Mutations of the serine phosphorylated in the protein phosphatase-1-binding motif in the skeletal muscle glycogen-targeting subunit

Jun LIU*, Jun WU*[1], Carey OLIVER†, Shirish SHENOLIKAR† and David L. BRAUTIGAN*[2]

*Center for Cell Signaling, University of Virginia School of Medicine, Box 577, West Complex MSB 7196, Charlottesville, VA 22908, U.S.A., and †Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, U.S.A.

Cellular functions of protein phosphatase-1 (PP1) are determined by regulatory subunits that contain the consensus PP1-binding motif, RVXF. This motif was first identified as the site of phosphorylation by cAMP-dependent protein kinase (PKA) in a skeletal muscle glycogen-targeting subunit (G_M). We reported previously that a recombinant fusion protein of glutathione S-transferase (GST) and the N-terminal domain of G_M [GST–G_M-(1–240)] bound PP1 in a pull down assay, and phosphorylation by PKA prevented PP1 binding. Here we report that substitution of either Ala or Val for Ser-67 in the RVS[67]F motif in GST–G_M-(1–240) essentially eliminated PP1 binding. This was unexpected because other glycogen-targeting subunits have a Val residue at the position corresponding to Ser-67. In contrast, a mutation of Ser-67 to Thr (S67T) in GST–G_M(1–240) gave a protein that bound PP1 the same as wild type and was unaffected by PKA phosphorylation. Full length G_M tagged with the epitope sequence DYKDDDDK (FLAG) expressed in COS7 cells bound PP1 that was recovered by co-immunoprecipitation, but this association was prevented by treatment of the cells with forskolin. By comparison, PP1 binding with FLAG-G_M(S67T) was not disrupted by forskolin treatment. Neither FLAG-G_M(S67A) nor FLAG-G_M(S67V) formed stable complexes with PP1 in COS7 cells. These results emphasise the unique contribution of Ser-67 in PP1 binding to G_M. The constitutive PP1-binding activity shown by G_M(S67T) opens the way for studying the role of G_M multisite phosphorylation in hormonal control of glycogen metabolism.

Key words: cAMP-dependent protein kinase, forskolin, PP1 binding protein, RVXF binding site.

INTRODUCTION

Protein phosphatase-1 (PP1) is implicated in control of multiple cellular processes in eukaryotic cells (see reviews [1,2]). The individual functions of this enzyme are dictated, in part, by association of the PP1 catalytic subunit with various regulatory subunits. Isolation of mammalian PP1 complexes and yeast two-hybrid screens with the PP1 catalytic subunit as bait have identified dozens of different eukaryotic PP1-binding proteins [3–5]. These proteins show a remarkable specificity in their association with PP1 catalytic subunit, compared with the closely related protein phosphatase 2A. Some PP1 subunits modify the substrate specificity of the enzyme, and many reduce sensitivity of PP1 to endogenous inhibitor proteins, such as inhibitor-1 and inhibitor-2. Several PP1-binding proteins have been shown to localize or target the enzyme to intracellular structures or organelles such as glycogen [4–8], ribosomes [9], microtubules [10], myofibrils [11,12] and RNA spliceosomes [13]. Some PP1 regulatory subunits are phosphoproteins and *in vitro* studies suggest that phosphorylation modifies their interaction with PP1 [14, 15]. The activity of the PP1 complexes may be modulated by hormones.

The first PP1-targeting subunit identified was the glycogen-binding subunit, called G_M or R_{G1}, which tethers PP1 to glycogen in skeletal muscle [6,7]. Our recent studies identified a glycogen-binding region of G_M that is highly conserved in bacterial and mammalian polysaccharide-binding proteins [16]. PP1 binding is dependent on a region of G_M containing the RVSF tetrapeptide motif, first identified as the site of cAMP-dependent protein kinase (PKA) phosphorylation [14]. Subsequent studies showed that PKA-mediated phosphorylation of Ser-67 in this motif promoted PP1 dissociation from G_M [15]. On the other hand, phosphorylation of G_M at Ser-48 reportedly regulates the activity of the G_M-PP1 complex in response to insulin [17].

A number of PP1-binding proteins appear to interact with the PP1 catalytic subunit through a RVXF motif, suggesting this is a highly conserved interaction site [18]. Screening a peptide display library, Zhao and Lee [19] demonstrated a preference for VXF-containing sequences with a preponderance of basic amino acids preceding this sequence. A comparison of the RVXF motifs in many different PP1 regulators has emphasised the variability in amino acids present in the X position. Even among the glycogen-targeting subunits expressed in different species and tissues, the X position is highly variable. For example, PTG, a widely distributed glycogen-targeting subunit [4], the nearly identical protein R5 [20], and U5 [5], a glycogen-targeting subunit identified from smooth muscle, have a valine residue in the X position. These glycogen-targeting subunits are not phosphorylated by PKA, and there is no evidence that their association with PP1 is regulated by cAMP or by phosphorylation [21].

To establish the role of G_M phosphorylation in modulating PP1 activity, we substituted different amino acid residues in place of Ser-67. The goal was to create a version of G_M that would not

Abbreviations used: PP1, protein phosphatase-1; G_M, skeletal muscle glycogen-targeting subunit; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; GST–G_M(1–240), fusion of GST to N-terminal 240 residues of G_M; FLAG, epitope tag of sequence DYKDDDDK; H89, *N*-{2-[(*p*-bromocinnamyl)amino]ethyl}-5-isoquinolinesulphonamide hydrochloride; DIG, digoxigenin; S67T, format for mutations in which (for example) the Ser residue at position 67 is changed to Thr; PTG, widely distributed glycogen-targeting subunit; DARP32, dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32000; M110, myosin-binding subunit; NIPP1, the nuclear inhibitor of PP1.

[1] Present address: Searle Research and Development, Mail AA3G, 700 Chesterfield Parkway North, St. Louis, MO 63198, U.S.A.

[2] To whom correspondence should be addressed (e-mail db8g@virginia.edu).

be phosphorylated in the PP1 binding motif, so the effects of phosphorylation at other sites could be tested. We analysed PP1 binding to recombinant G_M fusion proteins in biochemical assays and to epitope-tagged G_M in living cells. Substitutions of Ser-67 with Ala or with Val, a residue found in other glycogen-targeting subunits [4,5,20], were initially tested. The surprising results showed these proteins hardly bound any PP1. However, replacing Ser with Thr, another hydroxy amino acid, resulted in PP1 binding. Thus Ser-67 is critical in G_M binding to PP1. These studies also provide convincing evidence for PKA regulation of PP1/G_M association in living cells, and thus set the stage for future studies to understand the molecular basis for G_M function as a regulator of glycogen metabolism in skeletal muscle.

EXPERIMENTAL

Materials

Tissue culture reagents and the reverse-transcription kit (Preamplification System for First Strand cDNA Synthesis) were purchased from Life Technologies (Grand Island, NY, U.S.A.). pGEX vectors and glutathione-Sepharose were from Amersham-Pharmacia Biotech (Piscataway, NJ, U.S.A.). The fusion protein between glutathione S-transferase (GST) and the N-terminal domain of G_M [GST–G_M(1–240)] was prepared as previously described [22]. *N*-{2-[(*p*-Bromocinnamyl)amino]ethyl}-5-isoquinolinesulphonamide hydrochloride (H89) and Microcystin-LR were purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.). The anti-[DYKDDDDK epitope (FLAG)] antibody M2 was obtained from Sigma (St. Louis, MO, U.S.A.). Mouse anti-PP1C monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). FuGene 6 transfection reagent and digoxigenin (DIG) Protein Labelling Kit were purchased from Boehringer Mannheim. Restriction enzymes were from Promega Life Science (Madison, WI, U.S.A.). Oligonucleotides were synthesized by ITD (Coralville, IA, U.S.A.).

Cloning and mutagenesis of full-length rabbit G_M cDNA

First-strand cDNA synthesis was carried out using 2 μg of total RNA isolated from rabbit skeletal muscle and a 5′ end primer (5′-CGCGGATCCGAGCCTGGAAAGCCATTG-3′) as described in the manufacturer's protocol. After digestion with RNase H, three overlapping double-stranded cDNA products (corresponding to nucleotides 13–1175, 1089–2753, and 2213–3409; A, B, C, respectively) were synthesized by PCR using different pairs of forward/backward primers that added restriction sites as indicated below (outside the curly brackets). Nucleotide 13 corresponds to the start of the first codon from the reported mRNA. The fragments were assembled stepwise using unique restriction sites to yield a full-length G_M cDNA (covering nucleotides 13–3409). Briefly, *Cla*I digestion of the C product {2213–3409}-*Xho*I yielded a *Cla*I–*Xho*I fragment (nucleotides 2396–3409) that was ligated into pGEM-72f(+). Then *Bam*HI/*Xho*I double digestion of the pGEM-72f(+) containing the 2396–3409 insert gave a fragment that was ligated into the pGEX4T2 vector at the *Bam*HI and *Xho*I sites. The pGEX4T2 vector was digested with *Bam*HI/*Cla*I and the PCR product A, *Bam*HI-{13–1175}-*Cla*I, was inserted. This fused nucleotides 13–1175 to nucleotides 2396–3409. *Sph*I/*Cla*I digestion of the PCR product B gave nucleotides 1115–2396, which was inserted into the pGEX4T2 vector after *Sph*I/*Cla*I digestion. The final product was a *Bam*HI–*Xho*I insert encoding the full-length G_M, residues 1–1109. FLAG-tagged full-length G_M was constructed by transferring this insert into the FLAG2 mammalian expression vector. G_M was mutated using Stratagene Quick Change Mutagenesis Kit according to the manufacturer's protocol and sequences were confirmed by double-stranded DNA sequencing.

PP1C binding assays

The GST–G_M(1–240) fusion protein (2 μg) was bound to glutathione-Sepharose and incubated with NIH3T3 cell lysates as described previously [22]. The beads were washed extensively and the bound PP1C was visualized by immunoblotting with anti-PP1C monoclonal antibody. Content of GST fusion proteins in each assay was determined by staining with Coomassie Blue.

PP1C binding to GST–G_M was also analysed using a far-Western assay. Following the separation of fusion proteins on SDS/PAGE they were probed with DIG-derivatized recombinant human PP1Cα, prepared using the DIG Protein Labelling Kit as described previously [16]. The bound PP1C was detected using peroxidase-conjugated anti-DIG antibody.

Phosphorylase phosphatase assays

PP1 activity was assayed by the release of ^{32}P phosphate from phosphorylase *a* as described in Shenolikar and Ingebritsen [23]. PP1 was incubated with 10 μM phosphorylase *a* in 50 mM Tris/HCl, pH 7.0, 1 mg/ml BSA, 0.3 % (v/v) β-mercaptoethanol (total volume 60 μl) at 37 °C for 10 min. The reaction was terminated by addition of 0.2 ml of 20 % (w/v) trichloroacetic acid and 50 μl of BSA (10 mg/ml) and placed on ice for 5 min. Following centrifugation at 15000 × ***g*** for 5 min, the supernatant (200 μl) was analysed for ^{32}P release by liquid scintillation counting. In assays containing GST–G_M(1–240), the reaction was initiated by addition of enzyme to the substrate/G_M mixture, which was preincubated at 37 °C for 5 min.

G_M reaction with PKA

GST–G_M (5 μg of the purified fusion protein bound to glutathione-Sepharose) was incubated with 10 units of PKA in a reaction buffer containing 0.15 mM of ATP for 30 min at 30 °C [22]. The reaction was stopped by dilution and washing the beads with ice-cold PBS containing 1 mM DTT.

FLAG–G_M expression in COS7 cells

COS7 cells, grown in DMEM containing 10 % (v/v) newborn calf serum to 50–60 % confluence, were transfected with 10 μg of plasmid DNA and FuGene 6 reagent according to the manufacturer's instructions (Boehringer Mannheim). The cells were grown for 30 h prior to analysing PP1/G_M interaction by immunoprecipitation.

Cells expressing FLAG-tagged G_M proteins were treated with vehicle or 25 μM of forskolin in the presence or absence of 30 μM of H89 for 1 h. Cell lysates were prepared by solubilization in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 1 mM DTT, 1 mM Na_3VO_4, 1 μM Microcystin-LR, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min at 4 °C. The lysates were clarified by centrifugation at 10000 ***g*** for 10 min, and then incubated with the appropriate antibody for 1 h at 4 °C. The immunoprecipitates were adsorbed to protein G-Sepharose for 1 h at 4 °C, washed three times with the lysis buffer and subjected to SDS/PAGE.

For immunoblotting, the proteins were subjected to SDS/PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline containing 0.1 % Tween 20 and 5 % (w/v) non-fat dry milk. Membranes were then incubated with the appropriate antibodies,

washed as described previously [22], and the bound antibody was detected by enhanced chemiluminescence with a horseradish peroxidase-conjugated secondary antibody. Polyclonal anti-G_M antibody was generated by immunizing rabbits with recombinant GST–G_M(1–240).

RESULTS

PP1C Binding to GST–G_M(1–240) by pull down assay

GST–G_M(1–240) fusion proteins were expressed in *Escherichia coli*, purified on glutathione-Sepharose and analysed for PP1C binding in a 'pull-down' assay. The GST fusion proteins were incubated either with lysates of NIH3T3 cells, a source of native PP1C (Figure 1A), or with purified recombinant PP1C activated with Mn^{2+} (Figure 1B). Native PP1C, purified to homogeneity from rabbit skeletal muscle, also was used, yielding identical results (not shown). The affinity beads were extensively washed and the bound PP1C was analysed by immunoblotting. Wild-type GST–G_M(1–240) bound PP1C (Figures 1A and 1B, lane S) but no PP1 binding was observed with GST alone (result not shown). Substitution of Ser-67 in the GST–G_M(1–240) with either Ala (S67A) or Val (S67V) virtually eliminated PP1 binding (Figures 1A and 1B, lanes A and V, respectively). On the other hand, GST–G_M(1–240)-S67T and wild-type GST–G_M(1–240) bound PP1C the same (Figures 1A and 1B, lane T). Protein staining with Coomassie Blue (Figure 1, lower panels) demonstrated that equivalent amounts of fusion proteins were present in each pull-down. These results revealed that binding of PP1C was exceedingly sensitive to single residue substitutions for Ser-67.

Trypsin digestion of wild-type and mutated GST fusion proteins

The wild-type and mutated fusion proteins were subjected to digestion by trypsin as a means to probe their overall conformation. As shown in Figure 2, these GST–G_M(1–240) proteins were all rapidly digested, and the primary sites of trypsin digestion that produced fragments labelled A and B were exposed to the same extent. Indeed, the time-dependent partial proteolysis was indistinguishable for wild-type, S67T and S67V. By comparison, the S67A version of GST–G_M(1–240) was digested at the same overall rate, into the same-sized fragments but the subsequent digestion of fragment A appeared to be slower than for the other samples. These results showed that the overall conformation of the various fusion proteins was not significantly different. Moreover, GST fusion proteins mutated at S67 bound glycogen in a co-sedimentation assay (results not shown), further attesting to the conformational integrity of the G_M(1–240) domain.

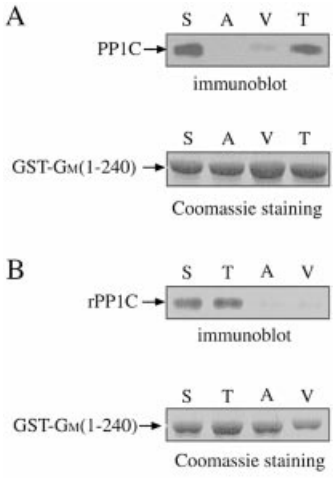

Figure 1 ***In vitro*** **binding of PP1C with GST–G_M(1–240) fusion proteins**

GST–G_M(1–240) wild-type and different mutant proteins (2 μg) were bound to glutathione-Sepharose beads and incubated with 750 μl of 3T3 cell lysate plus 350 μl of binding buffer (**A**), or 10 μg of purified recombinant PP1Cα (rPP1C) in 1 ml of binding buffer (**B**). PP1C was detected by immunoblotting (upper panels) and GST fusions were stained with Coomassie Blue (lower panels). The fusion proteins used were GST–G_M(1–240) wild-type (lane S), GST–G_M(1–240)-S67A (lane A), GST–G_M(1–240)-S67V (lane V), and GST–G_M(1–240)-S67T (lane T). The same results were obtained in multiple experiments with different preparations of the GST proteins.

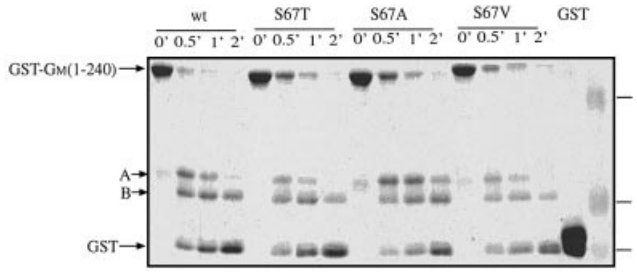

Figure 2 Trypsin digestion of different GST–G_M(1–240) fusion proteins

Wild-type (wt), the S67T, S67A or S67V mutant of GST–G_M(1–240) (2 μg) was incubated with 50 ng of trypsin at room temperature in 20 μl of 20 mM Tris/HCl, pH 7.5, for different periods of time (shown in min along the top). The digestion products were analysed by SDS/PAGE (10% w/v, acrylamide) and Coomassie Blue staining. A and B are the major fragments produced by trypsin digestion. Molecular size standards are shown as bars on the right hand side, from top to bottom: 47, 33, and 30 kDa.

PP1C Binding to GST–G_M(1–240) by far-Western assay

To test for PP1C binding to the GST–G_M(1–240) polypeptide, we also utilized an overlay or far-Western protocol (Figure 3), where the GST fusion proteins were denatured by heat plus SDS, resolved by SDS/PAGE, and transferred on to nitrocellulose. The filter was probed with DIG-conjugated recombinant human PP1Cα (DIG–PP1C) and the bound DIG–PP1C was detected with anti-DIG antibodies. This assay confirmed PP1C binding to wild-type GST–G_M(1–240) (Figure 3A, upper panel, lane 1) and S67T (lane 4) proteins, but little or no PP1 binding was seen with S67A (lane 2) or S67V (lane 3). Essentially identical amounts of fusion protein were present in all samples as seen by Coomassie staining (Figure 3A, lower panel). This overlay assay was used to compare relative binding capabilities by transferring various amounts of the GST fusion proteins on to the filters (Figure 3B). The amount of recombinant PP1C bound was quantified by densitometry and calculated as a percentage of the maximum, which was from the highest dose of wild-type protein. Over the 0.01–2.0 μg range of GST–G_M(1–240), binding of DIG–PP1C increased steeply. Compared with the wild-type, the S67T protein showed impaired binding of PP1C in this assay. The S67V protein had even lower apparent affinity for binding PP1C, with only approx. 20% maximum binding relative to the wild-type fusion protein. Together, the results of the different binding assays emphasised a strict requirement for hydroxy amino acid residues, either Ser or Thr, at the X position in the RVXF motif in G_M, and indicated that other amino acids, i.e. Ala and Val, could not support PP1C binding in this context.

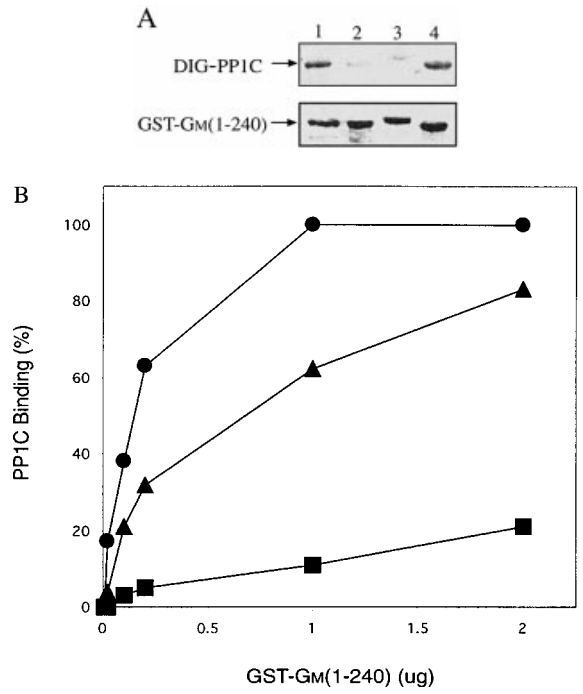

Figure 3 Far-Western assay of DIG–PP1C binding to GST–G_M(1–240) proteins

Purified proteins were transferred to PVDF filters after SDS/PAGE and probed in a far-Western assay with DIG–PP1C. (**A**) DIG–PP1C was detected by immunoblotting with anti-DIG antibodies. Coomassie Blue stain of a parallel gel showed the amount of the different GST fusion proteins in the assay. The fusion proteins used were GST–G_M(1–240) wild-type (lane 1), GST–G_M-(1–240)-S67A (lane 2), GST–G_M(1–240)-S67V (lane 3), and GST–G_M(1–240)-S67T (lane 4). (**B**) The far-Western blots were quantified by using NIH-IPLAB gel analysis program (National Institutes of Health, Bethesda, MD, U.S.A.). Binding by GST–G_M(1–240) wild-type (●), GST–G_M(1–240)-S67T (▲) and GST–G_M(1–240) S67V (■) is shown as an average of three different experiments. Comparison of PP1C binding was assessed in arbitrary units with the amount of PP1C bound to 2 μg of GST–G_M(1–240) wild-type as 100%.

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HTASSGGRRVSFADNFGFNLV GM  57-77
VQEKKVKKRVSFADNQGLALT GL  53-73
SPHNQAKKRVVFADSKGLSLT PTG 53-73
SSKSQKKKRVVFADMKGLSLT U5  58-78
CSHNQAKKRVVFADSKGLSLT R5  76-96
RPGCSQKLRVRFADALGLELA R6  94-114
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Figure 4 Conserved sequences in the PP1C binding domains of the glycogen-targeting subunits

Sequences from selected mammalian glycogen-targeting subunits were aligned using the Clustal method. The numbers indicate sequence positions in each protein. The residues that occur in two or more family members are shown in reverse type

Mutation G63K in GST–G_M(1–240)

The lack of PP1 binding to GST–G_M(1–240)-S67V could reflect sequence differences near the RVXF motif between G_M and other glycogen-targeting subunits (Figure 4) [4,5,7,8,20,24]. To test this idea we substituted Gly-63 of G_M with lysine, a basic residue found at this position in all other mammalian glycogen-targeting subunits. This mutation gave three consecutive basic residues, preceding VXF, as in the other subunits. The single residue mutation, G63K, in GST–G_M(1–240) had no discernible effect on PP1 binding (results not shown). Moreover, introduction of a lysine residue at position 63 did not compensate for mutation at position 67, because the purified recombinant GST–G_M(1–240)-G63K/S67V double mutant failed to bind PP1C in either the pull-down or overlay assay (results not shown). Thus the increased basic charge at the N terminus of the RVSF motif in G_M was not sufficient to overcome the specific requirement for Ser or Thr in this sequence.

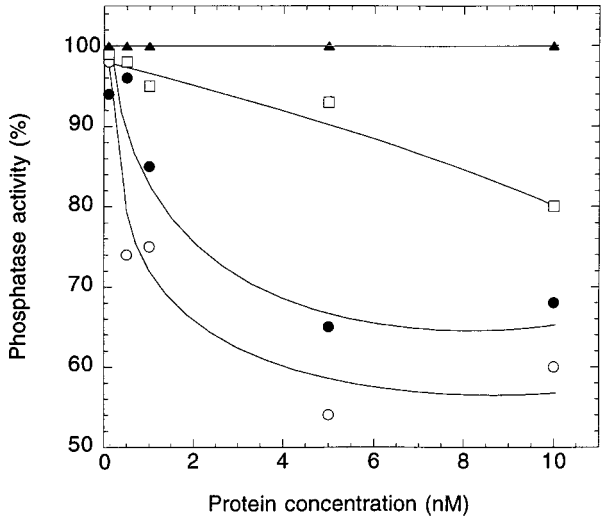

Figure 5 Effect of Ser-67 mutations in G_M(1–240) on phosphorylase phosphatase activity of bound PP1C

PP1C, purified from rabbit skeletal muscle, was assayed for phosphorylase phosphatase activity (as described in the Material and methods section) in the presence of increasing concentrations of GST–G_M(1–240) wild-type (○), S67T (●), S67V (□) or GST alone (▲). The assays were carried out in triplicate, and the individual assays varied by less than 5%. A representative figure from three independent experiments is shown.

Assay of GST–G_M(1–240) binding by changes in phosphorylase phosphatase activity

We analysed various GST–G_M(1–240) fusion proteins for their ability to modulate the phosphorylase phosphatase activity of PP1C. Association of PP1C with GST–G_L was previously reported to reduce activity with phosphorylase *a* by up to 50% [8]. Wild-type GST–G_M(1–240) reduced PP1C activity against phosphorylase in a dose-dependent manner (Figure 5). GST–G_M-(1–240)-S67T also was effective in suppressing phosphorylase phosphatase activity, but the mutants GST–G_M(1–240)-S67V and -S67A (results not shown) were much less effective over the same concentration range. Addition of GST alone had no effect on the phosphatase assay and served as control. Thus wild-type and S67T, but not S67V or S67A versions of GST–G_M(1–240), altered the reactivity of PP1C against phosphorylase. These results were consistent with the binding data described above.

Reaction with PKA and PP1C binding by GST–G_M(1–240)

Previous studies have established that PKA phosphorylates G_M on Ser-67 [14]. This reduced the affinity of G_M for PP1C, thereby promoting dissociation [15]. Hence, we examined the effect of *in vitro* reaction of GST–G_M(1–240) with PKA on PP1C binding. Wild-type GST–G_M(1–240) and the mutants S67T and S67A were incubated with MgATP and purified PKA catalytic subunit (Figure 6, lanes 2, 4, 6) or MgATP alone as control (Figure 6, lanes 1, 3, 5). The effect on PP1C binding to GST–G_M(1–240) was

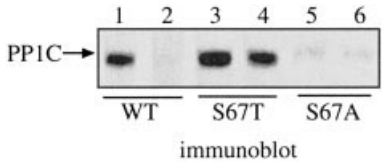

Figure 6 Binding of PP1C to GST–G_M(1–240)-S67T mutant protein is insensitive to PKA pre-treatment

Binding of PP1C from 3T3 cell lysate was assayed with GST fusion proteins pre-treated with MgATP alone (lanes 1, 3 and 5) or with MgATP + PKA (lanes 2, 4 and 6). PP1C was detected by immunoblotting. This is a representative result of three independent experiments.

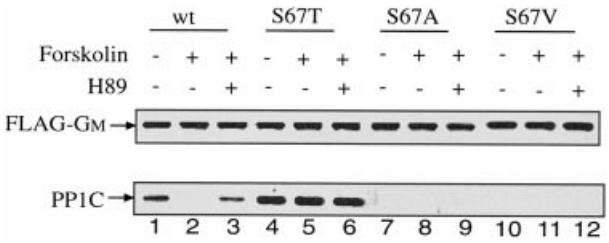

Figure 7 Forskolin inhibits *in vivo* binding of PP1C to the FLAG–G_M full-length wild-type but not to the S67T mutant

COS7 cells were transfected with FLAG-tagged G_M full-length wild-type, S67T mutant, S67V mutant, or S67A mutant plasmids. After treatment with vehicle alone, 30 μM of H89, or 25 μM of forskolin for 1 h, cells were lysed and anti-FLAG immunoprecipitates were analysed by anti-G_M (upper panel) and anti-PP1C immunoblotting (lower panel). The results were replicated in five independent experiments.

assayed using the GST pull-down assay. Reaction with PKA plus ATP eliminated PP1C binding to wild-type GST–G_M(1–240) (Figure 6, compare lanes 1 and 2). Incubation with PKA and MgATP did not affect the very low level of PP1C binding to GST–G_M(1–240)-S67A (Figure 6, lanes 5 and 6) or the near wild-type level of binding seen with GST–G_M(1–240)-S67T (Figure 6, lanes 3 and 4). These results showed that only when Ser was present at position 67 in the RVSF motif was PKA able to eliminate binding of PP1C.

Formation of PP1/G_M complexes in COS7 cells

To test for PP1 binding to G_M in living cells, we expressed FLAG-tagged full-length G_M (residues 1–1109) in COS-7 cells and examined PP1C co-immunoprecipitation, using the anti-FLAG antibody and anti-PP1 immunoblotting. Wild-type FLAG-G_M and the mutants S67A, S67V and S67T were expressed to similar levels with similar recoveries by the anti-FLAG immunoprecipitation (Figure 7, upper panel). The wild-type FLAG–G_M and FLAG–G_M-S67T bound PP1C (Figure 7, lower panel, lanes 1 and 4, respectively), but no PP1C binding was seen with the FLAG–G_M-S67A and -S67V mutants (Figure 7, lower panel, lanes 7 and 10). Treatment of cells with forskolin to elevate cAMP and activate PKA resulted in near complete loss of PP1C binding to wild-type FLAG–G_M (lane 2). Association of PP1C was partially restored by addition of the cell-permeable PKA inhibitor, H89 [25], which partially blocked PKA phosphorylation of the FLAG–G_M (lane 3). The recovery of PP1C with FLAG–G_M-S67T was slightly higher than with wild-type FLAG–G_M and was not modified by treatment of the cells with either forskolin or H89 (lanes 5 and 6). Neither FLAG–G_M-S67A nor -S67V bound PP1C in COS7 cells, and forskolin and H89 had no effect (lanes 7–12). It is important to note that essentially identical results were obtained by expressing wild-type and mutated FLAG-tagged N-terminal domain (1–240) of G_M in COS7 cells (results not shown). This validated the *in vitro* binding analyses described above that used the N-terminal domain of G_M (residues 1–240). The results suggested also that the C-terminal region of G_M (residues 241–1109) has little effect in binding to PP1 in cells.

DISCUSSION

Investigations of the prototypic PP1-targeting subunit, G_M, the glycogen-targeting subunit in rabbit skeletal muscle, have laid many of the foundations for current studies of PP1 regulation in eukaryotic cells. PP1C bound to G_M also associated with glycogen. This targets the phosphatase in close proximity to its substrates, glycogen synthase, phosphorylase kinase and phosphorylase, and facilitates the dephosphorylation of these glycogen-bound enzymes. Targeting of PP1 by regulatory subunits has become a recurrent theme in understanding the multiple functions of PP1.

Phosphorylation of G_M by PKA was shown to displace PP1 [15]. Phosphorylation was mapped to Ser-67 and the surrounding sequence RVSF was subsequently defined as a PP1-binding motif conserved in many other PP1 regulatory subunits. Mutational analyses [26] and co-crystallization of a G_M decapeptide with PP1 catalytic subunit [18] pointed primarily to the two hydrophobic residues, valine and phenylalanine, as critical for PP1 binding. These amino acids were also highlighted by the peptide screen that revealed the most common motif V/I-X-F/W in PP1-binding peptides [19]. The importance of this motif in PP1 binding and/or regulation has been established for inhibitor-1 [27], dopamine- and cAMP-regulated phosphoprotein of apparent M_r 32000 (DARPP32) [28,29], myosin-binding subunit (M110) [30], p53-binding protein ('p53BP2') [31], and the nuclear inhibitor of PP1 (NIPP1) [32]. The primary sequence of these proteins outside of the tetrapeptide PP1-binding motif differs significantly, as might be expected from their diverse biological functions. Among these regulatory subunits, the X position in the RVXF motif is occupied by a lysine residue in M110 and the p53-binding protein; a glutamine residue in the A-kinase anchoring protein of 220 kDa ('AKAP220') [33], inhibitor-1 and the related protein, DARPP32; a threonine residue in NIPP1; and an arginine residue in the RNA-splicing factor, PSF [13] and in the yeast glycogen-targeting subunit, ('GAC1') [34]. Limiting comparison to the mammalian glycogen-targeting subunits, shows a serine residue in the X position only for skeletal muscle G_M and liver G_L [8], with a valine residue occupying this position in most of the other variants (Figure 4).

Analysis of the three-dimensional structure of a decapeptide from G_M bound to the PP1 catalytic subunit revealed a hydrogen bond, via a water molecule, between the hydroxyl group of Ser-67 in G_M and the carbonyl of Thr-288 in PP1. This would be consistent with the sidechain of the residue in the X position of the RVXF motif contributing to stabilization of the PP1/G_M association. Substitution of Val, which is at position X in other subunits, for Ser-67 did not support G_M binding to PP1C. We substituted Thr for Ser to restore a hydroxy amino acid residue at position 67. Examining PP1/G_M association *in vitro* and *in vivo*, we obtained remarkably consistent results showing that Ser and Thr were both effective in mediating PP1 binding to G_M. The strict requirement for a hydroxy amino acid residue at the X position in the RVXF motif in G_M was surprising, given the variability in this position in peptides and proteins that bind

PP1C. Nonetheless, our results underscore the important contribution of position X in the PP1/G_M association. This presumably involves the water-bridged hydrogen bonding seen in the three-dimensional structure. Our results predict reduced affinity of RVXF for PP1C when X is not a hydroxy amino acid residue. Others have speculated that basic residues N-terminal to the RVXF motif may enhance binding to PP1C [35]. We tested this idea by introducing an additional basic residue at position 63, as seen in other family members with Val in the X position. The G63K mutation did not reinstate PP1 binding to a G_M fusion protein without Ser or Thr at position 67. Understanding the contributions of sequences surrounding the tetrapeptide motif will require more extensive analysis. Another possibility is that regions in various regulatory subunits some distance from the RVXF motif contribute to PP1C binding. Evidence for multiple PP1-interaction domains has been obtained with inhibitor-1 [27], DARPP32 [29], NIPP1 [32], M110 [30] and G_M [22,30]. Interactions at both sites are expected to contribute to overall stability of these complexes. A plausible explanation for the results reported here is that substitution of Val for Ser at position 67 reduces the relative affinity for this interaction site, thereby lowering the overall binding affinity of G_M for PP1C. In those members of the glycogen-targeting subunit family where Val is present at position 67, the weaker interaction at this site may be compensated by a relatively higher affinity interaction at the other site, which has not yet been identified.

Earlier studies showed that phosphorylation at Ser-67 in G_M impaired binding of PP1 and reduced glycogen synthase phosphatase activity [15]. Intravenous injection of adrenaline decreased the fraction of PP1 associated with rabbit skeletal muscle glycogen, with a corresponding increase in the PP1 activity in the cytosol [36]. In the present study, we provided evidence supporting G_M phosphorylation by PKA as a mechanism for dissociating PP1 from G_M. The ability of forskolin to dissociate or prevent the formation of PP1 complexes containing FLAG–G_M(1–240) and FLAG–G_M(1–1109) showed the unique C-terminal region of G_M (residues 240–1109) did not contribute significantly to the response. Partial reversal of the forskolin effects by the inhibitor H89 reinforces the conclusion that the effects were due to the phosphorylation of G_M by PKA. Interestingly, the S67T substitution in FLAG–G_M allowed constitutive association with PP1C that was not modulated by forskolin. Although PKA does phosphorylate some proteins on threonine residues, like inhibitor-1 and DARPP32, early work with synthetic peptides suggested that, even in an idealized consensus motif, PKA greatly preferred serine over threonine as a substrate. Peptides of inhibitor-1 were used to show that sequences beyond the known PKA recognition motif may be needed for the efficient phosphorylation of this protein by PKA.

Only G_M, not the other glycogen-binding subunits, like G_L in the liver [8,37] and PTG in adipose tissue [4,21], is phosphorylated by PKA. The PTG glycogen-binding subunit was not phosphorylated in cells metabolically labelled with ^{32}P [21]. G_M is phosphorylated at multiple sites by other protein kinases. This has raised questions about the physiological importance of G_M phosphorylation for hormonal control of glycogen metabolism. The S67T version of G_M, which shows constitutive PP1C binding and resistance to PKA, should greatly aid our understanding of the physiological relevance of PKA-dependent and -independent phosphorylation and hormonal control of PP1/G_M complexes and glycogen metabolism in mammalian skeletal muscle.

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