Identification of the separate domains in the hepatic glycogen-targeting subunit of protein phosphatase 1 that interact with phosphorylase *a*, glycogen and protein phosphatase 1

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Deletion and mutational analyses of the rat liver glycogen-targeting subunit (G_L) of protein phosphatase 1 (PP1) have identified three separate domains that are responsible for binding of PP1, glycogen and phosphorylase *a*. The glycogen-binding domain spans the centre of G_L between residues 144 and 231 and appears to be distinct from the glycogen-binding (storage) site of phosphorylase. The regulatory high-affinity binding site for phosphorylase *a* lies in the 16 amino acids at the C-terminus of G_L. The PP1-binding domain is deduced to comprise the -RVXF- motif [Egloff, Johnson, Moorhead, Cohen and Barford (1997) EMBO J. **16**, 1876–1887] located at residues 61–64 of G_L and preceding lysine residues at positions 56, 57 and 59. A possible approach for increasing glycogen synthesis in certain disorders is discussed.

INTRODUCTION

Protein phosphatase 1 (PP1) is a major protein serine/threonine phosphatase in eukaryotic cells, which regulates numerous distinct cellular processes. This is achieved by the interaction of the catalytic subunit of PP1 with a diverse range of targeting subunits that localize it to specific sites within the cell, modulate its activity towards particular substrates and allow its activity to respond to extracellular signals [1,2].

The family of proteins that target PP1 to glycogen and regulate its activity towards the enzymes of glycogen metabolism consists of four members, G_M/PPP1R3, G_L/PPP1R4, PPP1R5/PTG and PPP1R6 [3–8]. The liver-specific glycogen-targeting subunit, G_L, is a 33 kDa protein [5,9] that, when bound to PP1, enhances the rate at which the latter dephosphorylates and activates the rate-determining enzyme in glycogen synthesis, glycogen synthase, while suppressing the rate at which it inactivates glycogen phosphorylase. The stimulation of hepatic glycogenolysis by glucagon (acting via cAMP and protein kinase A) and α-adrenergic agonists (acting via Ca^{2+}) is achieved by activation of phosphorylase kinase, which increases the levels of the active phosphorylated form of glycogen phosphorylase (phosphorylase *a*). In addition, phosphorylase *a* binds to G_L and potently inhibits its glycogen synthase phosphatase activity, thereby inhibiting glycogen synthesis. Insulin lowers hepatic cAMP levels, causing a reduction in the level of phosphorylase *a* and alleviation of the phosphorylase *a*-mediated inhibition of the PP1–G_L complex, while the binding of glucose to phosphorylase *a* increases the rate at which phosphorylase is inactivated. These mechanisms contribute to the stimulation of glycogen synthesis by insulin and high blood glucose [10]. The inhibition of the PP1–G_L complex by phosphorylase *a* occurs at nanomolar concentrations and is thought to occur via an allosteric mechanism, since the K_m for phosphorylase *a* as a substrate is in the micromolar range [11]. This view is strengthened by the finding that phosphorylase *a* binds directly to G_L in protein-blotting experiments [5,9].

Recent studies identified conserved regions between the glycogen-targeting subunits G_M/PPP1R3 and G_L/PPP1R4 [5]. A peptide corresponding to one of these regions, G_M 63–75, was shown to bind PP1, and the N-terminal 38 residues of the myofibrillar-targeting subunit of PP1 were also demonstrated to interact with PP1 [12]. The G_M 63–75 peptide, which contains a small motif common to the myofibrillar-binding subunit and many other of the PP1-targeting subunits has been crystallized as a complex with PP1 and its structure solved to 2.8 Å resolution [2]. This motif, Lys/Arg-Val/Ile-Xaa-Phe/Trp, which has also been identified by a random peptide library approach [13], is found in all four glycogen-targeting subunits and is located at residues 60–64 of G_L. However, incubation of the PP1–G_L complex purified from hepatic glycogen–protein particles with a PP1-binding peptide from G_M failed to dissociate the PP1–G_L complex [12], even though the peptide abolished the suppression of phosphorylase phosphatase activity conferred on PP1 by association with G_L. These results suggest that a second PP1-binding site may be present on G_L.

Comparison of all four mammalian glycogen-targeting subunits of PP1 and a fungal glucoamylase identified a conserved region with a potential to be a polysaccharide-binding domain [6,8]. However, no experimental evidence for the binding of glycogen to this domain was available. In this paper, we examine the secondary interactions of PP1 with G_L, identify the domain in G_L that interacts with glycogen and a 16 residue region of G_L that interacts with the allosteric regulator, phosphorylase *a*. Location of these distinct regions in G_L provides molecular insight into the function of this and other glycogen-targeting subunits of PP1.

Abbreviations used: G_L, hepatic glycogen-targeting subunit; GST, glutathione S-transferase; PP1, protein phosphatase 1.

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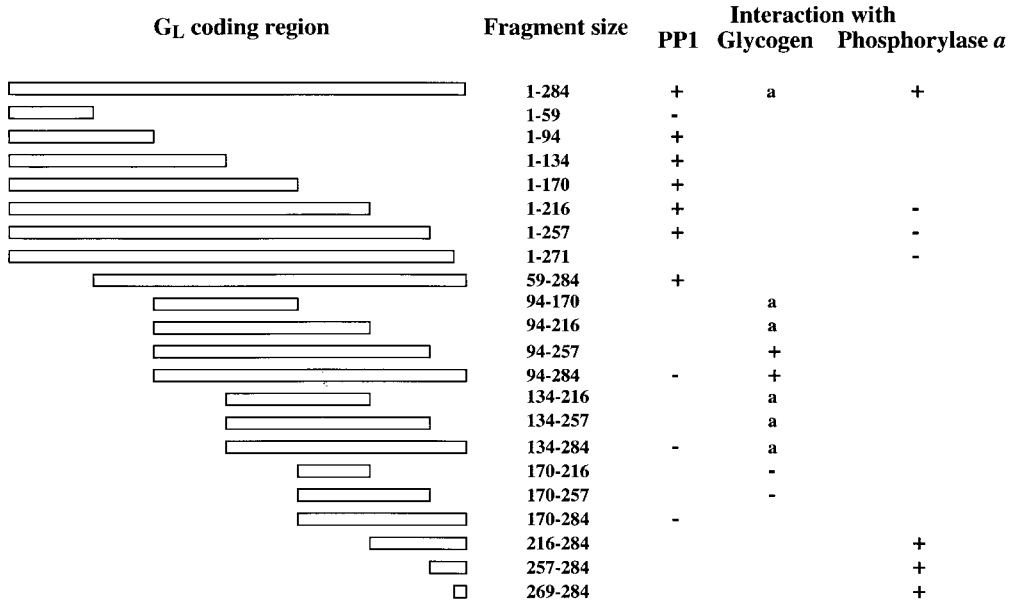

Figure 1 Schematic representation of G_L and truncated forms generated by PCR or restriction digestion

The ability of G_L and the G_L fragments to bind PP1, glycogen and phosphorylase *a* is indicated. + indicates that binding was observed; — denotes that binding was tested but not detected; 'a' indicates that aggregation of G_L and its derivatives precluded testing of glycogen binding; interactions that were not tested are left blank.

MATERIALS AND METHODS

Production of glutathione S-transferase–G_L fusion proteins

The pGEX–G_L construct containing the complete coding region of G_L [5] was used as a template in PCR using primer pairs to generate a variety of G_L coding region fragments (Figure 1). The 5′ coding primers all contained an *Nde*I site and the 3′ reverse primers contained the termination codon and an *Xho*I site, as described in [5]. The PCR products were then subcloned into the TOPO 2.1 PCR cloning vector (Invitrogen, Leek, The Netherlands) and verified by sequencing on an Applied Biosystems 373A automated DNA sequencer using *Taq* dye terminator cycle sequencing. The G_L coding region fragments were subsequently excised by restriction cleavage with *Nde*I and *Xho*I and ligated into the pGEX-AH vector digested with the same restriction enzymes. The G_L 1–94 and 1–170 truncations were generated by excision of a *Sac*I–*Sac*I and a *Hin*dIII–*Hin*dIII restriction fragment respectively from the pGEX–G_L construct, followed by religation of the plasmid. The G_L 94–170 truncation was generated by *Nde*I–*Hin*dIII digestion of pGEX-G_L 94–257 followed by ligation of the fragment into pGEX-AH vector digested with the same enzymes. The G_L 269–284 truncation was generated using complementary oligonucleotides encoding these amino acids, as described in [14], and ligated into the *Nde*I/*Xho*I sites of pGEX-AH. Site-directed mutation of the single mutants N152A, K157A, the double mutant N152A + K157A and the triple mutant K149A + N152A + L153A were generated as described in [15] using pGEX–G_L 94–257 as template (note the one-letter symbols for amino acids are used). The resultant constructs encoded glutathione S-transferase (GST) fused to G_L coding region fragments of various lengths.

Soluble GST–G_L fusion proteins were obtained by growing *Escherichia coli*, transformed with the various pGEX–G_L deletions constructs, in Luria–Bertani medium containing 100 μg/ml ampicillin and inducing expression in exponential-phase growth at an A_{600} of 0.5 with 0.2 mM isopropyl β-D-thiogalactoside. After 16 h further growth at 26 °C–28 °C, the *E. coli* were harvested and soluble GST–G_L fusion proteins were purified as described in [5].

Interaction of GST–G_L fusion proteins with digoxygenin-PP1γ and phosphorylase *a*

GST–G_L fusion proteins were separated on SDS/PAGE and transferred to nitrocellulose membranes. They were probed with digoxygenin-PP1γ as described [8]. Alternatively they were examined for binding of ^{32}P-labelled phosphorylase *a*. Non-specific binding to the membranes was blocked by incubation in 5% (w/v) Marvel dried-milk powder (obtained from Premier Beverages, Stafford, U.K.)/25 mM Tris/HCl, pH 7.5/500 mM NaCl for 16 h. The samples were then probed for 3 h with ^{32}P-labelled phosphorylase *a* (100 nM) in 25 mM Tris/HCl, pH 7.5/250 mM NaCl/1 mg/ml BSA. The membranes were subsequently washed (3 × 30 min) with 25 mM Tris/HCl, pH 7.5, before autoradiography.

Co-sedimentation of GST–G_L fusion proteins with hepatic glycogen

Protein-free glycogen was prepared by Dr G. Moorhead and Mr G. Browne (University of Dundee) according to the following protocol. Glycogen–protein particles were isolated from the livers of New Zealand White rabbits [16]. Protein was then stripped from the glycogen by boiling for 5 min in 1% (w/v) SDS. The suspension was cooled to room temperature and centrifuged for 60 min at 100000 ***g***. The 100000 ***g*** pellet was then resuspended in water, and the centrifugation and resuspension procedure was repeated a further two times in order to completely remove residual SDS and protein. Any contaminating nucleotides were removed by incubating the glycogen for 15 min with mixed-bed resin, AG 501-X8(D). The resin was removed by filtration

and the glycogen concentration was determined by the phenol/sulphuric acid method [17].

Protein-free glycogen (10 mg/ml) in 50 mM Tris/HCl, pH 7.5/150 mM NaCl, 0.1 % (v/v) 2-mercaptoethanol/0.02 % (w/v) Brij-35/0.1 mg/ml BSA was mixed with GST–G_L fusion protein (50 nM). After incubation on ice for 30 min, the samples were centrifuged for 90 min at 100000 ***g***. The supernatant and pellet fractions were denatured in SDS, subjected to SDS/PAGE and transferred to nitrocellulose. The membranes were incubated overnight in 25 mM Tris/HCl, pH 7.5/250 mM NaCl/0.1 % (w/v) Tween-20/10 % dried-milk powder before probing with affinity purified sheep anti-G_L protein antibodies [100 ng/ml in 25 mM Tris/HCl, pH 7.5/250 mM NaCl/0.1 % (w/v) Tween-20/3 % (w/v) dried-milk powder], followed by several washes in the same buffer (without the dried-milk powder) and incubation with horseradish peroxidase-conjugated anti-sheep antibodies (Pierce, U.K.). Immunoreactive bands were visualized using the enhanced chemiluminescence system (Amersham International, Bucks., UK.)

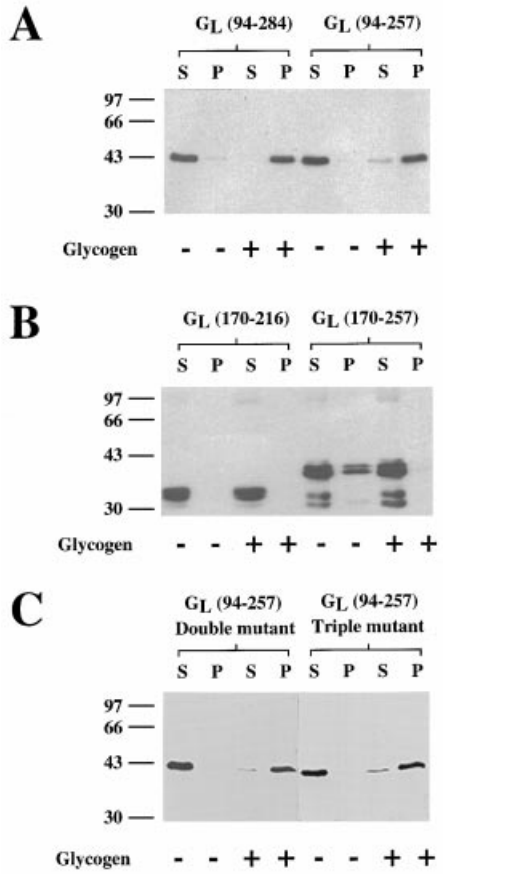

Figure 2 Identification of the region of G_L required for interaction with glycogen

GST–G_L fusion proteins containing truncated G_L coding regions were examined for their ability to co-sediment with glycogen as described in the Materials and methods section. The supernatant (S) and pellet (P) fractions obtained in the absence and presence of glycogen were subjected to SDS/PAGE on 12.5% polyacrylamide gels, transferred to nitrocellulose membranes and immunoblotted with affinity purified anti-G_L protein antibodies. (**A**) G_L truncations 94–284 and 94–257; (**B**) G_L truncations 170–216 and 170–257; (**C**) G_L truncation 94–257 containing the double mutation N152A + K157A and the triple mutation K149A + N152A + L153A. The positions of the standard marker proteins, glycogen phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa), are indicated.

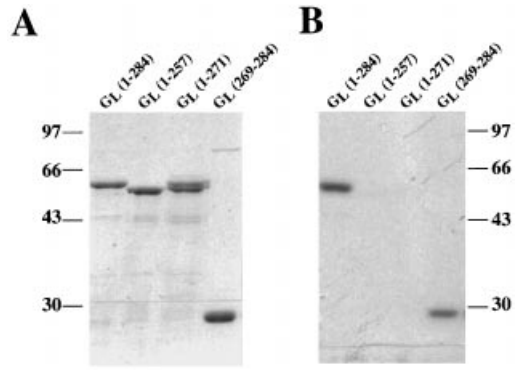

Figure 3 Identification of the binding site for phosphorylase *a* in the 16 C-terminal amino acids of G_L

GST–G_L (1–284) and GST fusions containing truncated G_L coding regions (2 μg) were separated on 12.5% SDS polyacrylamide gels and either (**A**) stained with Coomassie Blue or (**B**) transferred to nitrocellulose and probed with 100 nM ^{32}P-labelled phosphorylase *a*. The standard marker proteins are as in Figure 2.

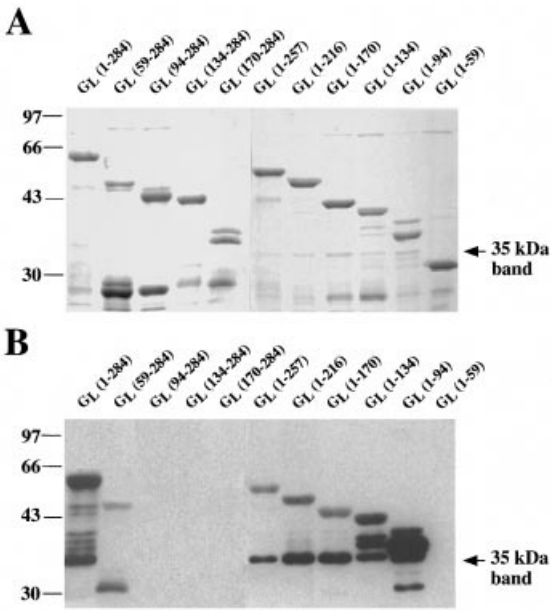

Figure 4 Identification of the region binding PP1 in G_L

GST–G_L (1–284) and GST fusions containing truncated G_L coding regions (2 μg) were separated on 12.5% SDS polyacrylamide gels and either (**A**) stained with Coomassie Blue or (**B**) transferred to nitrocellulose and probed with digoxygenin-labelled PP1γ. The standard marker proteins are as in Figure 2.

RESULTS

The glycogen-binding domain is located between residues 94 and 257

GST–G_L, containing the full-length G_L protein, and several of the GST–G_L truncations [GST–G_L(94–216), GST–G_L(94–170), GST–G_L(134–170), GST–G_L(134–216), GST–G_L(134–257), GST–G_L(134–284)] exhibited a strong tendency to aggregate and were pelleted at 100000 ***g*** for 1 h, even in the absence of glycogen. Therefore these constructs could not be tested for glycogen-dependent sedimentation. Of the GST fusion proteins that did not aggregate, GST–G_L(94–284) and GST–G_L(94–257) were both

detected predominantly in the 100000 ***g*** pellet obtained by centrifugation in the presence of glycogen (Figure 2A). In contrast, GST–G_L(170–216) and GST–G_L(170–257) did not bind to glycogen, being detected exclusively in the 100000 ***g*** supernatant fraction in the presence of glycogen (Figure 2B). GST–G_L(94–257) carrying the single mutations N152A or K157A (results not shown), the double mutation N152A + K157A, or the triple mutation K149A + N152A + L153A were all found to sediment in the presence of glycogen (Figure 2C).

The phosphorylase *a*-binding site lies in the C-terminal 16 amino acids of G_L

In order to identify the region of G_L that is responsible for the binding of phosphorylase *a*, GST–G_L and its truncated forms were transferred to nitrocellulose membranes and tested for their ability to bind ^{32}P-phosphorylase *a*. ^{32}P-Labelled phosphorylase *a* was found to bind to GST–G_L containing the entire G_L protein and to GST–G_L(216–284), GST–G_L(257–284) and GST–G_L(269–284), but not to GST–G_L(1–216), GST–G_L(1–257) and GST–G_L(1–271) (Figure 3 and results not shown). These results indicate that the phosphorylase *a*-binding domain lies in the C-terminal 16 amino acids of G_L.

The PP1-binding domain is situated between residues 59 and 94 of G_L

The GST–G_L truncations were also tested for their ability to bind digoxygenin-labelled PP1γ after transfer to nitrocellulose membranes. Figure 4 shows that digoxygenin-PP1 binds to GST–G_L(1–284), GST–G_L(1–94) and GST–G_L(59–284), but not to GST–G_L(1–59) or GST–G_L(94–284), GST–G_L(134–284) or GST–G_L(170–284). From these interactions, the principal PP1-binding domain must lie between residues 59 and 94 of G_L. Several proteolytically degraded fragments present in the preparations were also recognized by digoxygenin-PP1, in particular a minor 35 kDa Coomasssie Blue staining band that migrated slightly faster than GST–G_L(1–94) but slower than GST–G_L(1–59). Since this proteolytic fragment was retained on glutathione-Sepharose, it is likely to comprise GST linked to the first 75–80 residues of G_L. The strong signal with digoxygenin-PP1 may be explained by a more effective renaturation of this fragment from SDS on the nitrocellulose membrane.

DISCUSSION

Here we have identified three distinct functional domains on the rat liver glycogen-targeting subunit of PP1. The section comprising amino acids 59–94 is both necessary and sufficient for binding to PP1. This region contains the sequence, Arg-Val-Ser-Phe, which conforms to the consensus PP1-binding motif determined for other PP1-binding subunits (see the Introduction).

M_{110} (1-38)peptide	26	...PPVVKRQKTKVKF	38
G_L	52	...TVQEKKVKKRVSFADQGL...	69
G_M (63-93)peptide	63	GRRVSFADNFG...	73

Figure 5 Comparison of the sequences of rat M_{110} 1–38 peptide and rabbit G_M 63–93 peptide with G_L in the region of the RVSF (Arg-Ser-Val-Phe) motif (underlined)

Three basic residues (Lys or Arg) preceding the RVSF motif that are conserved in M_{110} and G_L but not present in the G_M 63–93 peptide are double underlined. No other clear sequence similarities between the M_{110} 1–38 peptide and G_L were apparent in the region preceding that shown.

The data provide further evidence for the importance of this short motif in the binding of PP1 to its targeting subunits. The results also indicate that no other domains outside of residues 59–94 can initiate and maintain an interaction with PP1 independently of the Arg-Val-Ser-Phe motif. Previous experiments [12] demonstrated that a peptide comprising the N-terminal 38 residues of the M_{110} subunit of the myosin-targeting subunit of PP1 (M_{110} 1–38) could disrupt the PP1–G_L complex, whereas a peptide comprising residues 63–93 of the skeletal muscle glycogen-targeting subunit (G_M 63–93) did not abolish binding of G_L to PP1. These results suggest that secondary sites of PP1–G_L interaction are likely to involve residues that are identical in M_{110} 1–38 and G_L but distinct (or not included) in G_M 63–93. A comparison of the sequences of the M_{110} 1–38 peptide and the G_M 63–93 peptide with G_L in the region of the Arg-Val-Ser-Phe motif identifies three basic residues (Lys or Arg) preceding the Arg-Val-Ser-Phe motif that are identical in M_{110} and G_L but are not present in the G_M 63–93 peptide (Figure 5). The basic residues in positions −2, −4 and −5 with respect to the Arg-Val-Ser-Phe motif may therefore provide the secondary interactions of G_L with PP1 that are not disrupted by the G_M 63–93 peptide. The crystal structure of PP1 complexed to the G_M 63–75 peptide reveals the presence of an acidic domain in PP1 that lies to the N-terminal end of the Arg-Val-Ser-Phe motif in the bound peptide and thus has the potential to interact with the basic residues in G_L and the M_{110} peptide [2].

The sequence similarity noted previously between the mammalian glycogen-targeting subunits and glucoamylase from *Rhizopus oryzae*, which binds starch, spans a region comprising amino acids 144–231 of G_L ([5,6] and Figure 6]. The present study demonstrates that the region 94–257 of G_L is capable of binding to glycogen, whereas the truncated GST fusion proteins GST–G_L(170–216) or GST–G_L(170–257) fail to bind to glycogen. The interaction of GST–G_L(94–170) or GST–G_L(134–170) with glycogen could not be tested due to the aggregation of these fragments. However, residues 148–163 of G_L show some sequence similarities (Figure 6) to the region in phosphorylase (398–437) that has been identified in crystallographic studies to bind to maltoheptaose and is believed to bind to glycogen *in vivo* [18,19]. The crystal structure of phosphorylase shows that the side-chains of conserved hydrophobic amino acids in this section form internal contacts and appear to be involved in maintaining the orientation of the α-helix that binds maltoheptaose. Of the residues shown to bind to maltoheptaose, only the Asn corresponding to Asn-152 in G_L is identical in the glycogen-targeting subunits and phosphorylase. However, mutation of N152A in GST–G_L(94–257) did not prevent this fragment binding to glycogen, nor did the double mutation N152A + K157A. The triple mutation K149A + N152A + L153A of residues which are conserved in the glycogen-targeting subunits and are aligned with those binding maltoheptaose in phosphorylase (Figure 6) also did not prevent the binding of GST–G_L(94–257) to glycogen. A peptide corresponding to this region (GST–G_L144–169) could not be tested because of insolubility. The results suggest that all of the conserved sections in G_L (144–231) are likely to be required for binding to glycogen, either by making direct contact with glycogen or by contributing to structural elements required for this binding. They further indicate that the binding site of the PP1 glycogen-targeting subunits and glucoamylase to polysaccharides is distinct from the glycogen-binding (storage) site of phosphorylase. Bork et al. [20] have recently produced an alignment of the starch-binding domain of bacterial and fungal amylases and a bacterial glycosyltransferase with some of the mammalian glycogen-targeting subunits. However, it is not clear that the bacterial amylases and *Bacillus circulans* cyclodextrin

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G_L   143   AIAGTVKVQNLAFEKVVKIRM--TFDTWKSFTD---FPCQ   177
R5    168   TVTGTVKVKNVSFEKKVQIRI--TFDSWKNYTD---VDCV   202
PTG   145   TVTGTVKVKNVSFEKKVQVRI--TFDTWKTYTD---VDCV   179
R6    188   GISGTVRVCNVAFEKQVAVRY--TFSGWRSTHE---AVAR   222
G_M   141   SIKGIIRVLNVSFEKLVYVRM--SLDDWQTHYD---ILAE   175
U5    151   ALSGTVKVRNIEYEKKVLVRI--TFDGWKSFRD---VSCQ   185
GAC1  257   KITGLVYVKNLSFEKYLEIKF--TFNSWRDIH-----YVT   289
PIG1  223   QIWGLIFVNNLNFEKKIEIKF--TLNNWADIH-----YIN   255
GIP2  438   LIAGRILVKNIFYDKRVVVRY--TWDSWRTTHEVECVYIS   475
PIG2  407   VIIGKVFVKNIYFDKKIIVRY--TWDAWRTFHESECVYFS   444
AMYL   44   TFSGKIYVKNIAYSKKVTVIYADGSDNWNNNGNT---IAA    80
Consensus     φ.G.V.V.Nφ.F.K.φ.V.φ.....W
                 I     Y     I
PHOS  398   RHLQIIYEINQRFLNRVAAAFPGDVDRLRRMSLVEEGAVK   437
                 ** ***                 * * *   *

G_L   178   YVKDTYAGSDRDTFSFDISLPEKIQSYER-----------   206
R5    203   YMKNVYGGTDSDTFSFAIDLPPVIPTEQK-----------   231
PTG   180   YMKNVYSSSDSDTFSFAIDLPRVIPTEEK-----------   208
R6    223   WRGPAGPEGTEDVFTFGFPVPPFLLELGSR----------   252
G_M   176   YVPNSCDG-ETDQFSFKIVLVPPYQKDGSK----------   204
U5    186   YMHSTYGSADMDTFSFELSLPKPSSSCKA-----------   214
GAC1  290   ANFNRTINSNVDEFKFTIDLNSLKYILLIKRIITME--KN   327
PIG1  256   AHYNKSVTPHVDEFKFIIDISALKLNLISKNLIYTNFFER   295
GIP2  476   DGDGILPGTNMDIFHFIIDDVSKVDPRGK-----------   504
PIG2  445   NANGILPGSNMDIFKFSIDDIHNPNDKDSNISQ-------   477
AMYL  81    SYSAPISGSNYEYWTFSASINGIK-----------------   104
Consensus             D.F.F
                      E W

G_L   207   -------MEFAVCYECNG-----QSYWDSNKGKNYRI      231
R5    232   -------IEFCISYHANG-----QVFWDNNDGQNYRI      256
PTG   209   -------IEFCISYHANG-----RIFWDNNEGQNYRI      233
R6    253   -------VHFAVRYQVAG-----AEYWDNNDHRDYSL      277
G_M   205   -------VEFCIRYETSV-----GTFWSNNNGTNYTF      229
U5    215   -------TEFCISFRCGQ-----KTHWDNNQGKNYKI      239
GAC1  328   TSSCPLNIELCCRYDVNN-----ETYYDNNNGKNYHL      359
PIG1  296   KTTCLLNLQFCCRYDVNG--FEYRSFYDNNDYKNYEI      330
GIP2  505   -------LEFCIHYSTRN-DYEREEYWDNNNGNNYKV      533
PIG2  478   -------LEFCIQYLTWGVDRSRKEYWDNNDSANYKI      507
AMYL  105   --------EFYIKYEVSG-----KTYYDNNNSANYQV      128
Consensus           F.φ.F.....W..N....Y.φ
                    L   Y         Y
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Figure 6 Comparison of the polysaccharide-binding domain of the mammalian, avian and yeast glycogen-targeting subunits and *R. oryzae* glucoamylase (AMYL) with phosphorylase (PHOS) glycogen-binding (storage) site

The sequences are rat G_L [5], human PPP1R5 [6], mouse PTG [7], human PPP1R6 [8], human G_M [4], *Saccharomyces cerevisiae* GAC1 [23], *S. cerevisiae* PIG1 and PIG2 [24], *S. cerevisiae* GIP2 [25], *R. oryzae* glucoamylase [26] and rabbit skeletal muscle glycogen phosphorylase [27]. A consensus sequence for the polysaccharide-binding domain of the glycogen-targeting subunits and glucoamylase is shown. Conserved residues are underlined and identical residues are double underlined. The maltoheptaose-binding residues in phosphorylase are marked with an asterisk.

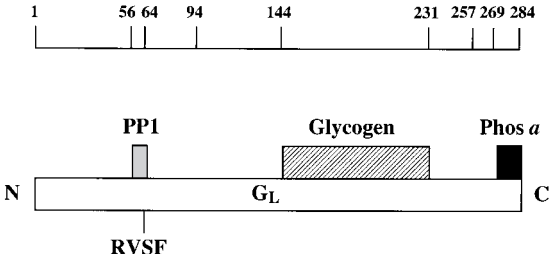

Figure 7 Schematic representation of the domains in G_L that interact with PP1, glycogen and phosphorylase *a*

PP1 binds to the Arg-Val-Ser-Phe motif located at residues 61–64. The basic sequence preceding this motif is also likely to be involved in binding PP1. Residues 144–231 include the conserved residues among the glycogen-targeting subunits that lie in the 94–257 fragment of G_L that co-sediments with glycogen. Phosphorylase *a* binds to the C-terminal terminal 16 amino acids of G_L. The relevant amino acid positions in G_L are indicated.

glycosyltransferase all bind polysaccharide in the same manner. Strikingly, in their alignment, the amino acids Gly-146, Asn-152, Lys-157, Asp-189, Phe-193, Tyr-213 and Asn-224 of G_L are not highly conserved in the bacterial and fungal enzymes, except for *R. oryzae* glucoamylase.

The allosteric inhibition of the glycogen synthase phosphatase activity of PP1–G_L by phosphorylase *a* is mediated by the binding of phosphorylase *a* to G_L [5,9–11]. The results presented in this paper demonstrate that the 16 amino acids at the C-terminus of G_L are essential for interaction with phosphorylase *a*. (Figure 7). However, although this short region is sufficient for the binding of phosphorylase *a*, it is likely that other regions of G_L are required to transmit the allosteric effect of this molecule to the active site of PP1. Nevertheless, the identification of a short sequence in G_L as crucial for the binding of phosphorylase *a*, and therefore also for inhibition of glycogen synthase phosphatase activity, provides a rationale for searching for small molecules that might block this inhibition. Raising the level of glycogen synthase phosphatase activity and consequently glycogen synthase activity may be useful in disorders, such as diabetes, where hyperglycaemia is a severe problem.

The other three glycogen-binding subunits, G_M/PPP1R3, PPP1R5 and PPP1R6 do not show significant sequence similarity to the C-terminus of G_L [5,6,8], which explains why G_M is not inhibited by phosphorylase *a* [10] and PPP1R5 and PPP1R6 have not been found to bind phosphorylase *a* after transfer to nitrocellulose membranes [6,8]. PTG, the mouse homologue of the human glycogen-targeting subunit PPP1R5 has been reported to bind to phosphorylase *a*, as well as to several other regulatory enzymes of glycogen metabolism [7]. If this is the case, then the amino acid sequence binding phosphorylase *a* in PTG is significantly different from that in G_L. However, experiments with PTG are stated to have been performed in a buffer containing 2 mg/ml glycogen [7,21]. Since phosphorylase *a*, phosphorylase kinase, glycogen synthase and PTG/PPP1R5 are all glycogen-binding proteins ([22]; G. Browne, P. T. W. Cohen and P. Cohen, unpublished work) it is not surprising that sedimentation of GST–PTG in the presence of glutathione–Sepharose and glycogen results in the sedimentation of other glycogen-binding proteins. It is clearly essential to repeat these experiments in the absence of glycogen.

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