# Phosphorylation of the skeletal muscle glycogen-targetting subunit of protein phosphatase 1 in response to adrenaline in vivo

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**Abstract The protein $G_M$, which targets protein phosphatase 1 (PP1) to the glycogen particles and sarcoplasmic reticulum (SR) of striated muscles, is known to be phosphorylated at Ser48 and Ser67 in vitro by adenosine 3′,5′ cyclic monophosphate-dependent protein kinase (PKA) and at Ser48 by MAP kinase-activated protein kinase-1 (MAPKAP-K1, also called p90 RSK). The phosphorylation of Ser48 increases the rate at which the glycogen-associated PP1.$G_M$ complex dephosphorylates (activates) glycogen synthase, but the phosphorylation of Ser67 has the opposite effect, suppressing the activity of PP1 toward glycogen-bound substrates. The phosphorylation of Ser67 overrides the activating effect of Ser48 phosphorylation because it dissociates PP1 from $G_M$. Here, we use two phospho-specific antibodies to demonstrate that the SR-associated form of $G_M$, as well as the glycogen-associated form of $G_M$, becomes phosphorylated at Ser48 and Ser67 in response to adrenaline, supporting the view that the PKA-mediated regulation of the PP1.$G_M$ complex plays a role in the adrenergic control of glycogen metabolism and SR function. In contrast, Ser48 is not phosphorylated significantly in response to insulin, and neither is Ser67. Thus the phosphorylation of $G_M$ at Ser48 by MAPKAP-K1 or other insulin-stimulated protein kinases is not involved in the activation of glycogen synthase by insulin.**



*Key words:* Protein phosphatase 1; Glycogen synthase; Glycogen phosphorylase; Adrenaline; Insulin

## 1. Introduction

Protein phosphatase 1 (PP1) is a serine/threonine-specific phosphatase involved in the regulation of numerous cellular processes. This diversity is achieved by the interaction of the catalytic subunit with more than 20 distinct targetting subunits, which localise it to specific subcellular sites, modulate its substrate specificity and allow its activity to be regulated by extracellular signals [1–3]. A targetting subunit known as $G_M$ plays a dual role in directing PP1 to both the glycogen particles [4] and the sarcoplasmic reticulum (SR) of striated muscles [5]. The PP1-binding domain is near the N-terminus of $G_M$ [2], followed by the glycogen-binding domain [6], the SR-binding domain being positioned near the C-terminus of this 124 kDa protein [7] (Fig. 1).

Two serine residues located at positions 48 and 67 of the rabbit muscle protein [7] are phosphorylated by adenosine 3′,5′ cyclic monophosphate (cAMP)-dependent protein kinase (PKA) in vitro [8]. Ser67 is located in the PP1-binding motif [2] and its phosphorylation disrupts the interaction of PP1 with $G_M$ and hence releases it from glycogen [8] and SR membranes [5]. Since the binding of $G_M$ to PP1 enhances the activity of the phosphatase towards glycogen-bound substrates (glycogen phosphorylase and glycogen synthase) the phosphorylation of $G_M$ at Ser67 provides a potential mechanism for selectively inhibiting the dephosphorylation of these enzymes [9]. In contrast, the phosphorylation of Ser48 increases the rate at which the glycogen-associated PP1.$G_M$ complex dephosphorylates (activates) glycogen synthase [10], but activation is overridden by the phosphorylation of Ser67 since it dissociates PP1 from $G_M$.

The hormone adrenaline, which elevates cAMP and activates PKA in skeletal muscle, induces the phosphorylation of glycogen-associated $G_M$ at Ser48 and Ser67 when injected intravenously into rabbits [11] and releases PP1 from $G_M$ [12]. This indicates that the PKA-catalysed phosphorylation of $G_M$ is indeed an in vivo mechanism for regulating the activity of glycogen phosphorylase and glycogen synthase in response to adrenaline. However, in these studies, the level of phosphorylation of Ser48 appeared to be high, even in the absence of adrenergic stimulation [11,13], raising the possibility that Ser48 is also phosphorylated by another protein kinase in vivo. The phosphorylation state of SR-bound $G_M$ was not analysed in these studies.

A protein kinase that is activated in vivo by mitogen-activated protein kinase (MAPK), and therefore termed MAPK-activated protein kinase-1 (MAPKAP-K1, also called p90 RSK), was found to phosphorylate $G_M$ at Ser48 much more rapidly than at Ser67 and hence increased the glycogen synthase phosphatase activity of PP1.$G_M$ in vitro [12–15]. Insulin is known to activate MAPKAP-K1 [10,16] and was reported to increase the phosphorylation of $G_M$ at Ser48 in rabbit skeletal muscle [10]. This suggested that the MAPKAP-K1-catalysed phosphorylation of Ser48 might be a mechanism by which insulin stimulated PP1.$G_M$ and hence the activation of glycogen synthase in vivo.

Several lines of evidence obtained subsequently have cast doubt on the hypothesis discussed above. Firstly epidermal growth factor, a stronger activator of the MAPK cascade than insulin, does not mimic a number of the actions of insulin in primary adipocytes, including the stimulation of glycogen synthase [17]. Secondly, in mice that overexpress a mutant insulin receptor with severely impaired function, insulin no longer activates the MAPK cascade but still triggers the activation of glycogen synthase [16]. Thirdly, PD 98059, a drug that blocks activation of the MAPK cascade and hence

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the activation of MAPKAP-K1 [18], does not prevent the insulin-induced activation of glycogen synthase in adipocytes or L6 myotubes, a skeletal muscle cell line [19].

For these reasons, we decided to reinvestigate the phosphorylation of $G_M$ at Ser48 and Ser67 in adult skeletal muscle using improved methodology. By generating antibodies that recognise $G_M$ only when it is phosphorylated at Ser48 or Ser67, we have established that adrenaline stimulates the phosphorylation of SR-bound as well as glycogen-bound $G_M$, while insulin fails to trigger the phosphorylation of either site under conditions where it stimulates the activation of glycogen synthase.

## 2. Materials and methods

### 2.1. Materials

A glutathione *S*-transferase fusion protein encoding the first 243 residues of human $G_M$ (GST-$G_M$[2–243]) [20] was expressed in *Escherichia coli* and purified by glutathione-Sepharose chromatography (Dr G. Moorhead) and PKA was purified from bovine muscle [21] (Dr C. Smythe) in this Unit by the investigators shown in parentheses. The peptides SPQPSRRGS*GSSED (corresponding to residues 40–53 of mouse $G_M$) and SASRRVS*FADSLG (corresponding to residues 61–73 of mouse $G_M$), where S* represents a phosphoserine residue, and the corresponding dephosphorylated peptides were synthesised by Dr G. Blomberg (Department of Biochemistry, University of Bristol, UK). Nitrocellulose was purchased from Schleicher and Schuell (Anderman and Co. Ltd, Surrey, UK), CH-Sepharose and the Enhanced Chemiluminescence immunoblotting kit from Amersham Pharmacia GB Ltd (Amersham, UK) and adrenaline from Sigma (Poole, UK). 'Complete' protease inhibitor tablets were obtained from Boehringer Mannheim (Lewes, UK) and rabbit anti-sheep IgG conjugated to horseradish peroxidase from Pierce (Chester, UK).

### 2.2. Production of anti-$G_M$ antibodies

Antisera were raised in sheep against PP1.$G_M$ purified from rabbit skeletal muscle in which the phosphatase catalytic subunit is complexed to a 103 kDa proteolytic fragment of $G_M$ [13] and affinity purified by chromatography on a CH-Sepharose column coupled to GST-$G_M$[2–243]. This antibody recognises dephosphorylated and phosphorylated $G_M$ equally well.

In order to raise antibodies that specifically recognise $G_M$ phosphorylated at either Ser48 or Ser67, the peptides SPQPSRRGS*GSSED and SASRRVS*FADSLG, corresponding to residues 40–53 and 61–73 of mouse $G_M$, respectively, were each conjugated separately to both bovine serum albumin and keyhole limpet haemocyanin and the conjugates injected into sheep at the Scottish Antibody Production Unit (Carluke, UK). The antibodies were purified from the antisera by chromatography on peptide-CH-Sepharose columns. The sera were first applied to the relevant dephosphopeptide Sepharose (to remove any antibodies that recognised dephosphorylated $G_M$) and the flowthrough fractions applied to the relevant phosphopeptide Sepharose. Antibodies that recognise $G_M$ phosphorylated at Ser48 or Ser67 were eluted with 50 mM glycine–HCl pH 2.5, neutralised by making the solution 0.2 M in Tris–HCl pH 8.0 and stored frozen in aliquots at −20°C.

### 2.3. Phosphorylation of GST-$G_M$[2–243] with PKA

The incubations (0.25 ml) were carried out for 1 h at 30°C and comprised GST-$G_M$[2–243] (5 μg), PKA (50 U/ml), 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 10 mM $Mg(CH_3COO)_2$ and 0.1 mM ATP. The reactions were terminated by the addition of 0.2 vol of 62.5 mM Tris–HCl, pH 6.8, 10% SDS, 50% glycerol, 0.005% (m/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol.

### 2.4. Preparation of rat skeletal muscle extracts

Adult male Wistar rats were anaesthetised by an intraperitoneal injection of sodium pentobarbital. The saphenous vein of one hind limb was exposed and injected with either 3 mg/kg propranolol, 3 mg/kg propranolol+1 U/kg insulin or 12 μg/kg adrenaline. After 5 min (propranolol or propranolol+insulin) or 30 s (adrenaline) the muscle from the limb that had not been injected was freeze-clamped in situ between aluminium tongs precooled to the temperature of liquid nitrogen and removed from the animal, which was then killed immediately. The frozen muscle was powdered finely under liquid nitrogen using a pestle and mortar, then homogenised at 4°C in 3 vol of ice-cold homogenisation buffer (25 mM Tris–HCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 1 μM microcystin-LR, 0.1% (v/v) 2-mercaptoethanol and 'Complete' protease inhibitor cocktail [one tablet per 25 ml]). The homogenates were centrifuged at $13\,000 \times g$ for 5 min at 4°C and the supernatants (cytosol) were snap-frozen and stored at −80°C until use. The myofibrillar pellets were washed twice in 2 vol of homogenisation buffer and then rehomogenised in 2 vol of the same buffer containing 1% (v/v) Triton X-100 in order to solubilise the SR membranes. The homogenates were again centrifuged at $13\,000 \times g$ for 5 min at 4°C and the supernatants (containing the SR-associated PP1.$G_M$ complex) snap-frozen and stored at −80°C until use.

### 2.5. Immunoblotting

Protein samples were separated by electrophoresis on 10% SDS/polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation for 2 h in buffer A (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% (v/v) Tween-20) and 5% (m/v) dried milk powder. The membranes were then incubated for 1 h with primary antibody diluted in buffer A plus 5% (m/v) dried milk powder, washed 5–6 times over the space of 1 h with buffer A, and incubated for 1 h with anti-sheep IgG coupled to horseradish peroxidase (0.1 μg/ml) in buffer A plus 5% (m/v) dried milk powder. Finally the membranes were washed 5–6 times over the space of 1 h with buffer A and immunoreactive bands visualised using the enhanced chemiluminescence (ECL) system.

### 2.6. Immunoprecipitation assay of protein kinase B

Protein kinase Bα (PKBα) was immunoprecipitated from control and insulin-stimulated muscle extracts as described [22,23].

## 3. Results

### 3.1. Production of antibodies that recognise $G_M$ phosphorylated at Ser48 or Ser67

Skeletal muscle has a low permeability to inorganic phosphate, making it difficult to $^{32}$P-label intracellular proteins to a high specific radioactivity, especially low abundance proteins like $G_M$ whose intracellular concentration is $< 1$ μM [4]. Phospho-specific antibodies were therefore raised that recognise $G_M$ only when it is phosphorylated at Ser48 or Ser67. These antibodies recognised bacterially expressed human GST-$G_M$[2–243] that had been phosphorylated with PKA, but did not recognise dephosphorylated $G_M$ (Fig. 2). The reactivity of the phospho-specific Ser48 antibody could be blocked by pre-incubation with the appropriate phosphopeptide immunogen, but not with the corresponding dephosphorylated peptide (Fig. 2) or the phosphopeptide corresponding to the sequence surrounding Ser67 (data no shown). Similarly the reactivity of the phospho-specific Ser67 antibody could be blocked by pre-incubation with the appropriate phosphopeptide immunogen, but not with the corresponding dephosphorylated peptide (Fig. 2) or the phosphopeptide corresponding to the sequence surrounding Ser48 (data not shown). In contrast, another antibody raised against a 103 kDa proteolytic fragment of rabbit $G_M$ recognised both the dephosphorylated and phosphorylated forms of $G_M$ with equal efficiency (Fig. 2A).

### 3.2. $G_M$ becomes phosphorylated in vivo at Ser48 and Ser67 in response to adrenaline, but not insulin

Adrenaline, acting via cAMP and PKA, is known to trigger the activation of glycogen phosphorylase and inactivation of

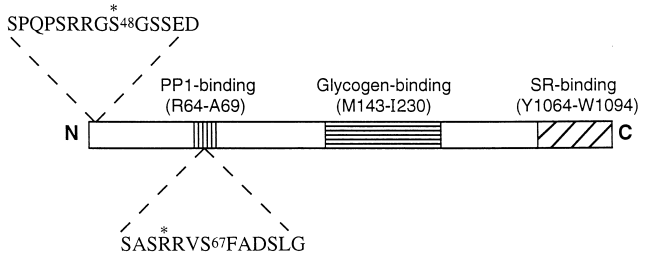


Fig. 1. Domain structure of $G_M$. The PP1-binding, glycogen-binding and SR-binding domains are indicated. The sequences of the phosphopeptides used to raise the phospho-specific antibodies that recognise $G_M$ phosphorylated at Ser48 or Ser67 are also shown, with the phosphoserine residues marked with asterisks. Ser67 is located in the PP1-binding domain, and Ser48 N-terminal to it. Sequences refer to murine $G_M$.

glycogen synthase within 1 min (reviewed in [24]). If the phosphorylation of $G_M$ plays a role in the adrenergic control of glycogen metabolism, it would therefore have to occur in the same time frame. Using the phospho-specific antibodies characterised above, we found that adrenaline induced a large increase in the phosphorylation of $G_M$ at both Ser48 and Ser67 after 30 s in rat skeletal muscle (Fig. 3A).

The results presented in Fig. 3 were carried out using skeletal muscle cytosol, which contains the glycogen-associated form of $G_M$, but not SR-bound $G_M$ which is pelleted with the myofibrils. We therefore also examined the effect of adrenaline on the phosphorylation of SR-bound $G_M$ which can be released from the myofibrils by extraction with Triton X-100 [5]. These studies showed that adrenaline also increases the phosphorylation of SR-bound $G_M$ at Ser48 and Ser67 within 30 s in rat skeletal muscle (Fig. 3B).

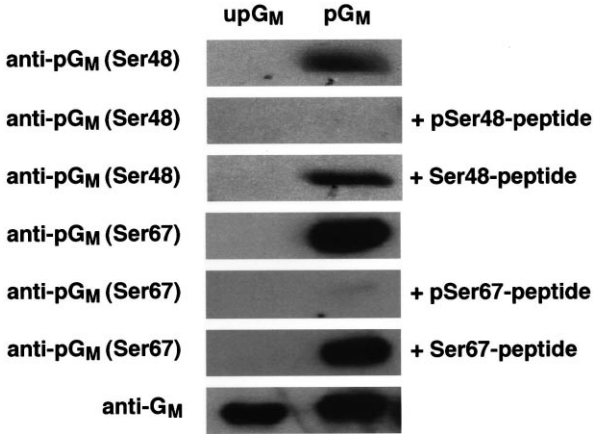


Fig. 2. Characterisation of antibodies that specifically recognise $G_M$ phosphorylated at Ser48 or Ser67. 100 ng of unphosphorylated GST-$G_M$[2–243] ($upG_M$) or PKA-phosphorylated GST-$G_M$[2–243] ($pG_M$) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was carried out using 3 μg/ml of an antibody raised against the peptide SPQPSRRGS*GSSED (anti-p$G_M$(Ser48)), 3 μg/ml of an antibody raised against the peptide SASRRVS*FADSLG (anti-p$G_M$(Ser67)) or 0.5 μg/ml of an antibody raised against the 103 kDa proteolytic fragment of $G_M$ (anti-$G_M$). Where indicated, the anti-p$G_M$(Ser48) antibody was incubated for 1 h at ambient temperature with 1 mM of the phosphopeptide SPQPSRRGS*GSSED (pSer48-peptide) or the dephosphopeptide (Ser48-peptide) prior to immunoblotting. Similarly, the anti-p$G_M$(Ser67) antibody was incubated, where indicated, with the phosphopeptide SASRRVS*FADSLG (pSer67-peptide) or the dephosphopeptide (Ser67-peptide) prior to immunoblotting.

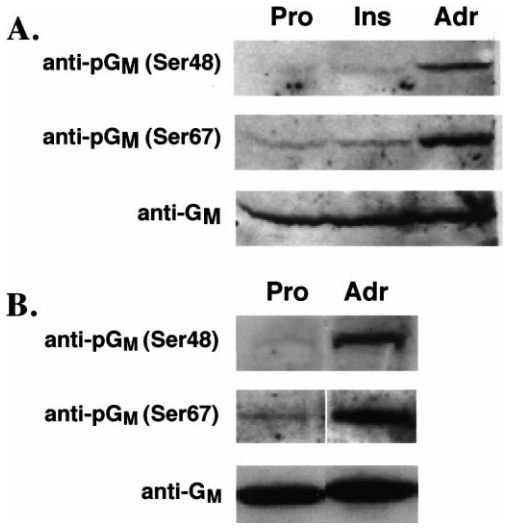


Fig. 3. Phosphorylation of $G_M$ in response to adrenaline, but not insulin. Cytosolic fractions (A) or Triton X-100 extracts of myofibrils (B) were prepared from the hindlimb skeletal muscle of rats injected with propranolol (Pro), propranolol+insulin (Ins) or adrenaline (Adr) as described in Section 2. Cytosol (0.5 mg protein) or Triton extract (75 μg protein) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was carried out using the anti-p$G_M$(Ser48), anti-p$G_M$(Ser67) and anti-$G_M$ antibodies, as described in the legend to Fig. 2.

Insulin induces the dephosphorylation and activation of glycogen synthase in skeletal muscle. This occurs with a half time of less than 5 min, is maximal after 15 min [23] and results from the dephosphorylation of three serine residues that are phosphorylated by glycogen synthase kinase-3 (GSK3) [25] and dephosphorylated by PP1.$G_M$ [9]. If the phosphorylation of $G_M$ at Ser48 plays a role in the stimulation of glycogen metabolism it should therefore occur in the same time scale. However, immunoblotting failed to detect a significant increase in the phosphorylation of Ser48 after stimulation for 5 min (Fig. 3A) or 15 min (data not shown) with insulin. Insulin stimulation also had no effect on the phosphorylation of Ser67 under the conditions studied (Fig. 3A). In contrast, insulin increased the specific activity of PKBα from $0.4 \pm 0.1$ mU/mg to $6.2 \pm 0.4$ mU/mg and $4.6 \pm 0.4$ mU/mg after 5 and 15 min, respectively (average of results from two experiments), similar to results reported previously [23].

## 4. Discussion

In this paper we use improved methodology to establish that adrenaline triggers an extremely rapid phosphorylation of glycogen-associated $G_M$ at Ser48 and Ser67 in rat skeletal muscle (Fig. 3A). We also demonstrate for the first time that SR-associated $G_M$ becomes phosphorylated at these two serine residues during adrenergic stimulation (Fig. 3B). The role of SR-associated PP1.$G_M$ in skeletal muscle is unclear. However, in cardiac muscle $G_M$ and the integral SR-membrane protein phospholamban may interact via their transmembrane domains [26] and biochemical evidence suggests that PP1.$G_M$ may be the major phospholamban phosphatase in the heart [27]. Phospholamban suppresses the activity of the cardiac SR calcium pump, which is relieved by the PKA-catalysed phosphorylation of phospholamban at Ser16 and the calmodulin-dependent protein kinase II-catalysed phosphorylation of

Thr17. This appears to be a major mechanism by which adrenaline stimulates the rate and force of cardiac muscle contraction [28,29].

The phosphorylation of Ser67 is known to trigger the dissociation of PP1 from $G_M$, leading to the release of the PP1 catalytic subunit from the SR membrane [5] as well as the glycogen particles [8,12]. It may therefore contribute to the adrenaline-induced phosphorylation of phospholamban at Ser16 and Thr17, as well as the activation of glycogen phosphorylase and the inhibition of glycogen synthase.

$G_M$ is phosphorylated at Ser48 by PKA in vitro at a similar rate to Ser67 [8], but Ser48 is dephosphorylated much more slowly in vitro by the major protein phosphatases PP1, PP2A or PP2B [8]. As a result, a form of $G_M$ is generated that is phosphorylated relatively specifically at Ser48. Interestingly, the PP1.$G_M$ complex in which $G_M$ is phosphorylated specifically at Ser48 is 2–3-fold more active than unphosphorylated PP1.$G_M$ in dephosphorylating glycogen synthase [10]. If Ser67 is dephosphorylated more rapidly than Ser48 in vivo, as it is in vitro, this could provide a potential mechanism for enhancing the rate of reactivation of glycogen synthase and hence the resynthesis of glycogen, as well as the dephosphorylation of phospholamban, in the recovery period after adrenergic stimulation ceases.

In earlier studies, we reported that the level of phosphorylation of Ser48 was very high (0.5–0.75 mol/mol) in rabbit skeletal muscle, even in the absence of adrenaline [11,13], which was not observed in the present study. The high level found in earlier studies is likely to be artefactual and probably explained by the failure to employ freeze-clamping procedures in these experiments. It seems likely that, in our earlier study, Ser48 was phosphorylated post mortem by a protein kinase(s) activated by the contraction of the muscle that occurred during its excision.

The acute insulin-induced activation of glycogen synthase in skeletal muscle results mainly from the dephosphorylation of serine residues that are phosphorylated by GSK3 [25] and thought to be dephosphorylated by PP1.$G_M$. This indicates that the action of insulin is likely to be mediated via an inhibition of GSK3 and/or an activation of PP1.$G_M$. It is now well established that insulin does indeed trigger an inhibition of GSK3 via a PI 3-kinase-dependent protein kinase cascade that has been dissected over the past few years (reviewed in [30]). This inhibition of GSK3 is likely to contribute to the insulin-induced activation of glycogen synthase, but the possibility that insulin also stimulates a glycogen synthase phosphatase is not excluded. In the present study, using improved methodology, we failed to confirm our original report that insulin induces the phosphorylation of $G_M$ at Ser48 [10], which would be expected to activate PP1.$G_M$ (see Section 1). However, it remains possible that insulin activates PP1.$G_M$ by another mechanism; for example by stimulating the phosphorylation of another site on $G_M$. Moreover, two other glycogen-associated forms of PP1 have subsequently been identified in skeletal muscle in which PP1 is complexed to homologues of $G_M$, termed R5 (or PTG) [31,32] and R6 [33]. Although present at much lower concentrations than PP1.$G_M$ in adult mammalian skeletal muscle (G. Browne, P. Cohen and P.T.W. Cohen, unpublished work), the possibility that insulin promotes the dephosphorylation of glycogen synthase by activating PP1.R5 or PP1.R6 remains a possibility.

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