$  or AdCMV- $\Delta G_M$  and incubated in the presence of 25 mmol/l glucose for 3 days. Cells were then switched to a glucose-deprived medium and collected at the indicated times. Whole cell extracts (30  $\mu$ g protein) were separated on a 12% gel, and  $G_M$  or  $G_M\Delta C$  were detected by immunoblotting. Representative autoradiograms of three (A) and two (B) independent experiments are shown.

sion and glycogen synthetic capacity in muscle of such individuals. As mentioned above, oppositely, overexpression of G<sub>M</sub>ΔC in primary rat hepatocytes caused enhanced glycogen accumulation with no evidence of degradation of the G<sub>M</sub>ΔC (23). This suggests that G<sub>M</sub>ΔC binding to cellular structures is not required for stability in the context of liver cells, or that a G<sub>M</sub>-targeted proteolytic activity is missing from such cells. Further studies will be required to resolve these issues.

In summary, our data indicates that G<sub>M</sub> is an important regulator of GS and GP that promotes glycogen storage while impairing glycogenolysis-ensuing glucose depletion. Our findings sustain the concept that G<sub>M</sub> is mainly involved in the activation of GS in glycogen-exhausted muscle, previously outlined in knockout mice (14), but not its role on early glycogen resynthesis stimulation. We also confirm that the effect of G<sub>M</sub> on GS and GP is reverted by elevated cyclic AMP, supporting the notion that G<sub>M</sub> action will be switched off during the adrenergic stimulation in exercise to unlock glycogenolysis and prevent glycogen synthesis. Remarkably, our data show that an intact COOH-terminal domain is required for G<sub>M</sub> protein stability in muscle, which may explain the association between G<sub>M</sub> truncation mutation and insulin resistance in human subjects.

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