Glycogen Synthase Association with the Striated Muscle Glycogen-targeting Subunit of Protein Phosphatase-1

SYNTHASE ACTIVATION INVOLVES SCAFFOLDING REGULATED BY β-ADRENERGIC SIGNALING

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Glycogen-binding subunits for protein phosphatase-1 (PP1) target the PP1 catalytic subunit (PP1C) to glycogen particles, where the enzymes glycogen synthase and glycogen phosphorylase are concentrated. Here we identify sites within the striated muscle glycogen-binding subunit (Gm) that mediate direct binding to glycogen synthase. Both PP1C and glycogen synthase were coimmunoprecipitated with a full-length FLAG-tagged Gm transiently expressed in COS7 cells or C2C12 myotubes. Deletion and mutational analysis of a glutathione S-transferase (GST) fusion of the N-terminal domain of Gm (residues 1–240) identified two putative sites for binding to glycogen synthase, one of which is the WNNXG-NXY(I/L) motif that is conserved among the family of PP1 glycogen-binding subunits. Either deletion of this motif or Ala substitution of Asn-228 in this motif disrupted the binding of glycogen synthase. Expression of full-length FLAG-Gm in cells increased the activity of endogenous glycogen synthase, but protein disabled in either PP1 binding or glycogen synthase binding did not produce synthase activation. The results show that efficient activation of glycogen synthase requires a scaffold function of Gm that involves simultaneous binding of both PP1C and glycogen synthase. Isoproterenol and forskolin treatment of cells decreased glycogen synthase binding to FLAG-Gm, thereby limiting synthase activation by PP1. This response was insensitive to inhibition by H-89, therefore probably not involving cAMP-dependent protein kinase, but did require inclusion of microcystin-LR during cell lysis, implying that phosphorylation was modulating binding of glycogen synthase. Phosphorylation control of binding to a scaffold site on the Gm subunit of PP1 offers a new mechanism for regulation of muscle glycogen synthase in response to β-adrenergic signals.

Protein phosphatase-1 (PP1)* is implicated in control of multiple cellular processes, including mitosis, muscle contraction, glycogen metabolism, protein synthesis, RNA splicing, and ion channel conductance (1, 2). Currently, it is thought that the individual functions are achieved by interaction of the PP1 catalytic subunit with various targeting/regulatory subunits. These subunits determine the subcellular location of subpopulations of the catalytic subunit of PP1.

The first PP1 targeting subunit identified was the muscle glycogen-binding subunit, called Gm or Gm, which tethers PP1 to glycogen particles and the sarcoplasmic reticulum in skeletal and cardiac muscle (3, 4). This localization presumably facilitates the dephosphorylation of glycogen-metabolizing enzymes (3). Recent studies identified separate domains in Gm for interaction with PP1 and glycogen (5). PKA-catalyzed phosphorylation of Ser-67 in the PP1 binding motif (RVSF) has been shown to prevent PP1 binding to Gm (6, 7), providing a mechanism by which the phosphatase would respond to β-adrenergic hormones such as adrenaline.

Since the discovery of Gm, several additional members of this protein family have been identified and cloned. Among these are the yeast homologue, GAC1 (8), and a liver protein, Gl (9), which show significant sequence identity with the N-terminal region of Gm. A search of the expressed sequence-tagged cDNA data base identified PPP1R5 (10) and PPP1R6 (11). A yeast two-hybrid screen of cDNA libraries using PP1C as the bait data base identified PPP1R5 and PPP1R6 (11). A yeast two-hybrid screen of cDNA libraries using PP1C as the bait identified another two glycogen-binding proteins, U5 (12) and PTG (13). PTG is nearly identical to PPP1R5 and is expressed abundantly in insulin-sensitive tissues such as skeletal muscle, liver, heart, and differentiated adipocytes (13). In addition to associating with PP1 and glycogen, PTG functions as a scaffold that binds glycogen synthase, phosphorylase, and phosphorylase kinase (13), the substrates for PP1 in the glycogen particles. This raises the possibility that other members of the PP1 glycogen-binding subunit family also fulfill a scaffolding role by directly interacting with PP1 substrates. Sequence comparison of six eucaryotic glycogen-binding subunits of PP1 and two yeast glycogen synthase-binding proteins, Pgi1p and Pgi2p (14), revealed two regions of conserved sequence. One is a VNXXFXEKXV motif that we found directly involved in glycogen binding (5). The other is a region of 15 residues with conserved residues Trp, Asn, Gly, Asn, Tyr, and Leu/Val that we designate as the WXXGXX(N/Y)(I/L) motif. However, no experimental evidence for a function for this motif is available.

In this study, we examined the binding of glycogen synthase with deletion and point mutants of Gm and identified specific (1109), epitope-tagged, full-length (1109 residues) version of Gm; Gl, liver-specific glycogen-binding subunit of PP1; PTG, protein targeting to glycogen subunit of PP1; DME, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; H-89, N-[2-[4-bromocinnamyl]amino]ethyl]-5-isouquinolinesulfonamide hydrochloride.
isoproterenol decreased the binding of glycogen synthase to and glycogen synthase is required for activation of glycogen synthase. Furthermore, stimulation of cells with forskolin and isoproterenol decreased the binding of glycogen synthase to wild type GM but in a PKA-independent manner. Results obtained with various mutated forms of GM suggest that the effects are mediated through change in phosphorylation of glycogen synthase, exposing a new mechanism for regulation by β-adrenergic signals in muscle.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture reagents and the kit for reverse-transcription (Preamplification System for first strand cDNA synthesis) were purchased from Life Technologies (Grand Island, NY). pGEX vectors and glutathione-Sepharose were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [U-14C]Uridine diphosphoglucose ([U-14C]UDP) was purchased from NEN Life Science Products (Boston, MA). Isoproterenol, propranolol, H-89, and microcystin-LR was purchased from CalBiochem-Novabiochem Corp. (La Jolla, CA). Forskolin, leupeptin, and trypsinin were from Sigma (St. Louis, MO). Pefabloc was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Mouse anti-PP1 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Dr. J. C. Lawrence, Jr. generously provided chick anti-glycogen synthase polyclonal antibodies and purified glycogen synthase. G, as antiserum was generated by immunizing rabbits with recombinant GST-GM(1–240) fusion protein. Restriction enzymes and bacterial cloning enzymes from Promega Life Science (Madison, WI). All the oligonucleotides were synthesized by ITD (Coralville, IA).

**Cloning and Mutagenesis of G, cDNA**—Rabbit cDNA encoding G, (1–240) was cloned and inserted into pGEX4T2 vector to produce recombinant GST-GM(1–240) fusion protein as described before (15). The G, (1–240) fragment was then subcloned into pCDNA3/F2 mammalian expression vector by BamHI/EcoRI double digestion to produce FLAG-tagged G, (1–240) protein. The entire coding sequence of rabbit G, was cloned into pGEX4T2 and pCDNA3/F2 vectors by reverse transcription-polymerase chain reaction and subcloning methods as described previously (16). Deletion mutants of G, and point mutations in G,(1–240) were made using the polymerase chain reaction method described elsewhere (15, 16). Point mutations in full-length GM were made by using Quick-Change Mutagenesis kit according to the manufacturer’s protocol. Mutations were all confirmed by DNA sequencing.

**Tissue Culture—** COS7 cells were maintained in DMEM containing 10% new born calf serum. C2C12 myoblasts were maintained in DMEM containing 20% fetal bovine serum until confluent, at which point differentiation was initiated by conversion to DMEM with 4% horse serum. Cell fusion was apparent after 48 h. C2C12 cells were routinely differentiated. DMEM with 4% horse serum and 20% FBS until confluent, at which point differentiation was initiated by conversion to DMEM with 4% horse serum and 20% FBS. Further truncation to GST-GM(97–240) interacted with glycogen synthase and PP1C, whereas GST alone bound neither enzyme and served as a negative control (not shown). The fusion protein GST-GM(1–218), truncated at the C terminus of the 1–240 domain, bound PP1C but not glycogen synthase, indicating that the region 219–240 was required for glycogen synthase binding. On the other hand, a fusion protein without the RVSF motif, GST-GM(77–240), failed to bind PP1C as expected but bound glycogen synthase the same as GST-GM(1–240). Further truncation to GST-GM(97–240) gave a protein that bound glycogen synthase to a much lesser extent, and with GST-GM(118–240) there was no detectable glycogen synthase binding. Coumassie Blue staining showed that an equal amount of the GST fusion proteins was added (Fig. 1B, lower panel), and the same amount of COS7 cell lysate protein was used in each assay. These observations suggested that binding of glycogen synthase involved two sites of interaction, as the peptides 219–240 and at 77–117 within the N-terminal domain of G,.

**RESULTS**

**Deletion Analysis to Define Regions of G, Required for Binding to Glycogen Synthase**—We linked glutathione S-transferase (GST) to various regions of the N-terminal domain of G, (Fig. 1A), expressed the fusion proteins in Escherichia coli, and purified them on glutathione-Sepharose. A “pull-down” assay was used to test for binding of glycogen synthase and PP1C from lysates of COS7 cells. The glycogen synthase and PP1C that bound to fusion proteins were detected by immunoblotting (Fig. 1B). Both GST-G,(1–240) and GST-GM(51–240) interacted with glycogen synthase and PP1C, whereas GST alone bound neither enzyme and served as a negative control (not shown). The fusion protein GST-GM(1–218), truncated at the C terminus of the 1–240 domain, bound PP1C but not glycogen synthase, indicating that the region 219–240 was required for glycogen synthase binding. On the other hand, a fusion protein without the RVSF motif, GST-GM(77–240), failed to bind PP1C as expected but bound glycogen synthase the same as GST-GM(1–240). Further truncation to GST-GM(97–240) gave a protein that bound glycogen synthase to a much lesser extent, and with GST-GM(118–240) there was no detectable glycogen synthase binding. Coumassie Blue staining showed that an equal amount of the GST fusion proteins was added (Fig. 1B, lower panel), and the same amount of COS7 cell lysate protein was used in each assay. These observations suggested that binding of glycogen synthase involved two sites of interaction, as the peptides 219–240 and at 77–117 within the N-terminal domain of G,.

**Single Residue Substitution Asn-228 → Ala in G, Disrupts Association with Glycogen Synthase**—In the 219–240 region of G, there is a WNXGXXNYXL/I motif comprised of several residues conserved among the family of glycogen-binding subunits. This motif also appeared in PIG1 and PIG2, two yeast proteins discovered in a two-hybrid screen using glycogen synthase as bait (14). We substituted alanine for the conserved

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2 The numbers in parenthesis indicate the residues of rabbit G, that were fused to GST.
residues with the smallest side chains to produce three mutant forms of GST-GM-(1–240): N224A, G226A, and N228A. In the pull-down assay, wild type GST-GM-(1–240), GST-GM-(1–240)-N224A, and GST-GM-(1–240)-G226A all bound equal amounts of glycogen synthase, but binding was essentially lost with GST-GM-(1–240)-N228A (Fig. 2A, upper panel). The same amount of purified fusion protein was used in each assay, shown by Coomassie Blue staining after SDS-PAGE (Fig. 2A, lower panel). None of the Ala substitutions reduced PP1C binding in this assay (Fig. 2A, middle panel), suggesting that the overall conformation of the various fusion proteins was not perturbed. As further evidence of similar tertiary structure, the wild type GST-GM-(1–240) and the mutant GST-GM-(1–240)-N228A yielded the same fragments during partial digestion with trypsin with the same kinetics of production (Fig. 2B). Therefore, the loss of glycogen synthase binding by Asn-228 → Ala substitution could not simply be attributed to misfolding of the mutant protein. Moreover, no significant difference in glycogen synthase binding was detected (Fig. 2A, upper panel) between wild type GST-GM-(1–240) and GST-GM-(1–240)-V150A/F155A, a double mutant that was shown previously to have reduced glycogen binding in a co-sedimentation assay (5). We concluded that glycogen synthase binding did not depend on glycogen binding to G_M. Taken together, these results indicated that Asn-228 in the WXXGXXXX(L/I) motif played a critical role in glycogen synthase binding. Furthermore, different sites were used by G_M to interact with glycogen, with PP1C, and with glycogen synthase.

Binding of Purified Glycogen Synthase to GST-GM-(1–240)—Besides using cell lysates as a source of glycogen synthase, we tested binding of purified skeletal muscle glycogen synthase to GST-GM-(1–240) to demonstrate a direct interaction between the proteins. Glycogen synthase was incubated with GST alone, GST-GM-(1–240), or GST-GM-(1–240)-N228A in the pull-down assay. Glycogen synthase bound GST-GM-(1–240) but did not bind to GST alone or GST-GM-(1–240)-N228A (Fig. 3A, upper panel). This result reinforced data with COS7 lysates as the source of glycogen synthase. Identical amounts of GST proteins were present in the samples, as seen in Coomassie Blue staining (Fig. 3A, lower panel). Next, this pull-down assay was used to measure relative binding affinities by incubating different concentrations of glycogen synthase with GST-GM-(1–240), or GST-GM-(1–240)-N228A. The relative amount of glycogen synthase bound was quantified by laser densitometry after immunoblotting. The amount of glycogen synthase specifically bound to GST-GM-(1–240) was corrected for background by subtracting the amount bound to GST alone and in densitometry by using exposures within the linear response range of the film. Although glycogen synthase specifically bound GST-GM-(1–240) with a K_d of 10–12 nM, GST-GM-(1–240)-N228A bound glycogen synthase in this assay with a K_d of >40 nM (Fig. 3B). We concluded that the single substitution Asn-228 → Ala in GST-GM-(1–240) caused a 4-fold decrease in the apparent affinity for glycogen synthase.

Glycogen Synthase Activation by Binding to the N-terminal Domain of G_M—We expressed the FLAG-tagged G_M N-termi-
nal domain in COS7 cells and examined glycogen synthase coimmunoprecipitation, using the anti-FLAG antibodies and anti-glycogen synthase immunoblotting. Cells were transfected either with empty vector or with a vector encoding wild type FLAG-GM-(1–240), FLAG-GM-(1–218), or mutant FLAG-GM-(1–240)-N228A. All three forms of GM were expressed at about the same levels, with similar recoveries by anti-FLAG immunoprecipitation (Fig. 4A, upper panel). Endogenous glycogen synthase was specifically coimmunoprecipitated with FLAG-GM-(1–240) but not with FLAG-GM-(1–218) or FLAG-GM-(1–240)-N228A (Fig. 4A, middle panel). All these fusion proteins bound PP1C, attesting to their conformational integrity (Fig. 4A, lower panel). The results showed that the GM-(1–240) domain interacted with glycogen synthase in living cells and that the Asn-228 in the WXXNXXYX(L/I) motif was required.

Transfected cells were lysed, and the glycogen synthase/glucose 6-phosphate activity ratio was measured in the cell lysate. Cells not expressing the GM-(1–240) domain had a low basal activity ratio of glycogen synthase (0.031 ± 0.003), and this increased 2.6-fold by expression of FLAG-GM-(1–240) (Fig. 4B). However, expression of either FLAG-GM-(1–218) or FLAG-GM-(1–240)-N228A had no significant effect on glycogen synthase activity (Fig. 4B). The results showed that only forms of FLAG-GM-(1–240) that bound to glycogen synthase caused its activation.

**Fig. 3.** Binding of purified glycogen synthase to GST fusion proteins. A, 3 μg of GST alone, wild type GST-GM-(1–240) or GST-GM-(1–240)-N228A were coupled to glutathione-Sepharose and incubated with 10 nM of purified glycogen synthase in 500 μl of binding buffer. Glycogen synthase was detected by immunoblotting, and GST proteins were stained with Coomassie Blue. B, different concentrations of glycogen synthase were used in the pull-down assay. The glycogen synthase bound to GST-GM-(1–240) (squares) and GST-GM-(1–240)-N228A (circles) was quantitated with laser densitometry and ImageQuaNT software, and was plotted against the concentrations of synthase used. Results are expressed as a percentage of the synthase bound at 80 nM of concentration and means ± S.E. for three separate experiments.

**Fig. 4.** Effects of GM N-terminal domain expression on endogenous glycogen synthase activity in COS7 cells. A, COS7 cells transiently transfected with vector alone, FLAG-GM-(1–240), FLAG-GM-(1–240)-N228A, or FLAG-GM-(1–218) were lysed, and immunoprecipitation was performed by using anti-FLAG affinity gel. Precipitated FLAG-GM and coprecipitated glycogen synthase and PP1C were detected by immunoblotting. B, COS7 cells expressing FLAG-tagged GM proteins were lysed, and glycogen synthase activity was assayed as described under “Experimental Procedures.” Shown are the means ± S.E. of three separate experiments.
FLAG-GM-(1109) with glycogen synthase was independent of PP1C binding. Glycogen synthase activity was measured in cells expressing various forms of FLAG-GM-(1109). Expression of the FLAG-GM-(1109) induced higher levels of glycogen synthase activity (6 glucose 6-phosphate) than did the FLAG-GM-(1–240) (compare Fig. 4B with 5B). This is the first hint of a functional effect of the large C-terminal region of GM (residues 241–1109) that is not conserved in other glycogen-binding subunits of PP1. Glycogen synthase activity was increased 5- to 6-fold in cells expressing either FLAG-GM-(1109) or FLAG-GM-(1109)-S67T (Fig. 5B). Expression of mutant proteins disabled in PP1C binding, such as FLAG-GM-(77–1109) and cocraptured glycogen synthase and PP1C were detected by immunoblotting. B, COS7 cells expressing FLAG-tagged GM proteins were lysed, and glycogen synthase activity was assayed as described under “Experimental Procedures.” Shown are the means ± S.E. of three separate experiments.

FIG. 5. Glycogen synthase activation in COS7 cells expressing full-length FLAG-GM-(1109). A, COS7 cells transiently transfected with vector alone, FLAG-GM-(1109), FLAG-GM-(77–1109), FLAG-GM-(1109)-N228A, FLAG-GM(1109)-S67T, or FLAG-GM-(1109)-S67A were lysed, and immunoprecipitation was performed by using anti-FLAG affinity gel. Precipitated FLAG-GM-(1109) and cocraptured glycogen synthase and PP1C were detected by immunoblotting. B, COS7 cells expressing FLAG-tagged GM proteins were lysed, and glycogen synthase activity was assayed as described under “Experimental Procedures.” Shown are the means ± S.E. of three separate experiments.

Modulation of Glycogen Synthase Binding to FLAG-GM-(1109) in Response to Forskolin and Isoproterenol—We previously demonstrated that in living cells dissociation of PP1C from FLAG-GM-(1109) can be induced by forskolin (16), a compound that promotes an increase in intracellular [cAMP], and consequently activates PKA. We stimulated COS7 cells transiently expressing FLAG-GM-(1109) with increasing concentrations of forskolin (16), a compound that promotes an increase in intracellular [cAMP], and consequently activates PKA. We stimulated COS7 cells transiently expressing FLAG-GM-(1109) with increasing concentrations of forskolin (16). Cells lysates were prepared, and anti-FLAG immunoprecipitation was performed as described under “Experimental Procedures.” Precipitated FLAG-GM-(1109) and cocraptured glycogen synthase and PP1C were detected by immunoblotting. B, serum-starved COS7 cells expressing FLAG-GM-(1109) were treated with or without 15 μM of isoproterenol for 30 min. Cells were lysed in the presence or absence of microcystin-LR. Immunoprecipitation was performed as described in A. C, serum-starved C2C12 myotubes expressing FLAG-GM-(1109) were treated with vehicle alone, 15 μM of isoproterenol, or 15 μM of forskolin for 30 min. Cells were lysed and immunoprecipitation was performed as described in A.

FIG. 6. Effect of forskolin and isoproterenol on coimmunoprecipitation of glycogen synthase with FLAG-GM-(1109). A, serum-starved COS7 cells expressing FLAG-GM-(1109) were treated with different concentrations of forskolin for 30 min. Cells lysates were prepared, and anti-FLAG immunoprecipitation was performed as described under “Experimental Procedures.” Precipitated FLAG-GM-(1109) and cocraptured glycogen synthase and PP1C were detected by immunoblotting. B, serum-starved COS7 cells expressing FLAG-GM-(1109) were treated with or without 15 μM of isoproterenol for 30 min. Cells were lysed in the presence or absence of microcystin-LR. Immunoprecipitation was performed as described in A. C, serum-starved C2C12 myotubes expressing FLAG-GM-(1109) were treated with vehicle alone, 15 μM of isoproterenol, or 15 μM of forskolin for 30 min. Cells were lysed and immunoprecipitation was performed as described in A.
We wondered whether an increase in PKA activity was sufficient to reduce binding of glycogen synthase to FLAG-GM-(1109) (Fig. 7A). Treatment of COS7 cells with forskolin, regardless of the co-association of PP1C, would directly bind enzymes involved in glycogen metabolism to promote reaction with PP1C. Here we present evidence that G_M does simultaneously associate with PP1C and glycogen synthase, even in the absence of glycogen. The glycogen interaction motif (VXNNFXEACKV) in G_M does not appear to be involved in the association with glycogen synthase, because the double mutant (V150A/F155A) that did not bind glycogen (5) nonetheless did bind glycogen synthase to the same extent as the wild type protein. The other family members PTG and GL bind glycogen phosphorylase (13, 26). However, studies in yeast with Gac1p and Pig1p suggested that besides association with glycogen, there were direct interactions of these PP1 targeting subunits with substrates such as Gsy2, a major form of glycogen synthase in Saccharomyces cerevisiae (14). Furthermore, PTG was discovered as another mammalian glycogen-binding subunit of PP1 and was shown to directly bind the PP1 substrates, glycogen synthase, phosphorylase, and phosphorylase kinase (13). Based on these reports we predicted that other members of the family of mammalian PP1 glycogen-binding subunits, specifically G_M, would directly bind enzymes involved in glycogen metabolism to promote reaction with PP1C. We here present evidence that G_M does simultaneously associate with PP1C and glycogen synthase, even in the absence of glycogen. The glycogen interaction motif (VXNNFXEACKV) in G_M does not appear to be involved in the association with glycogen synthase, because the double mutant (V150A/F155A) that did not bind glycogen (5) nonetheless did bind glycogen synthase to the same extent as the wild type protein. The other family members PTG and G_G bind glycogen phosphorylase (13, 26). However, in pull-down assays with 32P-labeled phosphorylase a and G_M-(11–240) we did not observe a specific interaction (not shown). Sequence comparison shows that the phosphorylase α-binding domain in G_M is not conserved in G_M (9, 26). Direct binding of phosphorylase α may be an important difference between G_M and family members such as G_G and PTG/R5.

We propose that high affinity binding of glycogen synthase to G_M is generated by the interaction of glycogen synthase with two sites (residues 77–118 and 219–240). Truncation of either site caused a loss of glycogen synthase binding in a GST pull-down assay. For this reason, we examined the effect of forskolin on the interaction between glycogen synthase and G_M.

### DISCUSSION

Starting over 30 years ago, glycogen particles have been studied as an example where kinases, phosphatases, and their substrate enzymes are organized into an assembly with properties that are lost upon dilution and dissociation (18–20). Targeting of PP1C into muscle glycogen particles involves a glycogen-binding subunit called G_G or R_{GL} (3, 4) that puts PP1C in proximity to its substrates, the glycogen-metabolizing enzymes. Glycogen synthase is the rate-limiting enzyme in glycogen synthesis (21–23) and is a major substrate for glycogen-phosphorylase, phosphorylase, and phosphorylase kinase (25). The enhanced phosphatase activity was attributed to a lower K_m, due to the association of both G_M and substrates with glycogen (3). However, studies in yeast with Gac1p and Pig1p suggested that besides association with glycogen, there were direct interactions of these PP1 targeting subunits with substrates such as Gsy2, a major form of glycogen synthase in Saccharomyces cerevisiae (14). Furthermore, PTG was discovered as another mammalian glycogen-binding subunit of PP1 and was shown to directly bind the PP1 substrates, glycogen synthase, phosphorylase, and phosphorylase kinase (13). Based on these reports we predicted that other members of the family of mammalian PP1 glycogen-binding subunits, specifically G_M, would directly bind enzymes involved in glycogen metabolism to promote reaction with PP1C. Here we present evidence that G_M does simultaneously associate with PP1C and glycogen synthase, even in the absence of glycogen. The glycogen interaction motif (VXNNFXEACKV) in G_M does not appear to be involved in the association with glycogen synthase, because the double mutant (V150A/F155A) that did not bind glycogen (5) nonetheless did bind glycogen synthase to the same extent as the wild type protein. The other family members PTG and G_G bind glycogen phosphorylase (13, 26). However, in pull-down assays with 32P-labeled phosphorylase a and G_M-(11–240) we did not observe a specific interaction (not shown). Sequence comparison shows that the phosphorylase α-binding domain in G_M is not conserved in G_M (9, 26). Direct binding of phosphorylase α may be an important difference between G_M and family members such as G_G and PTG/R5.

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down assay. Our study demonstrated that the 219–240 region of \( G_M \), containing the WXXGXXNY(L/I) motif, was required for glycogen synthase binding. Interestingly, a single Ala substitution of Asn-228 in this motif also was sufficient to prevent binding of glycogen synthase to \( G_M \). This leads us to suggest that the WXXGXXNY(L/I) motif is the primary or dominant site for interaction. Sequence alignment showed that the WX-

NGGXYXX(L/I) motif was highly conserved among the glycogen-
targeting subunits and the yeast glycogen synthase binding proteins, PIG1 and PIG2 (14). However, this motif alone does not seem sufficient for glycogen synthase binding, because a protein with the 219–240 sequence fused to GST did not bind glycogen synthase (results not shown). Recently, a structural model for the N-terminal domain of \( G_M \) has been proposed, based on homology to the microbial enzyme xylose isomerase, which has an \((\alpha/\beta)_\text{m}\) barrel structure (27). The model has an extended loop with RVSF for binding PP1C and a surface helix with the VPV residues exposed for binding to glycogen. Our proposed sites for interaction with glycogen synthase would lie in the spacer between putative helices \( \beta_6 \) and \( \alpha_6 \) (the WX-

NGGXYXX(L/I) motif) and a segment between helices \( \alpha_2 \) and \( \alpha_3 \) that includes beta sheet \( \beta_3 \) (residues 77–118). It appears that these two sites are on one side of the structure, opposite from the site for interaction with glycogen and separate from the loop for binding to PP1C.

Our data show that PP1C and glycogen synthase both must bind to \( G_M \) in order for \( G_M \) to mediate activation of glycogen synthase. Thus, \( G_M \) enhances PP1 activity toward glycogen synthase by recruiting PP1C and glycogen synthase into the same protein complex via direct binding. This may also involve conformational changes in the catalytic subunit and/or the substrate to induce or favor dephosphorylation. In this instance the scaffold subunit \( G_M \) would function as an allosteric activator. We pose the hypothesis that direct binding of substrates to target-binding sites may be a common mechanism to enhance substrate specificity of the PP1 catalytic subunit in vivo. Another example is the smooth muscle myosin phosphatase target-binding subunit that interacts with myosin directly, and two different regions in the myosin-targeting subunit were reported to be responsible for myosin binding (28, 29). These interactions enhance specificity of PP1C for myosin light chain as substrate. Many new PP1 targeting/regulatory subunits have been identified, but the physiological substrates for these PP1 holoenzymes are not yet defined. It will be important to define whether specific cellular phosphoproteins associate with each of these subunits. If so, then identifying and mapping the substrate binding sites on the PP1 subunits like we have done here will advance our understanding of how the specificity and activity of PP1 is regulated in cells. We imagine that, because we observed loss of function with a single residue substitution (N228A), pharmacological compounds could specifically target and disrupt the interactions between a substrate and a regulatory subunit (e.g. glycogen synthase and \( G_M \)). This would provide a way of selectively altering the state of phosphorylation, and hence the activity, of particular PP1 substrates.

Our results also expose a novel mechanism for glycogen synthase inactivation by \( \beta \)-adrenergic signals. We provide evidence that \( \beta \)-adrenergic signaling or elevation of [cAMP] by forskolin diminished glycogen synthase binding to \( G_M \). Microcystin was required in the lysates, implying that a phosphorylation accounted for the change and there was a rapid dephosphorylation by phosphatases of the PP1/PP2A family. Glycogen synthase dissociation from \( G_M \) seemed to be induced by phosphorylation of glycogen synthase that was cAMP-dependent, but resistant to inhibitors of PKA. Previous studies suggested that phosphorylation and inactivation of muscle glycogen syn-

thase by \( \beta \)-adrenergic hormones involved PKA (30–33). However, another report showed that Ser-10 of muscle glycogen synthase, not a PKA site, increased from 0.12 mol/mol to 0.54 mol/mol in vivo in response to adrenaline (38). After phosphorylation of Ser-7 by phosphorylase kinase, phosphorylation of Ser-10 by casein kinase I further inhibited glycogen synthase activity by over 2.5-fold (38). However, the activity of casein kinase 1 was unaffected by adrenaline in vivo or by incubation with PKA and MgATP in vitro. Nonetheless, one possibility is that glycogen synthase affinity for \( G_M \) is modulated by phosphorylation of Ser-10 by kinase(s) other than PKA. Although cAMP exerts many or most of its effects through PKA, cAMP also has some PKA-independent actions, including binding directly to cardiac pacemaker and other ion channels and to Rap1-specific guanine nucleotide exchange factor (34–36). It has been reported recently that mitogenic signals initiated by cAMP regulate PKB and membrane ruffling in a PKA-independent manner (37). Determining the site of phosphorylation in glycogen synthase that regulates affinity for \( G_M \) and the kinase that catalyzes the phosphorylation is important goals for future efforts.

We propose that \( \beta \)-adrenergic signaling via cAMP, but not PKA, reduces glycogen synthase association with its targeting sites in \( G_M \) that enhance PP1-mediated dephosphorylation. Reduced binding to \( G_M \) would be a means to prolong the phosphorylation and low activity state of glycogen synthase. This mechanism adds another piece to the regulatory mosaic for glycogen synthase, in addition to the traditional multisite phosphorylation and allosteric binding of metabolite effectors.

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