Protein Phosphatase Type-1 and Glycogen Bind to a Domain in the Skeletal Muscle Regulatory Subunit Containing Conserved Hydrophobic Sequence Motifs†

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ABSTRACT: This study identifies a 100-residue domain within the rabbit skeletal muscle regulatory subunit (PP1G) that binds both type-1 protein phosphatase (PP1C) and glycogen. An N-terminal portion of PP1G was cloned by RT-PCR, and different sized fragments were expressed in bacteria as glutathione *S*-transferase (GST) fusion proteins. A GST−PP1G fusion containing residues 51−240 bound both PP1C and glycogen, whereas GST alone or fusions containing residues 51−140 or 241−360 bound neither PP1C nor glycogen. The PP1C in whole cell lysates or partially purified PP1C from skeletal muscle, or a complex of PP1C−MCLR−biotin, all bound more effectively than Mn^{2+}-activated, recombinant PP1C purified from bacteria. Binding was enhanced by increasing the ionic strength and was disrupted by ethylene glycol, consistent with hydrophobic interactions being critical for stable association. Phosphorylation of the GST−PP1G fusion by cAMP-dependent protein kinase prevented completely association of PP1C. This domain of PP1G, from residues 141−240, contains two sequence motifs of hydrophobic residues: $Gx_8FEKx_{10}W$ and DxFxFxIxL, that are conserved among the known glycogen-binding PP1 regulatory subunits. These segments are predicted to form an α helix and a β sheet, and we propose that they are the sites for association with PP1C and glycogen, respectively.

Protein phosphatase 1 (PP1)[1] is one of the major serine/threonine protein phosphatases in mammalian cells. Based on genetic, pharmacological, and biochemical data, PP1 has a variety of functions including regulating glycogen metabolism and progression through mitosis [see reviews by Shenolikar (1994) and Wera and Hemmings (1995)]. The catalytic subunit of PP1 (PP1C) is a 37 kDa protein produced in isoforms termed α, γ1, γ2, and δ (Sasaki et al., 1990). The α and δ isoforms are abundant and widely distributed in mammalian tissues. The protein sequences from cDNA of these PP1C isoforms revealed that they were nearly identical, with most differences near the C-terminus. There are no distinctive differences between PP1C isoforms in biochemical assays for activity and susceptibility to inhibitors (Zhang et al., 1993).

It has been proposed that for a particular function the catalytic subunit of PP1 needs to interact with a regulatory subunit to target the enzyme to a specific location (Hubbard & Cohen, 1993). Different regulatory/targeting subunits of PP1C have been cloned and sequenced: a glycogen-bound regulatory subunit (PP1G) from human and rabbit skeletal muscle, plus a myosin-binding subunit of smooth muscle from a different species. In addition, PP1C binds the heat-stable proteins inhibitor-1 and inhibitor-2, and nuclear subunits called *sds22* and NIPP [see reviews by DePaoli-Roach et al. (1994) and Wera and Hemmings (1995)]. How PP1C is distributed among the various regulatory subunits is not understood. The PP1 regulatory subunits listed all are phosphorylated, and this is reported to influence their interaction with PP1C (Hiraga & Cohen, 1986; Ichikawa et al., 1996; Endo et al., 1996; Jagiello et al., 1995; DePaoli-Roach et al., 1994). Phosphorylation of PP1C itself may regulate its interaction with different proteins. Phosphorylation of Thr or Tyr in PP1C changed activity and substrate specificity in different assays (Johansen & Ingebritsen, 1986; Villa-Moruzzi et al., 1991; Belandia et al., 1994; Dohadwala et al., 1994; Yamano et al., 1994).

The glycogen-bound regulatory subunit PP1G was first identified by Cohen and colleagues (Stralfors et al., 1985) as a protein that copurified with PP1C from glycogen particles. Molecular cloning of full-length cDNA of PP1G revealed an open reading frame encoding 1109 amino acids (Tang et al., 1991). The PP1G mRNA was expressed most prominently in skeletal muscle and was not detected in liver (Tang et al., 1991). However, a protein with the same size, similar properties, and even immuno-cross-reactivity with PP1G was purified from liver, but the sequence, and therefore its identity, was not established (Wera et al., 1991; Zhao et al., 1996). Recently, a cDNA for a 33 kDa glycogen-bound PP1 regulatory subunit from liver ($PP1G_L$) was cloned (Doherty et al., 1995). The predicted amino acid sequence of $PP1G_L$ showed little identity with that of muscle PP1G, consistent with it being a separate gene product. The GAC1 protein, a yeast homologue of PP1G (Francois et al., 1992), diverges in sequence from both PP1G and $PP1G_L$. Despite the lack of identity among the entire sequence of these proteins, all three associate with PP1C and bind to glycogen, suggesting that they might share common motifs for these interactions, but these sites have not been identified.

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[1] Abbreviations: PP1, protein phosphatase 1; PP1C, catalytic subunit of protein phosphatase 1; PP1G, glycogen-binding regulatory subunit of PP1C; $PP1G_L$, liver glycogen-binding regulatory subunit of PP1C; AKAPs, A kinase anchoring proteins; GST, glutathione *S*-transferase; PKA, cAMP-dependent protein kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RT-PCR, reverse transcription polymerase chain reaction; DTT, dithiothreitol; HRP, horseradish peroxidase; MCLR, microcystin-LR.

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There are multiple phosphorylation sites in PP1G. Ser48 (site 1) and Ser67 (site 2) can be phosphorylated by PKA, and glycogen synthase kinase 3 (GSK-3) can also phosphorylate Ser40 and Ser44 *in vitro* but only after prior phosphorylation at site 1 (Dent et al., 1989). It was shown that injection of adrenaline *in vivo* or phosphorylation of PP1G *in vitro* by PKA promoted dissociation of PP1C from the glycogen-protein particles, which was thought to be caused by site 2 phosphorylation (Hiraga & Cohen, 1986; MacKintosh et al., 1988; Hubbard & Cohen, 1989).

PP1G is extremely susceptible to digestion by endogenous proteases during purification as it was recovered from muscle as a mixture of the intact protein and a set of fragments of various sizes. Analysis of individual fractions from gel filtration chromatography showed that a fragment of PP1G as small as 40 kDa was able to bind PP1C and glycogen (Hiraga et al., 1987). Far-Western blotting with PP1C and anti-PP1C antibodies in concert with an immunoblot with anti-PP1G antibody also supported the conclusion that the PP1C interaction site is in an N-terminal fragment of about 40 kDa of PP1G (Chi and Brautigan, unpublished observations). Here we used different GST fusion proteins and a solid-phase binding assay to identify a 100 residue domain within PP1G that binds PP1C and glycogen. This domain contains a putative α helix and a β sheet that is conserved in all different PP1 glycogen-bound regulatory subunits.

MATERIALS AND METHODS

Materials. Restriction enzymes were from Promega Life Science, Madison, WI. The kits for reverse-transcription (Preamplification System for First Strand cDNA Synthesis) and for PCR were from Life Technologies and Stratagene, respectively. pGEX vector was from Pharmacia. Glutathione−Agarose and glutathione were from Sigma Chemical Co., St. Louis, MO. All the oligonucleotides used for this research were synthesized by ITD (Coralville, IA). The rabbit polyclonal antibodies against PP1α catalytic subunit were from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit polyclonal antibodies against GST were provided by Dr. S. Shenolikar (Duke University, Durham, NC). The Vistra Fluorescence Western blotting kit was from Amersham Life Science, Inc., and the ECL Western Blotting kit was from DuPont NEN (Boston, MA). The DNA sequencing kit was from United States Biochemical (Cleveland, OH). The catalytic subunit of PP1 was partially purified by extraction from rabbit skeletal muscle and gradient elution from DEAE−Agarose (Brautigan et al., 1985). Homogeneous PP1C was purified from pig bladder as microcystin LR−biotin complex and generously provided by Dr. T. A. J. Haystead, University of Virginia (Haystead et al., 1995). Recombinant PP1α expressed in bacteria was purified to homogeneity (Zhang et al., 1992) and provided by Dr. Ernest Y. C. Lee, University of Miami School of Medicine.

RT-PCR of Rabbit PP1G cDNA. To clone the cDNA encoding the N-terminal 360 amino acids of PP1G, we used 5′-CGC GGA TCC ATG GAG CCT TCT GAA GTA C-3′ as forward primer and 5′-CGG AAT TCC CTT CTG TAA CTC CTC AGG-3′ as backward primer. These were designed to give 5′ *Bam*HI and 3′ *Eco*RI sites to facilitate subcloning. First-strand cDNA synthesis using 2 μg of total RNA from rabbit skeletal muscle was done according to the protocol provided by the manufacturer. After digestion with RNase H, double-stranded cDNA was synthesized using the forward primer, and the cDNA was amplified in the presence of forward and backward primers by PCR using the cycle of 95 °C 1 min, 55 °C 1 min, and 72 °C 1.5 min for 30 cycles. DNA was purified and digested, and cloned into the *Bam*HI and *Eco*RI sites of pGEX-4T-2. This fragment was used for subsequent PCR to make other fragments using primers 5′ CGC GGA TCC ATG GAG CCT TCT GAAA G 3′ as forward and 5′ CGG AAT TCC ATT CAA AAC TCG AAT ACC 3′ as backward for PP1Ga, the same forward primer and a backward primer of 5′ CGG AAT TCC TGG CTC AGG TTC CTT CTT 3′ for PP1Gb, and 5′ CGC GGA TCC GAG CCT GGA AAG CCA TTG 3′ as forward and 5′ CGG AAT TCC CTT CTG TAA CTC CTC AGG 3′ as backward for PP1Gc. Fragments were cloned into pGEX-4T-2 and primary structures determined to match PP1G by DNA sequencing. However, we found that instead of annealing to the 5′ end of the open reading frame, the forward primer annealed to a CTTCTGAAG sequence 150 nucleotides into the coding sequence and gave rise to DNA encoding a PP1G with deletion from amino acids 6 to 52. Because residues 4 and 5 are identical to residues 51 and 52, the final amino acid sequences of the GST fusion proteins have residues 1, 2, and 3 and then start from residue 51 of PP1G. Sequencing the pGEX inserts also revealed that nucleotides TAC at the 3′ end of the forward primer were missing from the constructs. Although the products of the PCR were not the ones expected, they were tested for binding to PP1C and glycogen.

Expression of GST Fusion Proteins in E. coli. The pGEX-4T-2 vectors containing fragments of interest were introduced into *E. coli* strain BL21. After overnight culture at 37 °C in LB medium containing 50 μg/mL ampicillin, the culture was inoculated 1:10 into fresh medium, cooled, and grown overnight at 30 °C in the presence of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) to induce protein expression. The bacteria were harvested by centrifugation at 6000*g* for 15 min at 4 °C and resuspended in 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA. Bacteria lysis was performed by application of 0.1% (w/v) lysozyme followed by 0.1% (v/v) NP-40, and then freezing and thawing and subsequent DNase I treatment (10 μg/mL). The protease inhibitors 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM benzamidine were added prior to lysis. The lysate was centrifuged at 15000*g* for 20 min at 4 °C, and the supernatant was mixed with glutathione−Agarose beads (0.2 mL/mL) at room temperature for 30 min. The beads were collected by centrifugation, washed 3 times with phosphate-buffered saline (20 mM PO_4, pH 7.4, 0.15 M NaCl), and then boiled in 1 × sample buffer. Proteins were separated by SDS−PAGE and stained by Coomassie Blue, and the molecular sizes were determined by comparison with prestained standards from Sigma.

Mammalian Cell Lysis. Swiss 3T3 cells, 10T1/2, or IV5 cells (a transformed 10T1/2 cell line with high expression of v-src protein) were grown to confluence and scraped off the dishes in cell lysis buffer (50 mM Hepes, pH 7.5, 0.15 M NaCl, 1.5 mM $MgCl_2$, 1 mM $MnCl_2$, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin) with or without the phosphatase inhibitors 1.0 mM sodium vanadate and 1 μM microcystin. After trituration and subsequent incubation in ice for 5 min, lysates

were centrifuged at 11000*g* for 30 min at 4 °C. The supernatant was stored frozen at −70 °C and used for further experiments.

PP1C Binding Assay. A GST fusion protein "pull down" previously described (Blaikie et al., 1994) was modified and used to perform the PP1C binding assay. Briefly, 1−2 μg of GST fusion proteins on glutathione−Agarose was incubated with cell lysates, or purified forms of PP1C in a binding buffer (20 mM Hepes, pH 7.5, 0.15 M NaCl, 0.1 mM $MnCl_2$, 60 mM 2-mercaptoethanol, 0.1 mg/mL BSA, 10% glycerol, 0.01% NP-40, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin) for 90 min at 4 °C. This was determined to be sufficient time to establish equilibrium binding. There was at least a 2-fold molar excess of PP1C over fusion protein in the assay, and this was determined to be sufficient to achieve saturation under conditions of the assay.

The suspension of beads was transferred into a Wizard miniprep column and the liquid removed by filtration on a ProMega Vac-Man vacuum manifold. The beads were washed 3 times with 1.0 mL of buffer containing 20 mM Hepes, pH 7.5, 1.0 M NaCl plus 10% glycerol. Some preliminary experiments also used a wash buffer of 20 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol, and 0.1% Triton X-100. Laemmli sample buffer was heated to 100 °C and then added to the column, and the column was heated for 1.5 min in a microwave oven. The column was placed on a microcentrifuge tube and centrifuged to collect the sample. The elution step was repeated twice, and the samples were pooled. The binding of PP1C was analyzed by Western blotting using ECL or FluorImaging as described below.

Chemical Modification of GST−PP1Gb. For lysine modification, the fusion protein was incubated with 1.0 mM succinic anhydride at pH 9.0 for 15 min at room temperature. For carboxylic acid modification, the fusion protein was incubated with 1 mM 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) at pH 4.5 for 15 min at room temperature, followed by addition of 0.1 M Tris, pH 8.5. After the beads were washed with binding buffer, the binding of PP1C from a 3T3 cell lysate was assayed. As a control, beads with GST−PP1Gb bound were incubated in the pH 4.5 and 9.0 buffers, without modification agents. Binding of PP1C was measured using Western blotting. Exposure of the fusion protein to pH 4.5 did reduce binding of PP1C, but the reaction with EDC produced no additional effect.

In Vitro Phosphorylation by PKA. Phosphorylation of GST fusion protein of PP1G by PKA was performed according to Ron and Dressler (1992) with the following modifications. GST−PP1G (5 μg) on glutathione−Agarose beads was shaken constantly in the reaction buffer containing 0.1 mM ATP, 50 mM potassium phosphate, pH 7.15, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM DTT, and 25 units of PKA (Sigma) for 30 min at room temperature. Parallel experiments using 0.1 mM [γ-^{32}P]ATP (1000 dpm/pmol) were performed under the same reaction conditions to monitor the extent of phosphorylation. The reaction was stopped by dilution and washing by centrifugation with cold PBS containing 1 mM DTT. The stoichiometry of phosphorylation was calculated from the amount of protein, estimated from Coomassie staining of a gel that also contained dilutions of BSA as a standard curve, and ^{32}P, determined by scintillation counting.

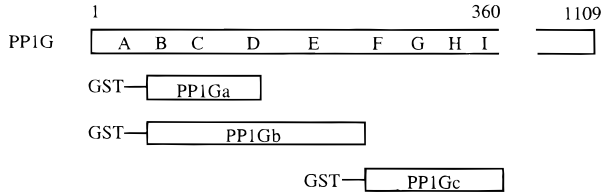

FIGURE 1: Expression of PP1G fragments as GST fusion proteins. The muscle PP1G protein of 1109 amino acids is depicted as the box in the top line, and the position of predicted α helices within the N-terminal 360 residues is shown by upper case letters A−I. Fragments encoding PP1Ga (residues 51−140), PP1Gb (residues 51−240), and PP1Gc (residues 241−360) were produced as GST fusion proteins which are depicted in the bottom portion of the figure. Residues 141−240 of PP1G contain only one putative helix (E) and only appear in the fusion protein that bound both PP1C and glycogen.

Western Blot and FluorImage Assays. For immunoblotting after SDS−PAGE, the gel was subjected to overnight transfer to a nitrocellulose membrane in a buffer containing 25 mM Tris-base, 192 mM glycine, and 20% methanol. The nitrocellulose membrane was air-dried and viewed after Ponceau S staining, and then washed with TBST buffer (25 mM Tris, pH 7.5, 0.15 M NaCl, and 0.1% Tween 20). After being blocked in TBST containing 0.1% each bovine serum albumin and chicken egg albumin for 2 h at room temperature, the membrane was incubated with the desired antibody for 2 h and washed 5 times, 5 min each, with TBST. The membrane was then incubated with a HRP-conjugated secondary antibody (Pierce Chemical Co., Rockford, IL) and subjected to ECL reaction, followed by exposure to Fuji-RX X-ray film and film development. For FluorImage analysis, proteins were transferred to a PVDF membrane. After blocking and subsequent blotting with primary antibodies as described, the membrane was incubated with fluorescein-linked anti-species antibody according to the protocol of the kit. The membrane was then incubated in anti-fluorescein antibody conjugated to alkaline phosphatase and developed with the AttoPhos substrate. The filter was then subjected to scanning on a Molecular Dynamics FluorImager for data analysis using ImageQuant software.

RESULTS

Expression of GST−PP1G Fusion Proteins. Our initial objective was to express and purify a GST fusion protein containing the entire 40 kDa N-terminus of PP1G (residues 1−360) and to analyze its binding to PP1C and glycogen. Using RT-PCR with rabbit skeletal muscle RNA, a DNA fragment of *ca.* 1 kb was amplified and subcloned into pGEX-4T-2. However, expression in *E. coli* and purification by glutathione−Agarose affinity chromatography yielded a protein of only 30 kDa by SDS−PAGE, essentially the same size as GST. No protein near the expected size of 68 kDa was detected, even with different cell lysis protocols and inclusion of multiple protease inhibitors in the extractions. As an alternative, the DNA recovered with primers for residues 1−360 was used as template with three additional primers designed to amplify 1−140, 1−240, and 241−360 fragments. These were subcloned in pGEX-4T-2 and expressed as separate GST fusion proteins, called GST−PP1Ga, GST−PP1Gb, GST−PP1Gc (Figure 1). When purified by glutathione−Agarose affinity chromatography and analyzed by SDS−PAGE, the apparent sizes of GST−PP1Ga, -b, and -c were 40, 49, and 45 kDa, respectively

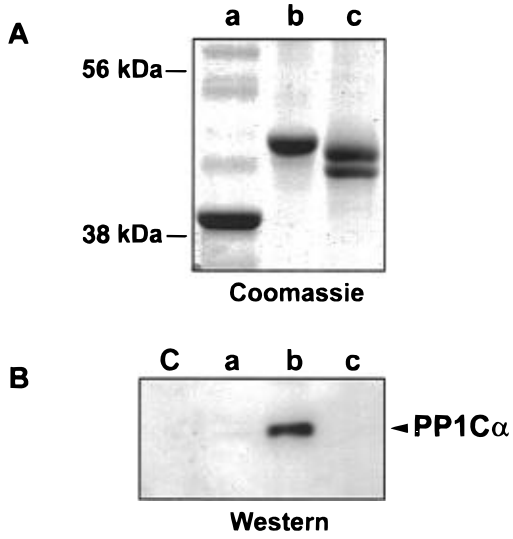

FIGURE 2: GST−PP1G fusion proteins and binding of PP1C. Different regions of the N-terminal of PP1G were expressed as GST fusion proteins: GST−PP1Ga, GST−PP1Gb, and GST−PP1Gc (see Figure 1). Panel A: The fusion proteins were purified by affinity chromatography, and 4 μg of each was subjected to SDS−PAGE and Coomassie staining (panel A). Their apparent molecular sizes were calculated based on migration relative to prestained standards (shown on the left): lane a, GST−PP1Ga, 40 kDa; lane b, GST−PP1Gb, 49 kDa; lane c, GST−PP1Gc, 45 kDa. Panel B: Different GST fusion proteins (1.5 μg) were bound to glutathione−Agarose beads and were incubated with 750 μL of 3T3 cell lysate plus 350 μL of binding buffer. PP1C was detected by Western blotting with ECL detection. The fusion proteins used were GST−PP1Ga (lane a), GST−PP1Gb (lane b), and GST−PP1Gc (lane c). GST alone was included as an additional negative control (lane C). Reblotting of the filter with anti-GST antibody showed the same amount of fusion protein present in each lane (not shown).

(Figure 2A), compared to the expected sizes of 43, 54, and 41 kDa. There were two proteins in the GST−PP1Gc preparation, presumably because of limited proteolysis of the C-terminus. The basis for GST−PP1Ga and GST−PP1Gb both being smaller than expected was discovered in the course of confirming the DNA sequence of the inserts in the pGEX-4T-2 vector. Instead of fusion with the N-terminus of PP1G, the GST fortuitously was fused in-frame starting at residue 51 of PP1G, so GST−PP1Ga and GST−PP1Gb contained residues 51−140 and 51−240.

Binding of GST−PP1Gb to PP1C and Glycogen. The GST−PP1G fusion proteins bound to glutathione−Agarose beads were incubated with 3T3 cell lysates. After washing and elution, Western blot analysis using anti-PP1Cα antibody showed that PP1C was bound only to GST−PP1Gb (Figure 2B). This result showed that fusion of a segment of PP1G containing residues 51−240 to GST yielded a functional protein capable of binding PP1C from a cell lysate. Protein staining of the samples revealed the fusion protein itself as the major component, indicating there was a low level of nonspecific protein adsorption. Neither GST alone nor fusion proteins containing residues 51−140 or 241−360 of PP1G bound detectable amounts of PP1C (Figure 2B). The results were replicated several times with different lysates. The other fusion proteins probably lack the sequences necessary for binding PP1C. Binding was specific, for in the GST−PP1Gb samples no PP2A was detected by Western blotting (not shown), even though it is closely related in sequence to PP1C and binds many of the same inhibitors.

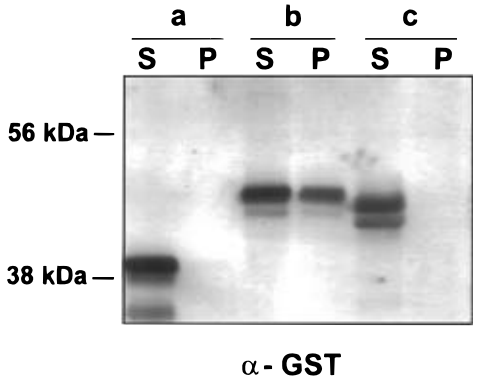

FIGURE 3: Sedimentation of GST−PP1G proteins with glycogen. Different GST−PP1G proteins were purified and incubated in a 2% glycogen solution. Following centrifugation at 100000*g*, of the supernatant (S) and resuspended pellet fractions (P) were subjected to Western blotting with an anti-GST antibody and FluorImage detection. The GST−PP1Ga, GST−PP1Gb, and GST−PP1Gc fusion proteins are detected in the lanes labeled a, b, and c, respectively. Only the GST−PP1Gb protein sedimented with glycogen.

The GST−PP1G fusion proteins were also tested for their ability to bind glycogen (Figure 3). The fusion proteins were purified on glutathione−Agarose and eluted with 10 mM glutathione, and 3.0 μg of each was incubated with 2% oyster glycogen in a total volume of 350 μL. After 20 min incubation, the samples were centrifuged at 100000*g* for 25 min in a Beckman Airfuge and the supernatant and pellets analyzed by SDS−PAGE and Western blotting with anti-GST antibodies. The GST−PP1Ga and GST−PP1Gc were recovered entirely in the supernatant fractions, and none was detected with glycogen in the pellets. In contrast, 40% of the GST−PP1Gb fusion protein was sedimented with glycogen in the high-speed pellet. The results were replicated in three independent experiments. From the binding activities of these GST fusion proteins, we propose that the region of PP1G from residues 141−240 binds glycogen as well as PP1C.

Binding of Different PP1C to GST−PP1Gb. We compared the binding of three different preparations of PP1C to GST−PP1Gb. First, PP1C was partially purified from rabbit skeletal muscle extracts by DEAE-Agarose chromatography. Second, PP1C was purified from pig bladder by ethanol precipitation and binding to MCLR−biotin. The complex was recovered using streptavidin−Agarose. The PP1C−MCLR−biotin was eluted and separated from PP2A−MCLR−biotin by ion-exchange chromatography. Third, rabbit PP1Cα was expressed in bacteria and purified as a Mn^{2+}-activated phosphatase. Similar to the results in Figure 2B, none of these PP1C preparations bound to GST alone or to the GST−PP1Ga or GST−PP1Gc fusion proteins. Binding was specific for the GST−PP1Gb protein containing residues 51−240. These results also allow the conclusion that binding was a direct interaction between PP1C and GST−PP1Gb, because the homogeneous proteins were the only components in the binding assay. In the binding assay with equivalent amounts of PP1Cα added (based on Western blotting), the GST−PP1Gb bound a higher proportion of added PP1C−MCLR−biotin complex relative to PP1C partially purified from muscle or recombinant PP1Cα from bacteria (Figure 4). The PP2A catalytic subunit−MCLR−biotin complex did not bind at all to GST−PP1Gb. Fur-

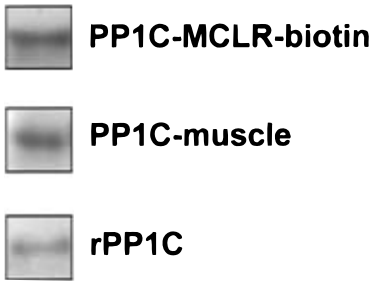

FIGURE 4: Binding of different PP1C preparations to GST−PP1Gb. Different preparations of PP1C described in the text were adjusted to the same PP1Cα concentration and used in a binding assay with the same amount of GST−PP1Gb. The bound PP1C was detected by Western blotting with the FluorImage assay. The amount of PP1C bound was different, with PP1C−MCLR−biotin > PP1C from rabbit muscle ≫ recombinant PP1Cα (rPP1C).

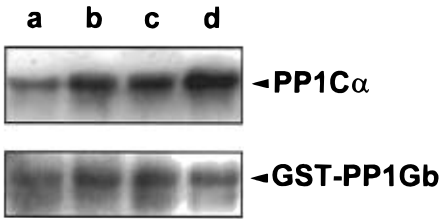

FIGURE 5: Increased ionic strength enhanced binding of GST−PP1Gb and PP1C. Binding of GST−PP1Gb (1.5 μg) to PP1C−MCLR−biotin (1.5 μg) was assayed in buffer containing 0.1 M NaCl (lane a), 0.5 M NaCl (lane b), 1.0 M NaCl (lane c), and 2 M $(NH_4)_2SO_4$ (lane d). PP1C was quantitatively detected by Western blotting in the FluorImage assay, shown in upper panel. Reprobing of the same membrane with an anti-GST antibody showed the same amount of GST−PP1Gb present in the samples (lower panel). The results were replicated in three independent experiments, and similar results were obtained with other PP1C preparations.

thermore, it is interesting that occupancy of the PP1C active site by the MCLR−biotin conjugate did not interfere with interaction between PP1C and the PP1Gb domain. The active site of PP1C must not be occluded by binding to PP1G because the heterodimer actively dephosphorylates proteins and the PP1C−$PP1G_L$ dimer binds to MCLR beads (Moorhead et al., 1994). The relatively poor binding of recombinant PP1Cα isoform to GST−PP1Gb is significant because it indicates the conformation of the Mn^{2+}-activated PP1C is different from native PP1C.

Hydrophobic Interaction Is Involved in the Binding of PP1C to PP1G. To investigate the nature of the protein−protein interactions between PP1C and PP1G, we carried out chemical modification of lysine ϵ-amino groups or carboxylic acid side chains of the GST−PP1Gb bound to glutathione−Agarose (see Materials and Methods). There was no discernible effect of either chemical modification on the binding of PP1C by GST−PP1Gb (not shown). These results, though negative, led us to suspect that ionic interactions might not have a significant role in stabilizing association. As a next step, we examined the effects of ionic strength. The binding buffer was adjusted to 0.1 M, 0.5 M, and 1.0 M NaCl, and the beads were washed with the same buffers. The amount of PP1C bound to GST−PP1Gb increased with increasing salt concentration (Figure 5, upper panel, lanes a, b, and c). As a control for the amount of GST−PP1Gb in the samples, the same filter was reprobed with anti-GST antibodies (Figure 5, lower panel). Increasing the ionic strength even further, by adjusting the solutions to 2.0 M $(NH_4)_2SO_4$, caused a significant further increase in the amount of PP1C bound to the 51−240 PP1G domain (Figure 5, lane d). Increased binding with increased NaCl was observed with homogeneous PP1C−MCLR−biotin (Figure 5) as well as with PP1C in cell lysates or the partially purified PP1C from rabbit skeletal muscle (not shown). The results have been replicated in several independent experiments.

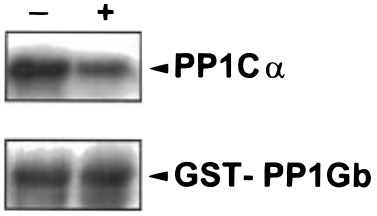

FIGURE 6: Elution of PP1C from GST−PP1Gb by ethylene glycol. Samples of GST−PP1Gb were incubated with purified PP1C in buffer containing 2 M $(NH_4)_2SO_4$. Complexes were recovered and incubated in 25% ethylene glycol (+) or buffer alone (−) for 15 min at room temperature. Western blotting and quantitative FluorImage analysis (upper panel) showed that 50% of the PP1C was eluted by ethylene glycol. Reprobing with an anti-GST antibody (lower panel) showed the same amount of GST−PP1Gb in both samples.

These results suggested that binding involved hydrophobic interactions between PP1C and the 51−240 domain of PP1G. Parallel samples of the PP1C complex with GST−PP1Gb were formed in the presence of 2.0 M $(NH_4)_2SO_4$, and the beads were recovered by centrifugation. One sample was incubated for 15 min in binding buffer without NaCl. The other sample was incubated in the same buffer, except with 25% (v/v) ethylene glycol. The samples were assayed for bound PP1Cα, and we found that addition of ethylene glycol resulted in elution of about 50% of the PP1C bound to GST−PP1Gb (Figure 6, upper panel). Blotting of the same filter with anti-GST showed that the amount of fusion protein was about the same in both samples (Figure 6, lower panel). This demonstrated that ethylene glycol eluted PP1C from GST−PP1Gb, and did not simply elute the fusion protein from the glutathione−Agarose beads. Additional evidence for a hydrophobic interaction was obtained by comparing binding at 4 and 24 °C. There was up to a 2-fold increase in the binding of PP1C from cell lysates but no difference in binding of the purified PP1C (not shown).

Phosphorylation of PP1Gb Eliminates Binding of PP1C. It is known from previous work that PP1G is phosphorylated by PKA on Ser48 and Ser67. The Ser67 phosphorylation has been reported to disrupt the binding of PP1C to PP1G (Hubbard & Cohen, 1989). We phosphorylated GST−PP1Gb with purified PKA, and as controls incubated parallel samples with buffer alone or with reaction buffer containing MgATP, without PKA. The GST−PP1Gb was phosphorylated by PKA, as determined by incorporation of ^{32}P. The stoichiometry was calculated as 1.7 and 2.2 mol of ^{32}P/mol of protein in separate experiments. It should be noted that GST−PP1Gb fusion included only residues 51−240, so Ser67 was the only expected site for phosphorylation by PKA. The location of other site(s) is not known. The phosphorylation of GST−PP1Gb completely prevented the binding of PP1C from cell lysate (Figure 7, top panel). There was no change in PP1C binding to the control samples incubated without PKA. The results reinforced the conclusion that the binding interaction was specific, rather than just indiscriminant hydrophobic association. The filter was

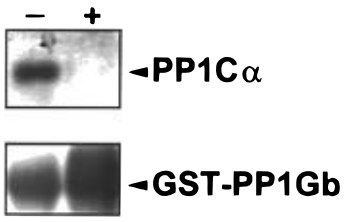

FIGURE 7: Phosphorylation by PKA prevents binding of GST−PP1Gb to PP1C. Binding of PP1C from 3T3 cell lysate was assayed with phosphorylated (+) or unphosphorylated (−) GST fusion. Western blotting with the FluorImage assay showed binding to the unphosphorylated, not the phosphorylated, GST−PP1Gb (upper panel). Reprobing of the filter with an anti-GST antibody showed the amount of GST−PP1Gb in the two samples. The result was replicated in an independent experiment, with a different preparation of GST−PP1Gb.

reprobed with anti-GST to show the amount of fusion protein in the binding assay (Figure 7, bottom panel). In the experiment shown, there was no PP1C binding detected, even though there was significantly more phosphorylated than unphosphorylated fusion protein in the binding assays.

DISCUSSION

Our results map PP1C and glycogen binding sites to a 100 residue region of PP1G. The GST fusion proteins of different regions of the N-terminal third of PP1G were expressed and used *in vitro* to bind PP1C in an assay using cell lysates or various preparations of PP1C. The association with PP1C was observed for GST−PP1Gb (residues 51−240) but not for GST alone or GST−PP1Ga (residues 51−140) and GST−PP1Gc (residues 241−360). We conclude that a site necessary for stable association of PP1C with PP1G lies between residues 141 and 240, and binding involved a direct protein−protein interaction between PP1C and PP1G. The same region of PP1G (residues 141−240) has the binding site for glycogen as well, shown by selective cosedimentation.

It is interesting to note that recombinant PP1C purified from bacteria showed less binding compared with PP1C proteins purified from tissues or PP1C in cell lysates. It has been suggested that the conformation of recombinant PP1C differs from the native protein (Egloff et al., 1995). There may also be reduced binding to PP1G because the recombinant PP1C protein lacks phosphorylation of Ser and/or Tyr residues. The phosphorylation of PP1C on Ser and Tyr was observed in v-src transformed *vs* parental 3T3 cells (Villa-Moruzzi & Puntoni, 1996). We observed that GST−PP1Gb binds more PP1C from lysates of v-src transformed cells, compared to lysates of parental 10T1/2 cells, when the same amount of total protein was used in the assays (unpublished observation). Thus, phosphorylation of PP1C is a potential mechanism that might increase binding of PP1C to PP1G. Increasing evidence has shown that phosphorylation of PP1C regulates its function, including substrate selection (Belandia et al., 1994), enzyme activity (Dohadwala et al., 1994), and cellular distribution (Villa-Moruzzi & Puntoni, 1996).

Our results are consistent with a hydrophobic protein−protein interaction between PP1G and PP1C. Indeed, there was enhanced binding even up to 2 M $(NH_4)_2SO_4$, which provided an ionic strength equivalent to 5 M NaCl. This seems opposite from first reports of properties of the PP1G−PP1C dimer, which was dissociated in 1 M NaCl (Stralfors et al., 1985). However, the conditions reported also specified 20 h incubation at <0.2 mg/mL at 4 °C, and these may have been more important than the ionic strength. Another PP1C interacting protein called p53BP2 (Helps et al., 1995) formed a complex with PP1C that was stable in 1 M NaCl. In contrast, p53 binding to p53BP2 was disrupted at 0.5 M NaCl or higher. An interesting possibility is that PP1G and p53PB2 bind the same hydrophobic site on PP1C.

Targeting of PKA by an anchoring protein involves binding of the R_{II} regulatory subunit with nanomolar affinity via an amphipathic helix (Coghlan et al., 1995), and we therefore originally suspected a helix in PP1G as a potential site for binding PP1C. The N-terminal domain of PP1G (residues 1−360) contains nine regions of putative α helix (see Figure 1), as predicted by the Chou−Fasman algorithm for secondary structure (Chou & Fasman, 1978). However, only one helix (E) lies in the region 141−240, at residues 146−169. Searching the protein database with the sequence of helix E using the BLASTp algorithm (Altshul et al., 1990), we uncovered a motif conserved in the four PP1G proteins known to date (human and rabbit PP1G, yeast GAC1 protein, and liver $PP1G_L$, which is only 33 kDa). The motif is characterized by a conserved FEK sequence, with a G residue N-terminal and a W residue C-terminal with the spacing: $Gx_8FEKx_{10}W$. Another segment of conserved residues within the 141−240 region of PP1G is at residues 188−196. This has a sequence motif DxFxFxIxL that is predicted to have a β sheet structure. We propose that these conserved motifs are sites for PP1C and glycogen binding, respectively. A point mutation in yeast PP1C changed R to C at position 75 (mammalian PP1C numbering) and prevented association with GAC1, the yeast PP1G homologue. From the 3D structure, R75 lies in the middle of helix B which has the sequence ^{69}QYYDLLRLFEY^{79} and is located on the "upper back" of the PP1C when facing the active site (Goldberg et al., 1995). We speculate that binding of PP1C to PP1G involves association of the hydrophobic faces of helix B in PP1C and helix E at 146−169 in PP1G.

It has been proposed that phosphorylation of PP1G has an important role in regulating the binding to PP1C *in vivo* (Dent et al., 1990). Experiments with yeast also suggested that PP1C might preferentially associate with un- or under-phosphorylated forms of GAC1 protein (Stuart et al., 1994). In our experiments, the phosphorylation of PP1G by PKA probably was at site 2 (Ser67), because site 1 (Ser48) was missing in the fusion protein. The other PKA phosphorylation site in GST−PP1Gb is not known, and sequence analysis does not predict other sites for PKA. It seems that phosphorylation prevented association between PP1C and residues 141−240 of PP1G. How this happens is still an unanswered question.

The PP1C−PP1G heterodimer is involved in the regulation of glycogen synthesis by activation of glycogen synthase, the rate-limiting enzyme in glycogen deposition in muscle and liver. We might expect some patients with non-insulin-dependent diabetes mellitus (NIDDM) to carry PP1G mutations which alter PP1C binding or activity of the heterodimer. Analysis of cDNA from 30 insulin-resistant NIDDM patients showed a C to A transversion at nucleotide 2792 that caused an alanine to glutamic acid substitution (Chen et al., 1994). However, whether this point mutation affects association of PP1G and PP1C in NIDDM patients has not been reported, and this mutation seems far removed in the sequence from the region implicated here.

The mechanisms for PP1C to regulate its interactions with a variety of different regulatory/targeting subunits might be more complicated than first thought. It seems likely that different proteins bind to different surface regions of PP1C. This seems the case at least for yeast PP1C, where point mutations in the yeast PP1C (called GLC7) interfere with binding to different partners. The *glc7-R73C* mutant protein does not bind *GAC1* protein (Stuart et al., 1994). The *glc7-T152K* mutant protein shows reduced binding with the *REG1* protein (Tu & Carlson, 1995). Some questions raised by the targeting hypothesis are how PP1C selects binding among different proteins, and once it binds to one protein, whether it still has the ability to bind other regulatory proteins. Experiments to answer these questions would help us understand more about the mechanisms to regulate the cellular functions of PP1.

After this manuscript was completed and reviewed, we found a report (Johnson et al, 1996) that assigned a PP1C interaction site in PP1G to the N-terminal 118 residues. Using a Far Western analysis against GST−PP1G fusion proteins with digoxygenin-labeled PP1Cγ as probe, residues 2−118 and 2−243, but not 100−350, showed an interaction. In the Far Western assay, the binding of a hydrophobic segment to the filter, or the necessary renaturation of the fusion protein, or even the modification of the PP1Cγ could have contributed to the lack of binding between PP1C and GST−PP1G(100−350). A peptide corresponding to conserved residues 63−93 enhanced phosphorylase phosphatase activity of PP1C−PP1G$_L$ in a dose-dependent manner, but without causing dissociation of PP1C. The authors propose that besides 60−93 there might be another site in PP1G for binding PP1C, but it was not identified. We did not see binding of PP1C to GST−PP1Ga (residues 51−141) perhaps because this region by itself does not afford stable association. The GST−PP1Gb (residues 51−240) that did bind PP1C could contain two separate sites that contact PP1C. An inviting synthesis of results is a two-site model for PP1C binding to PP1G: an interaction with the 63−93 region that is disrupted by phosphorylation of Ser67, as proposed by Johnson et al., in conjugation with binding to the 146−169 hydrophobic helix region as we suggest here.

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