A conserved domain for glycogen binding in protein phosphatase-1 targeting subunits

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Abstract The skeletal muscle glycogen-binding subunit (G_M) of protein phosphatase-1 (PP1) is the founding member of a family of proteins that tether the PP1 catalytic subunit (PP1C) to glycogen and promote the dephosphorylation of glycogen synthase. A hydrophobic sequence (called here the VFV motif) is conserved among G_M, the liver subunit G_L, and the widely expressed subunits, PTG, R5 and U5. This study analyzed the role of this VFV motif in binding to glycogen and PP1C. Glutathione *S*-transferase (GST) fusions with the N-terminal domain of G_M (GST-$G_{M(1–240)}$) and with the full length R5 protein (GST-R5) both bound to glycogen in a co-sedimentation assay. In contrast, GST itself did not bind to glycogen. A single residue substitution in GST-$G_{M(1–240)}$, F155A, reduced glycogen binding by 40%. Double residue substitutions V150A/F155A and F155A/V159A resulted in greater reductions (60–70%) in glycogen binding, showing these hydrophobic residues influenced the protein-glycogen interaction. The wild type and V150A/F155A fusion proteins were digested by trypsin into the same sized fragments at the same rate. Furthermore, the wild type and mutated GST-G_M proteins as well as GST-R5 bound equivalent amounts of PP1C, in either pull-down or far-Western assays. These results demonstrated retention of overall tertiary structure by the mutated fusion proteins, and indicated that glycogen and PP1C binding are independent of one another. A 68 residue segment of R5 encompassing the VFV motif was sufficient to produce glycogen binding when fused to GST. This motif, that is in bacterial and fungal starch metabolizing enzymes, probably has been conserved during evolution as a functional domain for binding glycogen and starch.

Key words: Protein sequence; Evolution; Hydrophobic residue; Starch binding

1. Introduction

The original physiological function described for protein phosphatase-1 (PP1) was the regulation of glycogen metabolism in skeletal muscle [1]. A glycogen-bound form of PP1 dephosphorylates glycogen synthase, phosphorylase kinase and phosphorylase to coordinate glycogen synthesis and breakdown. This PP1 was purified from rabbit skeletal muscle [2] as a heterodimer of the PP1 catalytic subunit (PP1C) with a 124 kDa glycogen binding subunit, termed G_M or R_{GL} [3]. The G_M subunit bound PP1C, associated with glycogen, and enhanced the dephosphorylation of glycogen synthase, even in the absence of glycogen.

Since the discovery of G_M, several additional members of this protein family have been discovered. Among these are the yeast homolog, GAC1 [4], and a liver protein, G_L [5], which show significant sequence homology with the N-terminal region of G_M. Search of expressed sequence tagged (EST) cDNA database identified PPP1R5 [6] (called here R5), which is nearly identical to PTG, a protein [7] cloned in a 2-hybrid screen using PP1C as the bait. A 2-hybrid screen of a chicken gizzard cDNA library identified yet another PP1 binding protein, U5, whose mRNA is expressed in smooth muscle, brain and ovary but not in skeletal muscle, heart or liver [8]. The sequence homology among these proteins and G_M suggests that they all represent glycogen targeting subunits of PP1.

In addition to association with PP1C and glycogen, the G_M homolog, PTG, functions as a scaffold and binds the PP1C substrates, glycogen synthase, phosphorylase and phosphorylase kinase [9]. This raises the possibility that other members of PP1 glycogen targeting subunit family also fulfill a scaffolding role to bring together PP1C and its substrates.

Co-crystallization of a synthetic decapeptide, residues 65–75 of G_M with PP1C [10], suggested that the R-V-S-F residues in G_M are involved directly in binding to PP1C. Interestingly, G_M (but not other family members) is phosphorylated by cAMP dependent protein kinase (PKA) on Ser^{67} which prevents association with PP1C [11]. Using PP1C to screen a phage display library yielded peptides resembling this 65–75 segment of G_M, supporting its identification as the primary site for association with PP1C [12]. A second site for PP1C association exists within residues 141–240 in G_M [13] but the specific residues involved have not been identified. Association with glycogen also mapped to the same 141–240 region in G_M [13]. This study analyzes the role of conserved hydrophobic residues in the 141–240 region of G_M for binding to PP1C and to glycogen.

Alignment of sequences of G_M, G_L, PTG, GAC1 and R5 drew our attention to a highly conserved segment in the 140–240 region of G_M, with the sequence, G-x_3-V-x-N-x_2-F/Y-E-K-x-V/L-x-V/I-R/K-x-S/T-x_3-W (see Fig. 1). In particular, we analyzed Ala substitution of residues in this sequence and found that substitutions of Val and Phe impaired glycogen binding, but did not alter PP1C binding. These results provide the first functional identification of a glycogen binding motif

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Abbreviations: G_M, the skeletal muscle glycogen targeting subunit for PP1C (also called R_{GL}); G_L, the liver glycogen targeting subunit for PP1C; PTG, protein targeting to glycogen subunit of PP1C; R5 and U5, targeting subunits of PP1C; PP1Cα, the α isoform of the type-1 protein phosphatase catalytic subunit; GST, glutathione *S*-transferase; GST-$G_{M(1–240)}$, a fusion of GST to the first 240 residues of the G_M subunit; GST-R5, a fusion of GST to full length R5; GST-$VFV_{(156–223)}$, a fusion of GST to residues 156–223 of R5

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in the G_M family of PP1C targeting subunits, a domain that seems to be conserved in enzymes across species.

2. Materials and methods

2.1. Cloning and mutagenesis

Total RNA was isolated from rabbit skeletal muscle tissue and cDNA encoding $PP1G_{M(1-240)}$ was cloned by RT-PCR as described [13] using 5′-CGC GGA TCC GAG CCT TCT GAA GTA CCT GGT CAG-3′ as forward and 5′-CGG AAT TCC TGG CTC AGG TTC CTT CTT-3′ as reverse primers. PCR product was subcloned into *Bam*HI and *Eco*RI sites of pGEX vectors and sequence was confirmed by automated DNA sequencing. Forward oligo CGC GGA TCC TCT GCC TTA AAA CAC CAC GAG and reverse CGG AAT TCC GGG TAA GTC AAT GGC AAA TGA GAA G were used for subcloning of a cDNA fragment that encodes the VFV motif in R5(156–223) fragment using the w19091 EST sequence as template (Research Genetics). PCR fragment was ligated into *Bam*HI and *Eco*RI sites of pGEX-4T2 vector. Mutations of $G_{M(1-240)}$ shown in Fig. 1 were created using oligos by PCR methods described elsewhere [13]. Mutations were all confirmed by double-stranded DNA sequencing.

2.2. Preparation of glycogen

A solution of type II oyster glycogen (Sigma) in water (200 ml of 5% (w/w)) was brought to the boil and cooled to room temperature. About 15 ml of Dowex MR3 was added. The solution was stirred occasionally for 20 min, decanted and mixed with 100 ml of ice cold 95% ethanol. The cloudy solution was centrifuged at 4°C at $5000\times g$ for 10 min and the supernatant discarded. The pellet was resuspended in 100 ml water and centrifuged at $300\,000\times g$ for 5 h. There were three layers formed by ultracentrifugation: the topmost, least viscous fraction of colorless liquid, a second fraction of dilute, golden colored glycogen, and the third, most viscous fraction (F3), that typically formed a honey-colored pellet which was collected and stored at 4°C.

2.3. Binding of GST fusion proteins to glycogen

Binding to glycogen was assayed by a modification of a published method [13]. The purified F3 glycogen was suspended in binding buffer and mixed with 2 μg of GST fusion protein with constant shaking for 90 min at 4°C in binding buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 150 mM NaCl, 1 mM EGTA). Proteins bound to glycogen were isolated by centrifugation in a Beckman Airfuge at $178\,000\times g$ for 90 min. Following centrifugation and collection of the supernatant, the pellet was washed immediately with two volumes of 100 μl of ice cold PBS. Both the supernatant and pellet were suspended in SDS-PAGE sample buffer and stored at 4°C until the pellet was dissolved, then were analyzed by SDS-PAGE and Coomassie staining. The GST fusion proteins were quantified by densitometry using ImageQuant software (Molecular Dynamics). The fraction of different fusion proteins bound to glycogen was calculated and normalized to the fraction of GST-$G_{M(1-240)}$ binding to glycogen.

2.4. Comparison of wild type and Ala substituted fusion protein conformation

Circular dichroic (CD) spectrometry measurements were performed essentially according to Shoelson {1208} using a Jasco J72 CD spectrophotometer. Fusion proteins were eluted with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 10 mM glutathione. Samples were dialyzed against 20 mM Tris-HCl, pH 7.0 for 24 h. Spectra in the ultraviolet from 200 to 250 nm were recorded at 4°C. Individual data points collected at various wavelengths for 10 s were plotted to give a spectrum. The spectra were offset by 2000 theta units to allow side by side comparison. For trypsin digestion GST fusion proteins were eluted from glutathione-Sepharose beads and 3 μg of wild type and V150A/F155A mutant proteins were resuspended in 20 μl of 20 mM Tris-HCl, pH 7.5, and 10 μl of 3 μg/ml trypsin in the same buffer was added to each sample at room temperature. Proteolysis was stopped

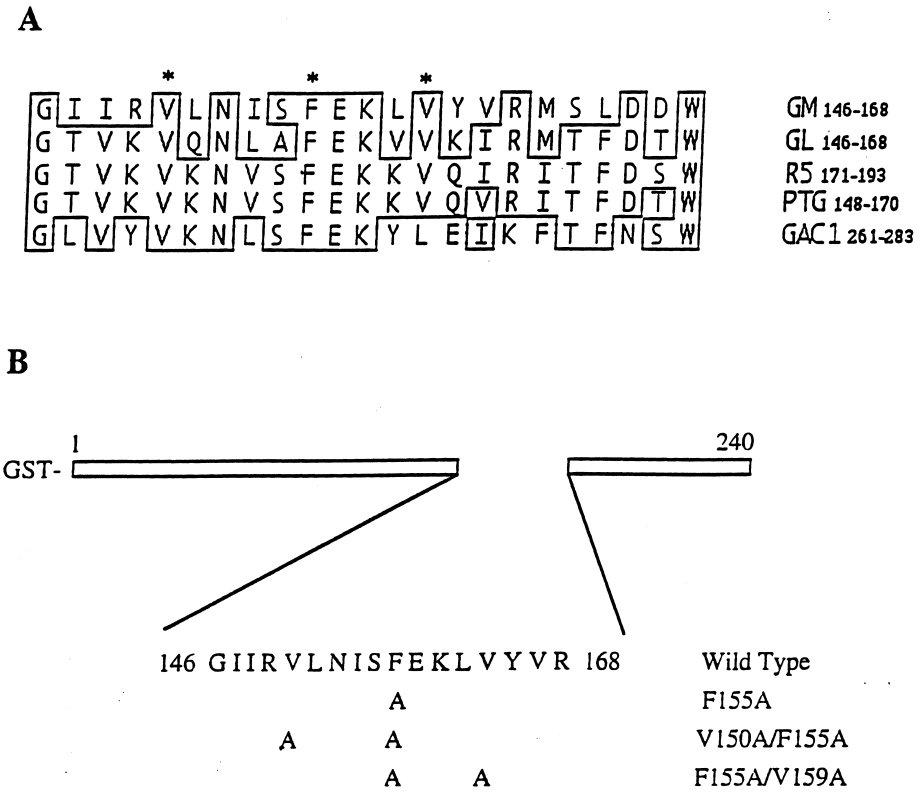

Fig. 1. Conserved sequences in the PP1C glycogen targeting subunits. A: Sequences from selected glycogen targeting subunits were aligned using the Clustal method. This alignment highlights the VFV motif conserved in glycogen targeting subunits from skeletal muscle (G_M), liver (G_L), and other tissues (R5 and PTG) as well the yeast homolog, GAC1. Subscript numbers indicate sequence positions in each protein. Identical residues are boxed, and the asterisks indicate the residues chosen for mutational analysis (see B). B: Single and double Ala substitutions in $PP1G_{M(1-240)}$ that were tested in this study.

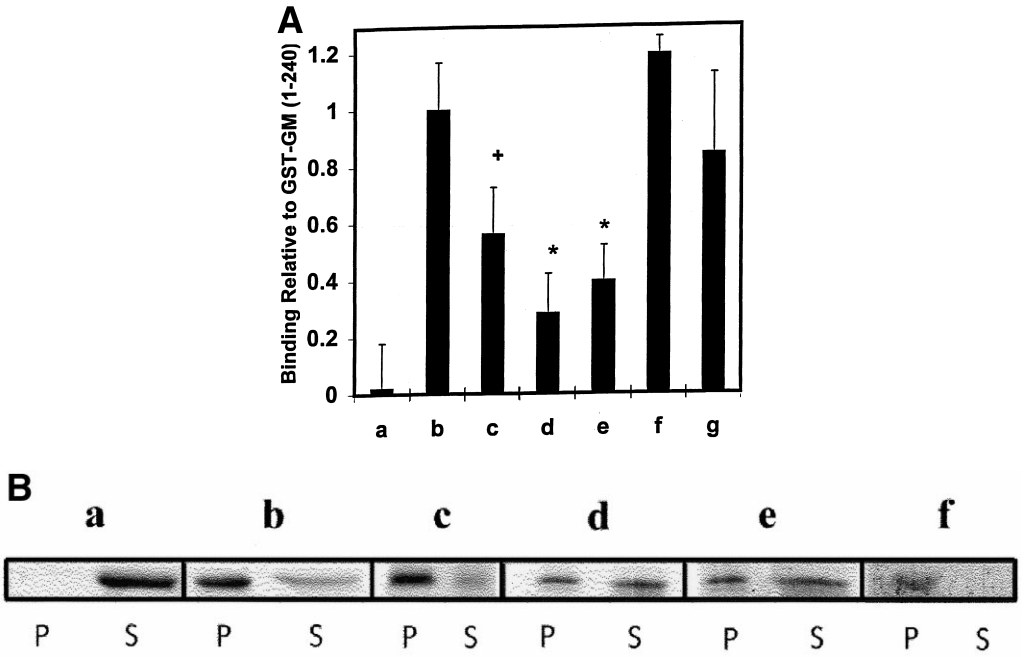

Fig. 2. Glycogen binding activity of GST fusion proteins: A. Using a co-sedimentation assay the following proteins were tested for association with glycogen, as described in Section 2: a, GST ($n=6$); b, GST-$G_{M(1-240)}$ ($n=9$) (defined as = 1.0); c, GST-$G_{M(1-240)}$ F155A ($n=9$); d, GST-$G_{M(1-240)}$ V150A/F155A ($n=6$); e, GST-$G_{M(1-240)}$ F155A/V159A ($n=4$); f, GST-VFV domain ($n=2$); g, GST-R5 ($n=5$). The fraction of total protein recovered in the glycogen pellet was normalized to wild type GST-$G_{M(1-240)}$. Values were calculated and plotted as the mean ± S.D., from the number of independent trials indicated, and results compared using Student's *t*-test. The F155A mutant was significantly different from wild type ($^{+}P<0.05$) and the double mutants (d, e) were also significantly different from wild type ($^{*}P<0.001$). The single mutant, F155A, was significantly different from the double mutant, V150A/F155A ($P<0.05$). GST alone was significantly different from all GST fusion proteins in this assay, including both of the double mutants ($P<0.05$). B: Images of Coomassie stained gels corresponding to columns b–f in A.

at different times by adding 15 μl of 3× sample buffer and heating at 95°C. Proteins were separated on 15% SDS-PAGE and visualized by Coomassie blue staining.

For analysis by trypsin digestion GST fusion proteins were eluted from glutathione beads and 3 μg of wild type and V150A/F155A double mutated protein were resuspended in 20 μl of 20 mM Tris-HCl, pH 7.5, and 10 μl of 10 mg/ml trypsin in the same buffer was added to each sample at room temperature. Proteolysis was stopped at different times by adding 7.5 μl of 5× sample buffer and heating at 95°C. Proteins were separated on 15% SDS-PAGE and visualized by Coomassie blue staining.

2.5. PP1C binding by GST fusion proteins

GST fusion proteins were tested for binding in a pull-down assay described previously [13] that used NIH 3T3 cell lysates as the source of PP1C, which was present in excess over the fusion protein. Far-Western experiments were performed according to the protocol of the DTG Protein Labeling Kit (Boehringer Mannheim). Briefly, PP1Cα expressed in bacteria [14] was purified and labeled with digoxigenin-3-*O*-succinyl-ε-amino-caproic acid-*N*-hydroxysuccinimide ester (DIG) and used as probe against GST fusion proteins bound to a nitrocellulose membrane following SDS-PAGE. Binding of DIG-labeled PP1Cα was detected by reaction with alkaline phosphatase-conjugated anti-DIG antibodies, followed by staining with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride.

3. Results

3.1. Glycogen targeting subunits as GST fusion proteins

The N terminal region of the skeletal muscle glycogen targeting subunit (G_M) has been expressed as a functional GST fusion protein [13]. In different glycogen targeting subunits there is a sequence segment with conserved hydrophobic residues Val, Phe, Val, between an invariant Gly and Trp, that is designated here as the VFV motif. To study the functional importance of these hydrophobic sidechains we produced GST fusions with wild type $G_{M(1-240)}$, as well as three alanine substituted forms: F155A, V150A/F155A and F155A/V159A (Fig. 1). In addition, a F192A substitution was made, but very little soluble protein was produced in bacteria, so this protein was not analyzed.

To compare the properties of another member of the glycogen targeting subunit family, the cDNA for R5, cloned by RT-PCR, was inserted into pGEX2T and expressed as GST-R5. A 68 residue fragment from R5 (residues 156–223) that encompasses the VFV motif was expressed as another fusion protein, GST-$VFV_{(156-223)}$. Wild type and mutated GST-$G_{M(1-240)}$, GST-R5, GST-$VFV_{(156-223)}$ and GST itself were purified from bacterial extracts by affinity chromatography on glutathione-agarose as described in Section 2.

3.2. Binding of fusion proteins to glycogen

To assay for glycogen binding to GST fusion proteins, we developed a co-sedimentation assay. Purified GST fusion proteins were incubated with purified oyster glycogen, and the glycogen was sedimented by ultracentrifugation. The fraction of GST fusion protein recovered in the pellet and in the supernatant was determined following SDS-PAGE, Coomassie staining, and densitometry. Fig. 2A shows that 70% of GST-$G_{M(1-240)}$ (column b) and 70% of GST-R5 (column g) bound glycogen with the remaining 30% recovered in the supernatant (see Fig. 2B). Thus, two different members of the G_M family showed the same binding to glycogen, and this was set as 1.0 in the assay. In contrast, GST alone was

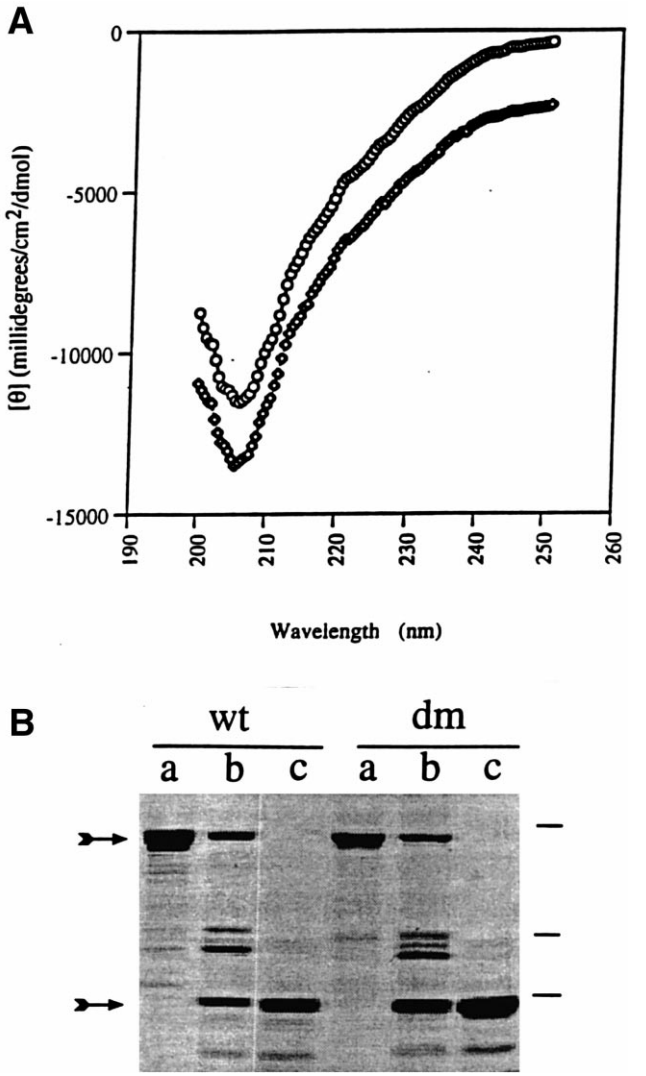

Fig. 3. Comparison of spectra and digestion of wild type and F155A substituted GST-PP1G$_{M(1-240)}$. A: The CD spectra for wild type and F155A fusion proteins were recorded as described in Section 2 and are displayed offset by 2000 theta units to allow side by side comparison. B: Timed digestion by trypsin shows the same pattern of fragmentation for wild type (wt) and double mutant (dm; V150A/F155A) forms of GST-PP1G$_{M(1-240)}$. Experimental conditions are described in Section 2: a, no trypsin; b, 1 min; c, 3 min. Upper arrow, full length GST-PP1G$_{M(1-240)}$ full length; lower arrow, GST. Molecular size standards shown as bars on the right, from top to bottom: 47.7 kDa, 34.6 kDa, and 28.3 kDa.

found almost exclusively in the supernatant fraction. Dilution of the glycogen over a 10-fold range did not have a significant effect on the fraction of protein that co-sedimented. Different preparations of glycogen gave 55–70% of the protein recovered in the pellets, and the relative differences between wild type and mutants were preserved.

Alanine substitutions for hydrophobic sidechains significantly reduced the binding of GST-G$_{M(1-240)}$ to glycogen. Binding of F155A protein was lowered by 40% ($P < 0.05$) compared with wild type GST- G$_{M(1-240)}$ (Fig. 2A, column c). Proteins with double substitutions, V150A/F155A (Fig. 2A, column d) and F155A/V159A (Fig. 2A, column e) showed 60–70% loss of glycogen binding when compared to wild type GST-G$_{M(1-240)}$. Therefore, the VFV hydrophobic residues are needed for binding to glycogen.

Glycogen binding also was examined using the GST-VFV protein. This fusion protein was readily degraded during isolation and storage, presumably by bacterial proteases. We analyzed only freshly prepared GST-VFV$_{(167-223)}$ for binding to glycogen in the co-sedimentation assay. This fusion protein, with only 68 residues of R5, bound to glycogen the same as the full length fusion protein (Fig. 2g). In independent experiments, there was no significant difference in glycogen binding between GST-VFV$_{(167-223)}$, GST-R5, and GST-G$_{M(1-240)}$ (Fig. 2, columns g, f, and c). These data show that the VFV$_{(167-223)}$ domain was sufficient to confer glycogen binding to GST.

3.3. Conformational integrity of mutant and wild type fusion proteins

The Ala substituted fusion proteins retained the same overall conformation as the wild type fusion protein. CD spectra in the near UV of wild type and the F155A fusion proteins were essentially identical (Fig. 3A). The wild type GST-G$_{M(1-240)}$ and the double mutant V150A/F155A proteins yielded the same fragments with the same kinetics during partial digestion with trypsin (Fig. 3B), indicative of similar tertiary structures. In addition, the Ala substituted GST-G$_{M(1-240)}$ were stoichiometrically phosphorylated by PKA

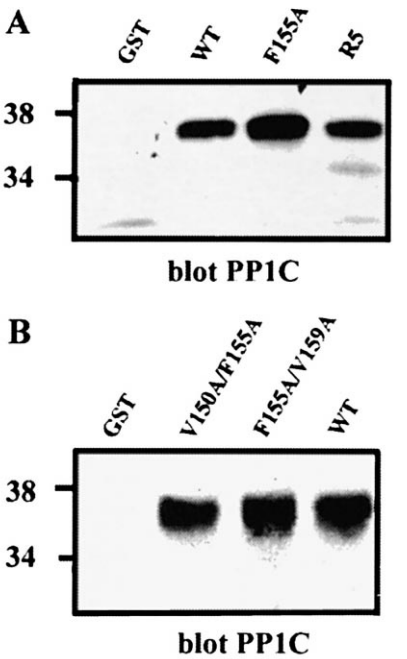

Fig. 4. Binding of PP1Cα to wild type and Ala substituted GST-PP1G$_{M(1-240)}$ and GST-R5. GST fusion proteins immobilized on glutathione-Sepharose were tested for binding of PP1C in a pull-down assay, as described in Section 2, and Western blots for PP1Cα using an anti-peptide antibody are shown. Identical amounts of fusion proteins were immobilized on the filters, as judged by staining with Ponceau S (not shown). A: Binding of PP1C to GST alone as a negative control (GST), wild type GST-PP1G$_{M(1-240)}$ (WT), the F155A single residue mutant of GST-PP1G$_{M(1-240)}$, and GST-R5 (R5). B: Binding of PP1C to GST (another control), to the V150A/F155A and F155A/V159A double mutants of GST-PP1G$_{M(1-240)}$, and to wild type (WT), GST-PP1G$_{M(1-240)}$. Migration of molecular size standards is shown on the left of the panels.

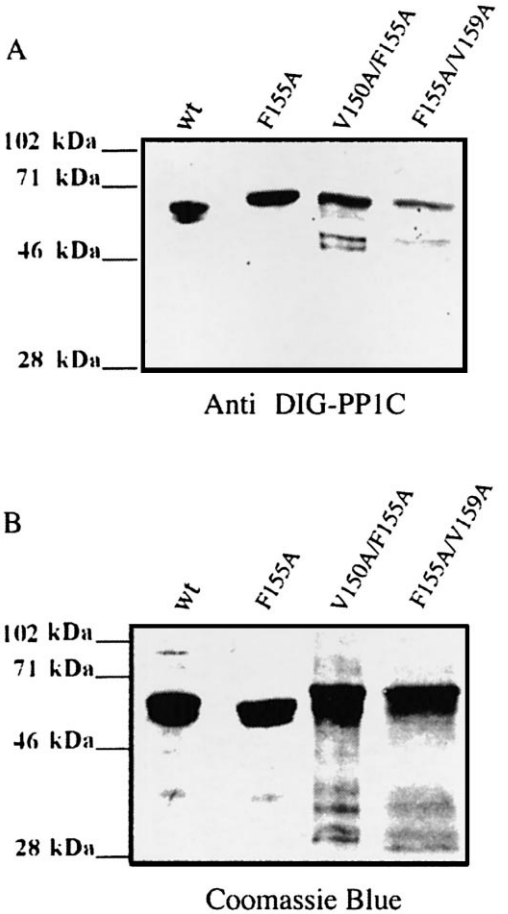

Fig. 5. Far-Western assay of DIG-PP1C binding to GST-PP1G$_{M(1–240)}$ proteins. Purified GST fusion proteins were transferred to filters after SDS-PAGE and probed in a far-Western assay with DIG-PP1Cα, as described in Section 2. A: Immunoblotting with anti-DIG to detect DIG-PP1Cα bound to fusion proteins on the filter. B: Coomassie stain of a parallel gel showing the amount of the different proteins in the assay. There were no significant differences in the amount of DIG-PP1Cα bound per unit of GST fusion protein. Migration of molecular size standards is shown on the left of the panels.

with similar kinetics (not shown) and were digested by trace proteases into similar fragments. The loss of glycogen binding by Ala substitutions could not simply be attributed to loss of tertiary structure of the fusion proteins.

3.4. Binding of PP1C to fusion proteins

The VFV motif lies in a region that has been implicated in binding to PP1C, therefore we analyzed the GST fusion proteins for PP1C binding, using two different assays. GST alone served as a control and did not bind PP1C. One assay (Fig. 4) analyzed the ability of GST fusion proteins immobilized on glutathione beads to bind to an excess of PP1C in a pull-down assay. This assay demonstrated equivalent binding of PP1C to wild type GST-G$_{M(1–240)}$ and GST-R5 (Fig. 4A, lanes 2 and 4). None of the single or double Ala substitutions, F155A, V150A/F155A and F155A/V159A, had any influence on PP1C binding in this assay (Fig. 4B). The same amount of purified fusion protein was used in each case, and this was confirmed by Coomassie staining following SDS-PAGE.

The second assay (Fig. 5) used a far-Western protocol in which the GST fusion proteins were transferred onto nitrocellulose filters and incubated with DIG-conjugated PP1C. PP1C binding was then detected using anti-DIG antibody and peroxidase-linked secondary antibody. Wild type GST-G$_{M(1–240)}$ was analyzed alongside the single and double Ala substitutions in the VFV motif and showed no significant difference in PP1C binding. These results indicate that the VFV motif is not required for PP1C binding to glycogen targeting subunits.

4. Discussion

Isolation and characterization of G$_M$, the glycogen targeting subunit of PP1 from rabbit skeletal muscle, established a new paradigm for the regulation of the phosphatase. G$_M$ mediated the subcellular localization of PP1C on the glycogen granules and also restricted its broad in vitro substrate reactivity. The enzymes involved in glycogen synthesis and breakdown became preferred substrates for the G$_M$:PP1C dimer. Subsequently a number of proteins related in sequence to G$_M$ have been identified, making a family of glycogen targeting subunits for PP1C. Some subunits show tissue specific expression, such as G$_M$ and G$_L$, but various tissues show expression of multiple subunits. Curiously, of the known family members only the G$_M$ protein is phosphorylated, and phosphorylation of Ser^{67} by PKA eliminates PP1C binding, identifying at least one site of interaction.

Members of this family all presumably have sites for interaction with PP1C, with glycogen, and with PP1C substrates, but these sites are not yet fully delineated. Deletion analysis of G$_M$ defined the region between residues 140–240 as mediating its binding to glycogen [13]. In this study, we provide experimental evidence that a hydrophobic sequence motif in this 140–240 region is the site for interaction of the G$_M$ family of PP1 targeting subunits with glycogen. The glycogen binding domain was localized to a 68 residue fragment from the R5 subunit, which was sufficient to confer glycogen binding to GST. Though the VFV motif is conserved, overall this segment of R5 shows less than 50% sequence identity with the corresponding region of G$_M$. We expect that the residues identical between G$_M$ and R5 are primarily responsible for glycogen binding. The greatest sequence identity between the glycogen binding subunits, G$_M$, G$_L$, R5, PTG, U5 and yeast GAC1, exists in a 23 residue segment bounded by invariant glycine and tryptophan (see Section 1). Our studies show that the substitution of conserved VFV hydrophobic residues within this segment severely impaired the ability of GST-G$_M$ to bind glycogen. An alternative explanation for the severely reduced binding of the substituted proteins to glycogen is that most of the protein is misfolded and only a small fraction folds in a conformation that binds glycogen. This implies that the hydrophobic residues are not directly binding glycogen, but influence the conformation of the binding site. However, the substituted proteins showed similar CD spectra and trypsin digestion patterns to wild type G$_M$, and bound PP1C similarly, arguing that hydrophobic alanine substitutions at least did not seriously perturb the overall structure.

A sequence that precedes the VFV motif in G$_M$ and R5 shares some homology with two yeast proteins, PIG1 and PIG2 [15], that were identified in a 2-hybrid screen using glycogen synthase as bait. This led to the speculation that the sequences conserved in PIG1 and PIG2 and some glyco-

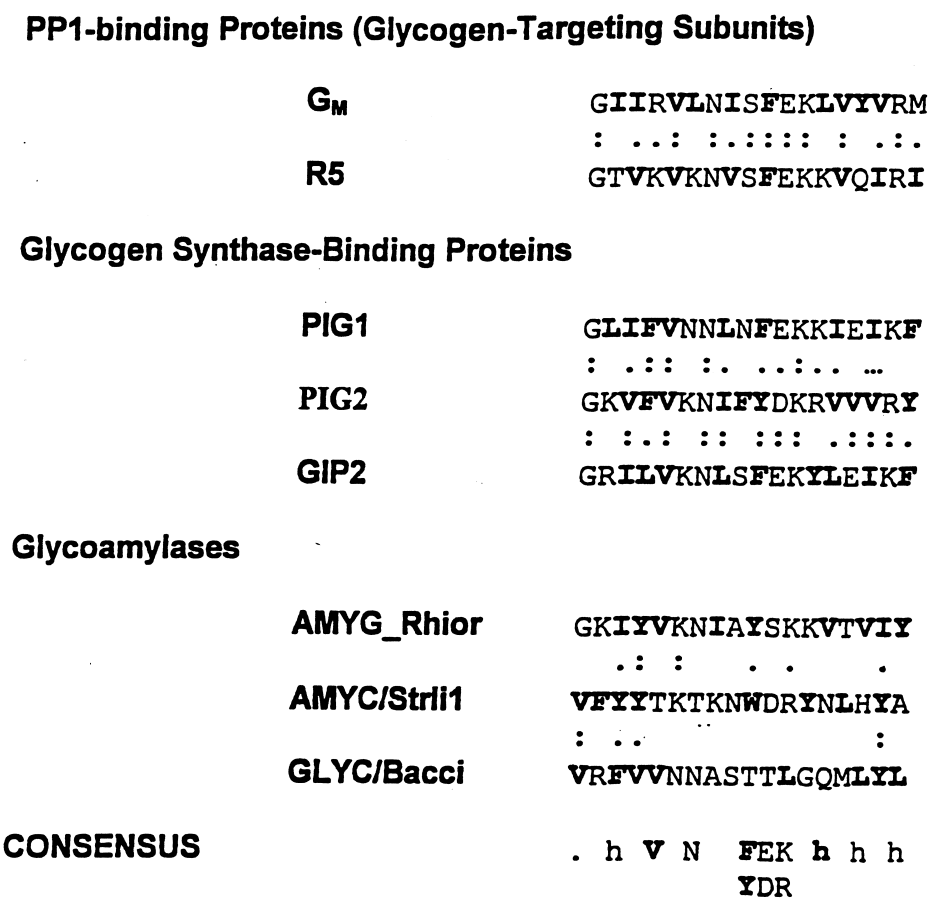

Fig. 6. Alignment of sequences from polysaccharide-binding enzymes and proteins. The VFV motif in G_M and R5 was aligned with the yeast glycogen synthase binding proteins and bacterial glycoamylases [17]. The putative carbohydrate binding domains contain numerous hydrophobic amino acids (in bold) and show some conservation (a colon indicates identity; a dot indicates conservative substitutions) of the residues analyzed in G_M for glycogen binding.

gen targeting subunits of PP1 represent sites for interaction with glycogen synthase. Indeed, the G_M homolog, PTG, binds glycogen synthase [9]. We tested for association of G_M and R5 with glycogen synthase using two distinct assays. However, we observed no measurable binding to skeletal muscle glycogen synthase phosphorylated in vitro with PKA (not shown). Based on these results, we pose an alternative scenario, i.e. that the sequence similar in PIG1 and PIG2, and related to the VFV motif present in the G_M family of proteins, binds to glycogen, which accounts for association with glycogen synthase. One of the PIG1 clones that bound glycogen synthase in the 2-hybrid assay did not contain the specific VFV motif, suggesting other sequences are involved in binding to glycogen synthase [15].

An earlier speculation that the VFV region of G_M, GAC1, and G_L might interact with glycogen was based on some resemblance to the hydrophobic sequence in glycogen phosphorylase that interacts with glycogen [16]. The VFV motif also shows remarkable homology with the starch binding domains of glycoamylases [17] from bacteria and fungi (Fig. 6). Alignment of these sequences shows a high degree of conservation, particularly in the three hydrophobic residues analyzed in this study. This may indicate that the glycogen binding motif present in the PP1C glycogen targeting subunits evolved from a common ancestral domain utilized by metabolic enzymes for binding polyglycans. A recent prediction [17] based on multiple sequence alignments and modeling of 3D structural data emphasizes hydrophilic sidechains and offers a putative glycogen binding domain of about 80–90 residues. Our experimental results define a much smaller segment that is sufficient for glycogen binding, and highlights the importance of at least three hydrophobic sidechains for glycogen binding.

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