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Amino acid sequence and expression of the hepatic glycogen-binding (G_L)-subunit of protein phosphatase-1

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Abstract A full-length cDNA encoding the putative hepatic glycogen-binding (G_L) subunit of protein phosphatase-1 (PP1) was isolated from a rat liver library. The deduced amino acid sequence (284 residues, 32.6 kDa) was 23% identical (39% similar) to the N-terminal region of the glycogen-binding (G_M) subunit of PP1 from striated muscle. The similarities between G_M and G_L were most striking between residues 63–86, 144–166 and 186–227 of human G_M (~40% identity), nearly all the identities with the putative yeast homologue GAC1 being located between 144–166 and 186–227. The cDNA was expressed in *E. coli*, and the expressed protein transformed the properties of PP1 to those characteristic of the hepatic glycogen-associated enzyme. These experiments establish that the cloned protein is G_L.

Key words: Protein phosphatase; Targetting subunit; Glycogen; Glycogen metabolism; Cyclic AMP-dependent protein kinase

1. Introduction

Protein phosphatase-1 (PP1) is one of the major protein serine/threonine phosphatase catalytic subunits found in eukaryotic cells. In recent years, evidence has accumulated that this enzyme is regulated by 'targetting' subunits which tailor its properties to a particular cellular function. The interaction of PP1 with a targetting subunit not only directs it to a specific location, but may enhance its activity towards some substrates, decrease its activity towards others and/or confer critical regulatory properties upon it. Targetting subunits have been identified which direct PP1 to glycogen particles and membranes, the muscle contractile apparatus and nuclei, but many more are likely to be identified in the future (reviewed in [1]).

A remarkable feature of this system is that the targetting subunits which direct PP1 to the same location in different tissues are tissue specific, and alter the specificity and regulatory properties of PP1 in different ways. For example, the M_{110}-subunit which targets PP1 to the myofibrils of smooth muscle enhances the rate at which PP1 dephosphorylates smooth muscle myosin, but not the rate at which it dephosphorylates skeletal muscle myosin. In contrast, the structurally related M-subunit which targets PP1 to the myofibrils of striated muscles, enhances the rate at which PP1 dephosphorylates skeletal muscle myosin far more than it enhances the rate of dephosphorylation of smooth muscle myosin [2,3].

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**These investigators have made equally important contributions to this study.

It has long been recognised that the subunits which target PP1 to the glycogen particles of striated muscle (G_M) and liver (G_L) must be distinct proteins. G_M is a 124–125 kDa protein [4,5] which targets PP1 to the membranes of the sarcoplasmic reticulum (SR) as well as to glycogen [6]. The glycogen-binding and PP1-binding sites are located in the amino-terminal third of G_M [7], while the membrane association domain appears to comprise a sequence of 32 consecutive hydrophobic residues located near the C-terminus [4]. Phosphorylation of human G_M at Ser-46 by an insulin-stimulated protein kinase(s) enhances the rate at which PP1 dephosphorylates (activates) glycogen synthase and dephosphorylates (inactivates) phosphorylase kinase, and is thought to contribute to the stimulation of glycogen synthesis and inhibition of glycogenolysis by insulin [8]. In contrast, the phosphorylation of human G_M at Ser-65 by cyclic AMP-dependent protein kinase (PKA), which occurs in response to adrenalin, triggers the dissociation of PP1 from G_M. As a result PP1 is released from glycogen particles or SR membranes, impairing its ability to dephosphorylate glycogen-associated (glycogen synthase, glycogen phosphorylase [9–11]) or SR-associated [6] substrates. This represents one of the mechanisms by which adrenalin inhibits glycogen synthesis and stimulates glycogenolysis in striated muscle [12].

In contrast to G_M, the G_L subunit suppresses the rate at which PP1 dephosphorylates (inactivates) glycogen phosphorylase and enhances the rate at which it activates glycogen synthase. As a consequence, the glycogen synthase phosphatase:phosphorylase phosphatase activity ratio of hepatic PP1G is far higher than that of muscle PP1G under the standard assay conditions [13–15]. Moreover, incubation of hepatic glycogen particles with PKA and Mg-ATP is reported not to dissociate PP1 from G_L [16]. Instead, G_L confers upon PP1 a remarkable property, namely inhibition of the dephosphorylation of glycogen synthase by phosphorylase *a* (the active, phosphorylated form of glycogen phosphorylase). In the presence of glycogen, this inhibition occurs at nM concentrations, about 1000-fold lower than the K_m for phosphorylase *a* as a substrate, indicating that it is an allosteric effect [15,17]. Inhibition of the dephosphorylation (activation) of glycogen synthase by phosphorylase *a* provides an alternative mechanism for inhibiting glycogen synthesis when glycogenolysis is activated which, in striated muscle, is achieved by the PKA-catalysed phosphorylation of G_M. In the liver, the level of phosphorylase *a* is elevated by hormones that increase the intracellular concentration of cyclic AMP (i.e. glucagon or adrenalin acting via β-receptors) or calcium ions (vasopressin, angiotensin II or adrenalin acting via α-receptors) [18,19] and decreased by insulin (which reduces the level of cyclic AMP) or by glucose. When the circulating level of glucose rises, this sugar binds to hepatic phosphorylase *a* inducing a conforma-

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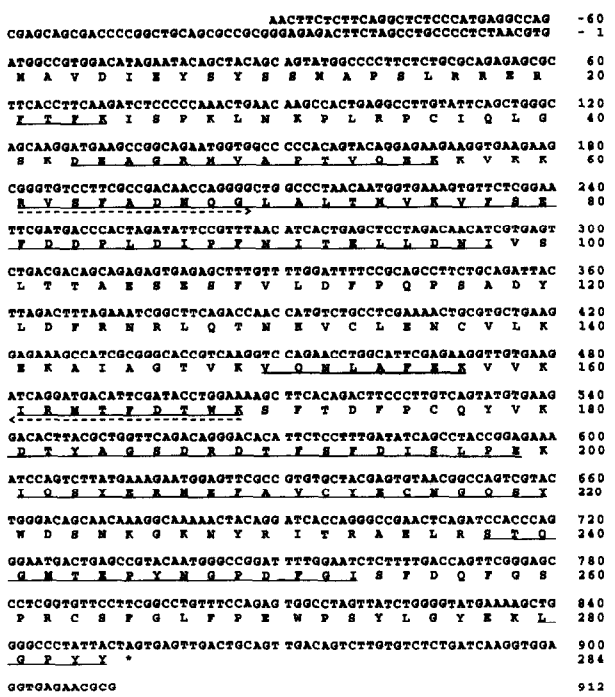

Fig. 1. Complementary DNA and predicted protein sequence of the glycogen-binding G_L subunit of rat liver PP1G. The sequences of peptides isolated from the purified protein by digestion with either trypsin or Lys-C endoproteinase are underlined, and the broken lines show the two peptide sequences used to construct oligonucleotides for PCR.

tional change that allows it to be dephosphorylated (inactivated) more rapidly [20,21]. Moreover, the sensitivity of hepatic PP1G to phosphorylase *a* is itself highly regulated. It is greatly potentiated by glycogen [14,22], and may therefore only operate in the fed state.

We recently purified hepatic PP1G to apparent homogeneity [23] by microcystin-Sepharose affinity chromatography [24]. The isolated enzyme retained the characteristic properties of hepatic PP1G and was composed of the PP1 catalytic subunit complexed to a 33-kDa protein. The latter appeared to be G_L, because it bound to glycogen and to phosphorylase *a*, while two tryptic peptides derived from it were similar to sequences found in the N-terminal domain of G_M [23]. The small size of G_L was surprising, since G_M has a molecular mass of 124 kDa [4] and the presumed G-subunit homologue in *S. cerevisae* (GAC1) is an 88 kDa protein [25]. However, the 33 kDa protein did not appear to be a proteolytic fragment of a larger precursor, because it retained all the known properties of G_L and no other PP1 binding protein was detectable in rat or rabbit hepatic glycogen particles which had been isolated rapidly at 0–4°C in the presence of a proteinase inhibitor 'cocktail' [23]. Here we have determined the amino acid sequence of the 33-kDa protein from cloned cDNA and established by reconstitution with PP1 that it is G_L.

2. Materials and methods

2.1. Materials

PP1G was purified from rabbit skeletal muscle [26] and the PP1 catalytic subunit dissociated from G_M by incubation for 2 h in 2 M LiBr and purified by gel filtration on Superose 12 in the presence of 0.5 M LiBr [2]. Sources of other Materials are given in [23].

2.2. Production of anti-G_L antibodies

The peptides EAGRMVAPTVQEK and TQGMTEPYNGPDF corresponding to residues 44–56 and 239–251 of G_L were conjugated to bovine serum albumin, and injected into a sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire, UK). The antibodies were affinity purified by chromatography of the antiserum on peptide-CH-Sepharose columns.

2.3. Cloning of the rat liver glycogen-binding subunit of PP1

Polymerase chain reactions were performed in 100 μl reactions using

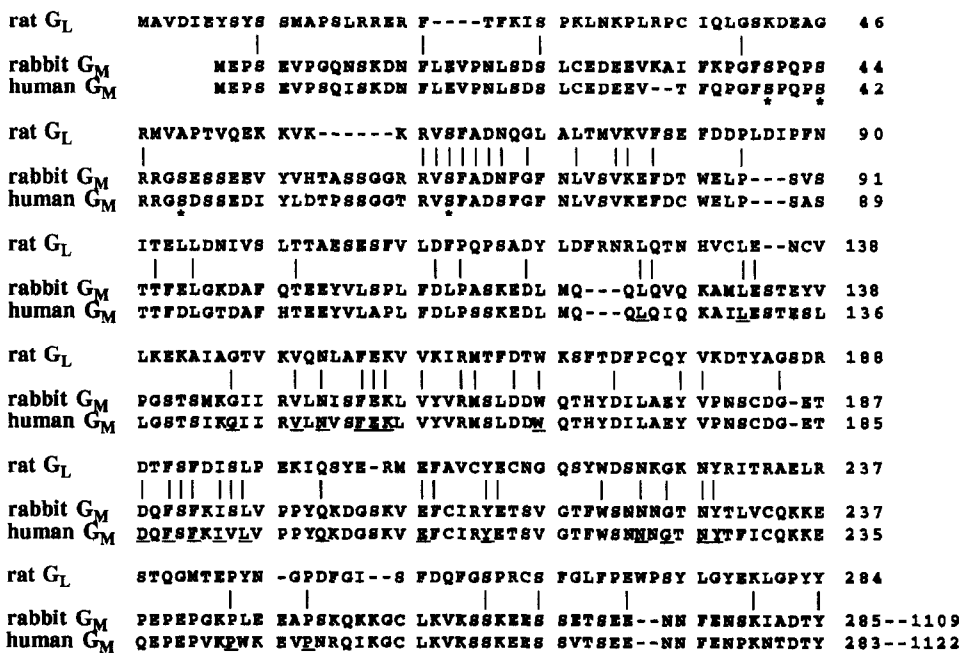

Fig. 2. Comparison of the amino acid sequence of rat liver G_L with the N-terminal regions of G_M from rabbit and human skeletal muscle. Identities are shown by vertical lines. The serine residues in G_M that are phosphorylated by PKA (serines 46 and 65 in human G_M) and by glycogen synthase kinase 3 (serines 38 and 42 in human G_M) are marked by asterisks. Residues that are identical in GAC1, G_M and G_L are underlined.

oligonucleotide-1 (5′-GTITCITT(T/C)GCIGA(T/C)AA(T/C)CA(A/G)GG-3′) and *oligonucleotide-2* (5′-TTCCAIGT(A/G)TC(A/G)AAIGTCATICT(A/G/T)AT-3′) (0.5 μM each primer), 1×10^7 bacteriophage (1 μl), 1.5 mM $MgCl_2$ and Taq DNA polymerase Perkin-Elmer Cetus (Bucks, UK) as described by the suppliers. The major PCR product was purified by excision and electroelution from a 1% agarose gel and sequenced directly using oligonucleotides 1 and 2 as primers. The λZAP II rat liver cDNA library was hybridised at 55°C with the [α-^{32}P]dATP labelled [27] PCR product, followed by washing in 1 × SSC (15 mM Na-citrate, 150 mM NaCl, pH 7.0), 0.1% SDS at 65°C. Two positively hybridising clones were purified and plasmids containing the cDNA inserts (both 2.3 kb) were excised according to the manufacturer's protocol (Stratagene, La Jolla, CA). DNA sequencing was performed in both directions on double-stranded plasmid DNA using an Applied Biosystems 373A automated DNA sequencer and Taq dye terminators.

2.4. Expression of a glutathione-S-transferase-G_L fusion protein in E. coli

The open reading frame of G_L was amplified by PCR using oligonucleotide 5′-CGCCCATATGGCCGTGGACATAGAATAC-3′, which creates an *Nde*I site (underlined) at the initiating methionine codon and oligonucleotide 5′-GCGCCTCGAGCACTAGTAATAGGGCCC-CAG-3′ which creates a *Xho*I site (underlined) just 3′ of the termination codon. Digestion of the PCR product with *Nde*I and *Xho*I allowed the complete coding region of G_L to be cloned into *Nde*I-*Xho*I sites of the prokaryotic gene fusion expression vector pGEX-KG [28] which had modified to include a *Nde*I site in the linker region (C.G. Armstrong and P.T.W. Cohen, unpublished data). The final construct termed pGEX-G_L, encoded glutathione *S*-transferase, followed by a thrombin cleavage sequence and the complete open reading frame of G_L. After verifying that the sequence of the pGEX-G_L construct was correct, the plasmid was transformed into the *E. coli* strain BL-21(DE3)pLysS and transformants selected on LB plates containing ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml). Single colonies were grown to an A_{600} of 0.4 at 37°C and expression of the fusion protein was induced at 28°C by addition of IPTG to a final concentration of 200 μM for 16–20 h. The bacteria were harvested by centrifugation, lysed by sonication in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 0.02% (v/v) Brij-35. Insoluble protein was removed by centrifugation for 30 min at 40,000 × *g* and the fusion protein purified on glutathione-Agarose as in [29]. Only 75–150 μg of soluble G_L protein was isolated from each litre of bacterial culture, most of the expressed protein being insoluble and present in inclusion bodies.

2.5. Preparation of phosphorylated proteins and phosphatase assays

^{32}P-Labelled rabbit skeletal muscle phosphorylase *a* (containing 1.0 mol phosphate per mol subunit) was prepared by phosphorylation with phosphorylase kinase [30], and thiophosphorylase *a* was obtained in an identical manner except that unlabelled adenosine 5′-[γ-thio]triphosphate replaced [γ-^{32}P]ATP. ^{32}P-Labeled rabbit skeletal muscle glycogen synthase was phosphorylated in the site-3 region to 1.5 mol/mol subunit with glycogen synthase kinase-3 [31]. The dephosphorylation of phosphorylase *a* (10 μM) and glycogen synthase (1 μM) were carried out using standard procedures [30]. One unit of PP1 activity (U) was that amount which catalysed the release of 1 μmol of phosphate from phosphorylase *a* in 1 min.

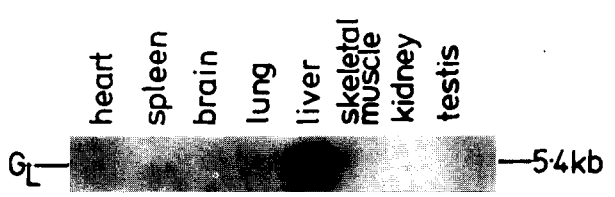

Fig. 3. Tissue distribution of the G_L mRNA. A blot (Clonetech) of poly(A)$^+$ RNA from different rat tissues was hybridised with a G_L cDNA probe at 42°C according to the supplier's protocol. The figure shows an autoradiograph of the blot developed after 24 h. Each lane contained approximately 2 μg of poly(A)$^+$ RNA.

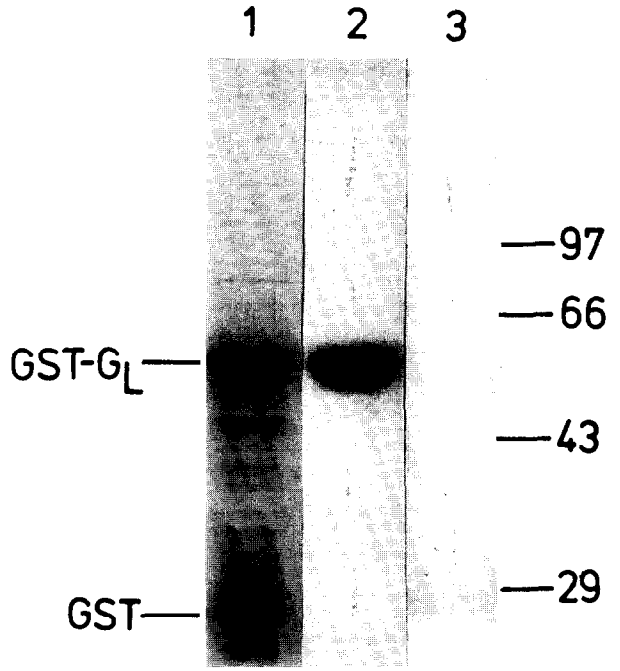

Fig. 4. The GST-G_L fusion protein binds to phosphorylase *a* specifically. The GST-G_L fusion protein was electrophoresed on a 10% SDS/polyacrylamide gel and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose membranes and probed with 100 nM ^{32}P-labelled phosphorylase *a* (lane 2) as described in [23]. In lane 3, the nitrocellulose membrane was probed with phosphorylase *a* containing 5 mM magnesium acetate and 3 mM 5′-AMP. The arrows show the positions of the GST-G_L fusion protein, GST and the molecular mass markers phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

3. Results

3.1. Cloning of cDNA encoding the 33-kDa subunit of hepatic PP1G from a rat liver library

The purified 33-kDa subunit was digested with either Lys-C endoproteinase or trypsin and a number of peptides were purified and sequenced. Two peptides, RVSFADNQGLALTMVK and IRMTFDTWK, showed significant sequence similarities to residues 65–80 and 161–169 of G_M, respectively, and were used to design suitable degenerate oligonucleotides for PCR (section 2.3). PCR using a rat liver cDNA library and these two oligonucleotides gave a 309 base pair fragment. The internal sequence of this fragment encoded amino acid sequences identical to two other peptides isolated from the 33 kDa subunit, indicating that the PCR product did indeed encode a fragment of this protein. The PCR product was therefore used as a probe to screen the library, and 2 of the 18 positive clones identified (out of 500,000 phage screened) were purified and found to contain an identical open reading frame of 852 nucleotides encoding a 284 amino acid protein (Fig. 1). The molecular mass of this protein (32.56 kDa) is identical to the apparent molecular mass of the purified protein estimated by SDS/polyacrylamide gel electrophoresis [23]. Every peptide isolated (a total of 133 residues) was found in the deduced 284 amino acid sequence (Fig. 1), including the C-terminal peptide LGPYY. A stop codon precedes the initiating ATG and the termination codon is followed almost immediately by two in-frame stop codons.

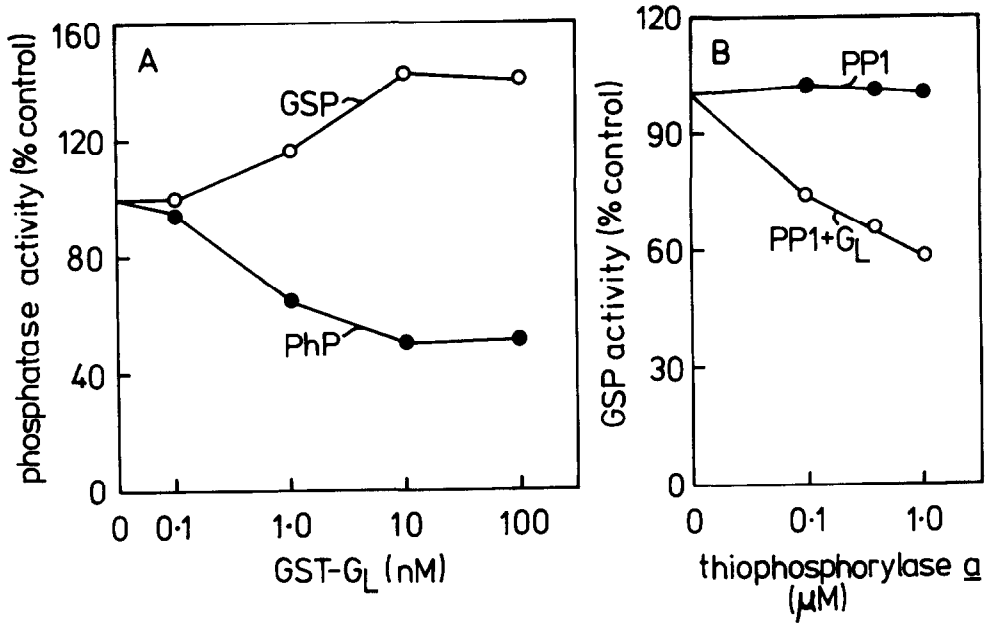

Fig. 5. The GST-G_L fusion protein transforms the catalytic subunit of PP1 to a species with properties resembling those of hepatic PP1G. (A) The catalytic subunit of PP1 was diluted in assay buffer to 1.5 U/ml (1.4 nM), and a 0.01 ml aliquot was incubated for 15 min at 30°C with 0.01 ml of the indicated concentrations of purified bacterially expressed GST-G_L fusion protein diluted in the same buffer. Phosphorylase phosphatase (PhP; closed circles) or glycogen synthase phosphatase (GSP; open circles) assays were then initiated with 0.01 ml of substrate. (B) The catalytic subunit of PP1 was incubated in the absence (closed circles) or presence (open circles) of GST-G_L for 10 min at 30°C and then for 5 min with thiophosphorylase *a* before initiating the asssays with ^{32}P-labelled glycogen synthase. The final concentrations in the assay were: PP1 (0.5 nM), GST-G_L (10 nM), glycogen synthase (1 μM), thiophosphorylase *a* (as indicated). Activities are presented as a percentage of those measured in the absence of thiophosphorylase *a*.

The amino acid sequence of the 33-kDa protein showed 23% identity to residues 1–284 of the G_M subunit from human skeletal muscle (Fig. 2), and 39% similarity if the most conservative substitutions are included. The most striking regions of similarity were those corresponding to residues 63–86, 144–166 and 186–227 of human G_M (~40% identity).

Northern blotting demonstrated that the 5.4 kb RNA encoding the 33-kDa protein was specific to liver, being undetectable in brain, heart, kidney, lung, skeletal muscle, spleen and testis (Fig. 3).

3.2. *Expression of the 33-kDa protein in* E. coli *and reconstitution with PP1*

The 33-kDa protein was expressed in *E. coli* as a GST fusion protein, and after affinity chromatography on glutathione-Agarose, the preparations showed two protein-staining bands with apparent molecular masses of 57 kDa and 24 kDa (Fig. 4). The 57-kDa protein was recognised by an anti-GST antibody as well as by antibodies raised against two peptides from the 33 kDa protein (section 2.2), while the 24-kDa species (which comigrated with GST) was only recognised by the anti-GST antibody (data not shown).

The 57-kDa fusion protein transformed the properties of the PP1 catalytic subunit to those resembling hepatic PP1G. The 57-kDa protein suppressed the phosphorylase phosphatase activity of PP1 by 50% and enhanced its glycogen synthase phosphatase activity by 40% with maximal effects at 10 nM (Fig. 5A). Moreover, only after addition of the 57-kDa protein, did the dephosphorylation of glycogen synthase become sensitive to inhibition by phosphorylase *a* (Fig. 5B). The 57-kDa protein interacted with ^{32}P-labelled phosphorylase *a* (Fig. 4), and the interaction was specific because neither the 24-kDa protein nor any of the molecular mass standards (one of which is phorphorylase *b*) were recognised by the phosphorylase *a* probe (Fig. 4). Moreover, the interaction of phosphorylase *a* with the 57 kDa species was prevented by 5′-AMP (Fig. 4), which binds to phosphorylase *a* and is known to cancel the allosteric inhibition of hepatic PP1G by phosphorylase *a* [20,21].

4. Discussion

We have previously isolated PP1G from the glycogen-protein particles of rat liver and shown that the final preparation consists of the catalytic subunit of PP1 complexed to a 33 kDa glycogen-binding protein [23]. Here we have cloned the cDNA encoding the 33 kDa protein, deduced its amino acid sequence, and provided definitive evidence that this protein is indeed G_L. When expressed in *E. coli*, GST-G_L bound to phosphorylase *a* specifically (Fig. 4) and transformed the properties of PP1 to those characteristic of hepatic PP1G (Fig. 5). The dephosphorylation of phosphorylase *a* was suppressed, the dephosphorylation of glycogen synthase enhanced and the glycogen synthase phosphatase activity became sensitive to inhibition by phosphorylase *a*. However, the dephosphorylation of phosphorylase *a* is only suppressed by 50% compared to 80% in native hepatic PP1G, and the concentration of phosphorylase *a* which inhibited the dephosphorylation of glycogen synthase by 50% (about 1 μM in the absence of glycogen – Fig. 5) was 5-fold higher than for native hepatic PP1G [14]. These differences were not explained by the presence of GST at the N-terminus of the expressed protein, because removal of the GST-tag by cleavage with thrombin, did not enhance the ability of G_L to modify the properties of PP1 (data not shown). It is more likely that these differences are related to the highly aggre-

gated state of expressed G_L, which could be pelleted by centrifugation for 30 min at 200,000 × *g* (data not shown). We have noticed that rat hepatic PP1G also has a strong tendency to aggregate in the absence of glycogen, and this property may explain, at least in part, the progressive loss of its distinctive properties during purification by conventional procedures, a problem which prevented its isolation for many years.

G_L and G_M are structurally related, but the overall sequence similarity (23% identity, 39% similarity over the first 284 residues of human G_M) is surprisingly low (Fig. 2). G_L lacks Ser-46 of human G_M whose phosphorylation activates muscle PP1G (see section 1), as well as Ser-38 and Ser-42 of human G_M which are phosphorylated by glycogen synthase kinase-3 in vitro (the functional significance of these phosphorylations is unclear) [7,32,33]. G_L does retain a residue equivalent to Ser-65 of human G_M whose phosphorylation by PKA triggers the dissociation of PP1 from G_M [9–11] (Fig. 2). However, G_L is poorly phosphorylated by PKA in vitro (G.M., unpublished experiments), perhaps because the sequence surrounding this residue is KRVSF and not RRVSF, and incubation with PKA under phosphorylating conditions is reported not to release PP1 from hepatic glycogen particles [16]. Further work is needed to find out whether this serine residue in G_L is phosphorylated by another protein kinase in vivo. G_L lacks the membrane-association domain found near the C-terminus of G_M [4]. This is consistent with an earlier finding that the form(s) of PP1 associated with hepatic microsomes is distinct from hepatic PP1G [14].

The regions showing most conservation between G_L and G_M (~40% identity) correspond to residues 63–86, 144–166 and 186–227 of human G_M (Fig. 2) suggesting that they may represent part of the PP1 and/or glycogen-binding domains of these proteins. GAC1, which may be the homologue of G_M/G_L in *S. cerevisiae* [26,34], shows weak homology to G_M/G_L. Nearly all the identities between GAC1 and G_M/G_L occur between residues 144–166 and 186–227 of human G_M with no similarity to residues 63–86 of G_M/G_L (Fig. 2) [5,25]. Work is in progress to identify the PP1 and glycogen binding domains on G_M/G_L, the high affinity binding site for phosphorylase *a*, and the region(s) responsible for modulating the substrate specificity of PP1.

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