Purification of the hepatic glycogen-associated form of protein phosphatase-1 by microcystin-Sepharose affinity chromatography

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Abstract The form of protein phosphatase-1 associated with hepatic glycogen (PP1G) was purified to near homogeneity from rat liver by affinity chromatography on microcystin-Sepharose and gel-filtration. The enzyme is a heterodimer consisting of the catalytic subunit of PP1 (the α and β isoforms) complexed to a 33 kDa glycogen-binding (G_L) subunit. The G_L subunit binds phosphorylase *a* with high affinity, and is responsible for the enhanced dephosphorylation of glycogen synthase by PP1G and its allosteric inhibition by phosphorylase *a*.

Key words: Microcystin; Protein phosphatase; Glycogen; Phosphorylase; Glycogen synthase

1. Introduction

The subcellular location, substrate specificity and regulation of protein phosphatase-1 (PP1) is determined by its interaction with targetting subunits (reviewed in [1]). Two targetted forms of PP1 have been isolated from striated muscles, termed PP1G [2] and PP1M [3,4], in which the catalytic subunit is complexed to G and M subunits, respectively. The G subunit directs PP1 to both glycogen–protein particles [2,5] and the membranes of the sarcoplasmic reticulum [6], facilitating the dephosphorylation of glycogen metabolising enzymes [7] and SR proteins [8], respectively, while the M subunit targets PP1 to myofibrils, greatly enhancing the dephosphorylation of myosin and suppressing activity towards the glycogen-metabolising enzymes [3]. PP1 interacts with at least two proteins in nuclei, an inhibitor NIPP1 [9] and the *Saccharomyces cerevisiae* protein $sds22^+$, which is essential for exit from mitosis [10]. PP1G can be activated or inhibited in striated muscles depending on whether it is phosphorylated by an insulin-stimulated protein kinase [11] or by cyclic AMP-dependent protein kinase [1,12–14], while the ability of NIPP1 to inhibit PP1 is prevented by its phosphorylation in vitro [15]. These observations suggest that the modification of targetting subunits may be a common device for regulating different forms of PP1 selectively.

The targetting subunits of PP1 are tissue specific. The M subunit of striated muscle is structurally [4] and functionally [3] distinct from that found in smooth muscle [16,17]. The former enhances the dephosphorylation of skeletal muscle myosin much more than the dephosphorylation of smooth muscle myosin, while the latter stimulates the dephosphorylation of smooth muscle myosin, but not skeletal muscle myosin [16]. The hepatic glycogen-binding subunit (G_L) is also distinct from that present in striated muscles (G_M). Hepatic PP1G has greatly enhanced activity towards glycogen synthase, while activity towards phosphorylase is strongly suppressed. Thus the glycogen synthase phosphatase (GSP):phosphorylase phosphatase(PhP) activity ratio of hepatic PP1G is far higher than that of muscle PP1G or the PP1 catalytic subunit [18]. Hepatic PP1G is also remarkable in that its GSP activity is inhibited by the active form of phosphorylase (phosphorylase *a*) at a concentration (IC_{50} = 5 nM) 1000-fold lower than the K_m for phosphorylase *a* as a substrate [19], suggesting that inhibition occurs by an allosteric mechanism. The GSP activity of hepatic PP1G is also inhibited by physiological concentrations of calcium ions [20]. Hepatic PP1G is inhibited by phosphorylase *a* [21] or Ca^{2+} [20] much more potently in the presence of glycogen.

Several years ago, hepatic PP1G was purified by a procedure involving chromatography on cyclodextrin-Sepharose [22], the final preparation containing four silver staining bands of 160, 54, 36 and 32 kDa. The 160 kDa component co-migrated with the G_M subunit on SDS-PAGE and was suggested to be G_L, while the 36 and 32 kDa species were though to represent native and proteolysed forms of PP1. Peptide sequencing revealed that the 54 kDa band was α-amylase [22]. We have recently developed a microcystin-Sepharose affinity column for purifying protein serine/threonine phosphatases and exploited it to isolate PP1M from skeletal muscle [4]. Here we use this method to isolate hepatic PP1G and identify the G_L subunit as a 33 kDa protein.

2. Materials and methods

2.1. Materials

PP1M from chicken gizzard [17] was provided by Miss D. Johnson, PP1G from rabbit skeletal muscle [2] and bacterially expressed PP1γ [22a] by Dr. D. Alessi, phosphorylase *b*, phosphorylase kinase and glycogen synthase by Miss F. Douglas and glycogen synthase kinase-3 by Dr. C. Sutherland in this laboratory. Microcystin (MC)-LR was purified and used to synthesise aminoethanethiol-microcystin-LR-Sepharose (hereafter called MC-Sepharose) as in [4]. ^{125}I-Labelled MC-YR (a MC variant provided by Dr. P. Thiel, Research Institute for Nutritonal Diseases, Tygerberg, South Africa) was prepared as in [4]. Superose 12 (HR 10/30) was from Pharmacia (Milton Keynes, UK), alkylated trypsin from Promega (Southampton, UK), endoproteinase Lys-C and anti-digoxygenin antibodies from Boehringer (Lewes, UK) and alkylated Triton X-100 from Calbiochem (Nottingham, UK). Other chemicals were from BDH Chemicals (Poole, UK) or Sigma (Poole, UK).

2.2. Preparation of phosphorylated proteins and phosphatase assays

^{32}P-Labelled phosphorylase *a* (containing 1.0 mol phosphate per mol subunit) was prepared by phosphorylation with phosphorylase kinase [23], and thiophosphorylase *a* was obtained in an identical manner except that unlabelled adenosine 5′ [γ-thio]triphosphate replaced ATP. ^{32}P-Labelled glycogen synthase was phosphorylated at site 2 to 0.8 mol/mol subunit with trypsin-activated phosphorylase kinase [24] or at

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site 3 to 1.5 mol/mol subunit with GSK3 [25]. The deposphorylation of phosphorylase *a* (10 μM) and glycogen synthase (1 μM) was carried out as in [23] and one unit of activity (U) was that amount which catalysed the release of 1 μmol of phosphate from each substrate in 1 min.

2.3. Isolation of hepatic glycogen-protein particles

These were isolated from the livers of adult Wistar rats or fed New Zealand White rabbits according to [21]. After the final centrifugation at 100,000 × *g*, the microsomal pellet overlaying the glycogen–protein pellet was removed by vortexing gently with buffer followed by aspiration. Glycogen-protein particles were also isolated from the livers of 300 g Wistar rats that had been deprived of food for 16 h and injected with glucagon (0.35 mg) 30 min prior to removal of the livers to ensure total depletion of glycogen [26]. In this procedure, livers were processed as for fed rats up to and including high speed centrifugation. The 100,000 × *g* supernatant was then made 5 mg/ml in glycogen and again centrifuged for 90 min at 100,000 × *g*.

2.4. Purification of rat liver PP1G

MC-Sepharose (0.5 ml, containing 0.15 mg of MC) was added to the glycogen–protein pellet from one rat liver suspended in 3 ml of 50 mM Tris-HCl, pH 7.0, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol (buffer A) plus the proteinase inhibitors 1 mM benzamidine, 0.5 mM phenylmethanesulphonyl fluoride, 0.5 mM tosylphenylchloromethyl ketone and 4 μg/ml leupeptin. After mixing for 30 min at 4°C, the suspension was poured into a small column and the matrix washed with buffer A containing 0.5 M NaCl. When the protein concentration of the eluate was ≤0.005 mg/ml, one void volume of buffer A containing 3 M sodium isothiocyanate was passed through the column and the flow stopped for 30 min. PP1 was then eluted with buffer A containing 3 M sodium isothiocyanate and the active fractions dialysed overnight against buffer A.

2.5. Identification of the PP1-binding subunit using [^{125}I]microcystin-YR-PP1 and digoxygenin-PP1 as probes

The PP1γ isoform (30 μg) was labelled with [^{125}I]MC-YR as in [4] and bovine serum albumin included at 1 mg/ml. The solution was reduced from 1 ml to 50 μl by ultrafiltration through a Centricon 30 membrane, diluted to 1.5 ml with 25 mM Tris-HCl (pH 7.5) containing 250 mM NaCl (buffer B), reconcentrated to 50 μl, and this procedure repeated twice to remove MC not coupled to PP1. PP1γ (30 μg) was also labelled with the ester of digoxygenin-3-*O*-methylcarbonyl-ε-aminocaproic-acid-*N*-hydroxysuccinimide (Boehringer) and separated from excess reagent according to the instructions of the manufacturer.

Protein samples containing hepatic PP1G were separated by SDS-PAGE, transferred to nitrocellulose membranes, and non-specific sites blocked by incubation for 16 h at ambient temperature with 5% (w/v) skimmed milk powder in 25 mM Tris-HCl (pH 7.5) and 500 mM NaCl. The nitrocellulose membranes were incubated for 4 h at ambient temperature with either digoxygenin-labelled PP1γ or [^{125}I]MC-labelled PP1γ diluted to 2.5 μg/ml in buffer B plus 1 mg/ml bovine serum albumin, then washed several times with buffer B over a 3 h period to remove unbound probe. Blots were either autoradiographed ([^{125}I]MC-PP1γ) or probed for 45 min with anti-digoxygenin antibodies conjugated with peroxidase (0.03 U/ml in buffer B plus 1 mg/ml bovine serum albumin), then washed for 3 h with buffer B and visualised with the enhanced chemiluminescence system (Amersham International, Bucks, UK).

2.6. Identification of the G_L subunit as a phosphorylase a*-binding protein*

This was carried out as in section 2.5 except that after SDS-PAGE and transfer to nitrocellulose, hepatic PP1G was probed with 10 μg/ml ^{32}P-labelled phosphorylase *a* (10^6 cpm/nmol) [23], washed three times in 25 mM Tris-HCl, pH 7.5 (30 min per wash), and autoradiographed.

2.7. Amino acid sequencing

The Coomassie blue-stained subunits of hepatic PP1G (about 4 μg) were excised from SDS-PAGE gels, washed five times with water and brought to near dryness in a vacuum concentrator. The gel pieces were suspended in 0.3 ml of 50 mM Tris-HCl, pH 8, 0.01% (v/v) alkylated Triton X-100 and digested for 24 h with 1 μg of alkylated trypsin, or suspended in 0.3 ml of 50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.01% (v/v) alkylated Triton X-100 and digested for 48 h with Lys-C endoproteinase, 1.25 μg of proteinase being added at time zero and after 24 h. The supernatants were removed and the gel pieces extracted for a further 5 h with 0.2 ml of buffer. The combined supernatants from each digest were either chromatographed on Aquapore RP-300 (100 × 2.1 mm) or on a Vydac C_{18} column (150 × 2.1 mm) which were connected to an Applied Biosystems 140B HPLC system equilibrated in 0.1% (v/v) trifluoroacetic acid. Both columns were developed with linear acetonitrile gradients in 0.1% trifluoroacetic acid with an increase in acetonitrile concentration of 0.5% per min. Peptides detected by absorbance at 214 nm were collected and analysed on an Applied Biosystems 476A Sequencer.

3. Results

3.1. Purification of glycogen-associated PP1 from rat liver

Glycogen-protein particles isolated from the livers of fed rats contain four major proteins of 160, 97, 86 and 70 kDa, corresponding to glycogen debranching enzyme, glycogen phosphorylase, glycogen synthase and glycogen branching enzyme, respectively (Fig. 1, lane 1), but the concentration of hepatic PP1G at this step is too low to be detected by staining with Coomassie blue. PP1G associated with liver glycogen–protein particles was retained by MC-Sepharose and eluted with 3 M isothiocyanate, as reported for PP1M from skeletal muscle myofibrils [4]. The PP1 activity which eluted in the first two fractions (Pool 1, Fig. 2) had a PhP/GSP activity ratio of 15.4 ± 1.6 (±S.E.M. for four preparations) similar to the value of 14.7 ± 0.6 (±S.E.M. for four preparations) in glycogen–protein particles. The GSP activity of Pool 1 was inhibited by phosphorylase *a* with an IC_{50} (11 nM) similar to that of PP1G in glycogen–protein particles (8 nM) (Fig. 3). These properties indicated that the PP1 in Pool 1 contained the subunit(s) which confers the distinctive properties of hepatic PP1G. Pool 1, which contained 0.5% of the protein applied to MC-Sepharose, was concentrated and analysed by SDS-PAGE. Debranching

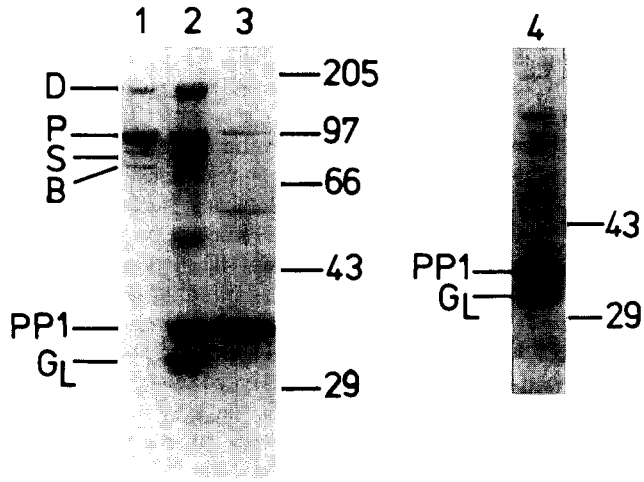

Fig. 1. SDS-10% polyacrylamide gel-electrophoresis of different fractions obtained during the purification of hepatic PP1G. The gels were stained with Coomassie blue and migration is from top to bottom. The positions of debranching enzyme (D), phosphorylase (P), glycogen synthase (S), branching enzyme (B) and the standard marker proteins myosin (205 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) are indicated. Protein phosphatase-1 (PP1) is eluted as a doublet at 38/37 kDa corresponding to the α and β isozymes, respectively (see section 3.2) and its glycogen-binding (G_L) subunit is eluted at 33 kDa. Lane 1, glycogen–protein particles from fed rats; lane 2, Pool 1 from MC-Sepharose (Fig. 2); lane 3, Pool 2 from MC-Sepharose; lane 4, PP1G further purified from Pool 1 by gel-filtration on Superose 12.

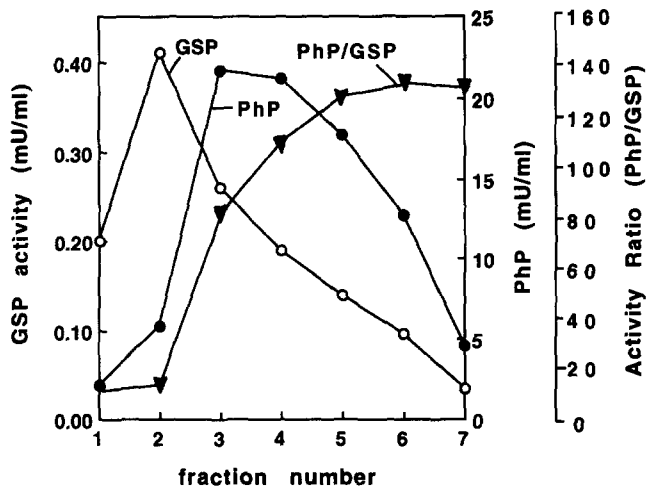

Fig. 2. Affinity chromatography of rat liver glycogen particles on microcystin-Sepharose. Glycogen–protein particles isolated from the liver of a fed rat were chromatographed on MC-Sepharose and PP1 eluted with buffer A plus 3 M isothiocyanate (see section 2.4). Fractions of 1.0 ml were collected and assayed for phosphorylase phosphatase (PhP) activity (●) and for glycogen synthase phosphatase (GSP) activity (○) with glycogen synthase labelled at site 2. The PhP/GSP activity ratio is shown by the filled triangles. Fractions (1 and 2) with a low PhP/GSP activity ratio and fractions (4–7) with a high PhP/GSP ratio were pooled separately and termed Pool 1 and Pool 2, respectively.

enzyme, phosphorylase, glycogen synthase and branching enzyme were still present, but there was a striking enrichment of a 33 kDa protein and a 38/37 kDa doublet when compared to glycogen–protein particles (compare lanes 1 and 2 in Fig. 1). The 38 and 37 kDa proteins were present in similar amounts in every preparation and continued to co-purify with the 33 kDa band, and with hepatic PP1G, through gel filtration, the higher molecular mass contaminants being largely removed at this stage (Fig. 1, lane 4). PP1G eluted from Superose 12 as a broad peak centred at 60 kDa (data not shown).

3.2. *Identification of the 38/37 kDa doublet as the α and β isoforms of PP1 and the 33 kDa band as the G_L subunit*

The cyclic heptapeptide MC binds covalently to active PP1 [4] and radiolabelled MC–PP1 complexes can therefore be identified after SDS-PAGE and autoradiography. [^{125}I]MC-YR labelled the 38/37 kDa doublet in Pool 1 or in glycogen–protein particles (Fig. 4), indicating that these bands correspond to the PP1 catalytic subunit. To establish this point definitively, the 38/37 kDa doublet was excised from the gel, digested with trypsin and several of the peptides generated were purified and sequenced. All the peptides analysed were derived from PP1. However, one peptide (IVQMTEAEVR) was unique to PP1β (residues 26–35) while another isolated in similar amounts (YGQFSGLNPGGR) was specific to PP1α (residues 306–317) [27]. Immunoblotting with isoform-specific antibodies revealed that the apparent molecular masses of PP1α and PP1β on SDS-PAGE are 38 kDa and 37 kDa, respectively [28], suggesting that the 38 kDa and 37 kDa bands in hepatic PP1G might correspond to PP1α and PP1β, respectively. Consistent with this interpretation, the catalytic subunit of PP1G from skeletal muscle, which is PP1β [29], co-migrated with the lower (37 kDa) band of the doublet (data not shown).

The 33 kDa band was not labelled with [^{125}I]MC-YR, indicating that it was not the active PP1 fragment which can be generated by proteolysis from the native 37 kDa species [15]. However, the 33 kDa band was the only protein that became labelled when Pool 1 was subjected to SDS-PAGE and blotted with [^{125}I]MC-YR-PP1γ (Fig. 5A, lane 1). The specificity of this probe for PP1-binding subunits was established by the finding that it labels only the 130 kDa PP1-binding M subunit of smooth muscle PP1M (Fig. 5A, lane 2), and not the 37 kDa

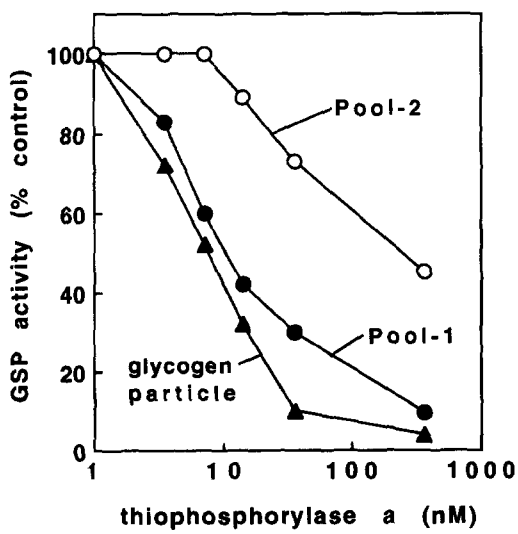

Fig. 3. The sensitivity of the glycogen synthase phosphatase activity of hepatic PP1G to thiophosphorylase *a* is unaffected by affinity purification on microcystin-Sepharose. Glycogen synthase phosphatase (GSP) activity was assayed in the presence of 5 mg/ml glycogen with glycogen synthase labelled at site 3, using hepatic glycogen–protein particles from a fed rat liver (closed triangles), Pool 1 from MC-Sepharose (●) and Pool 2 from MC-Sepharose (○) (see legend to Fig. 2).

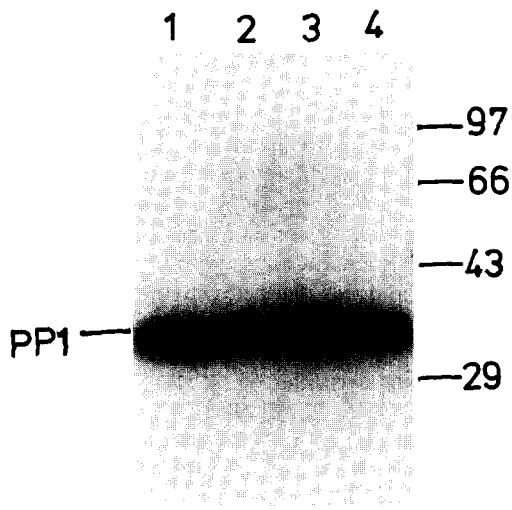

Fig. 4. Labelling of the protein phosphatase-1 catalytic subunit with [^{125}I]microcystin-YR. Fractions containing PP1 were incubated for 2 h (lane 2) and 16 h (lanes 1, 3 and 4) with [^{125}I]MC-YR as in [4], denatured in SDS, subjected to electrophoresis on a 10% polyacrylamide gel and autoradiographed. Lane 1, bacterially expressed PP1γ; lanes 2 and 3, Pool 1 from MC-Sepharose (Fig. 2); lane 4, hepatic glycogen–protein particle preparation from the liver of a fed rat. The marker proteins are as in Fig. 1.

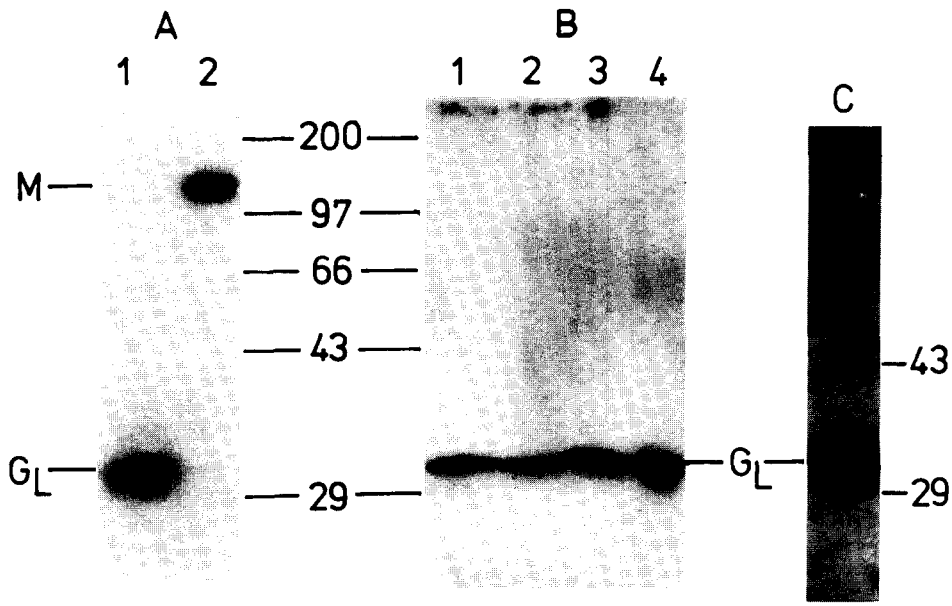

Fig. 5. Identification of the G_L subunit using [^{125}I]microcystin-YR-PP1γ, digoxygenin–PP1γ and ^{32}P-labelled phosphorylase *a* as probes. The PP1-containing fractions from Fig. 2 were denatured in SDS, electrophoresed on 10% polyacrylamide gels, transferred to nitrocellulose and blotted with the labelled PP1 probes (see section 2.5) and ^{32}P-labelled phosphorylase *a* (see section 2.6). (A) Pool 1 from chromatography of rat hepatic glycogen–protein particles on MC-Sepharose (lane 1) and purified PP1M from chicken gizzard smooth muscle (lane 2) were blotted with [^{125}I]MC-YR-PP1γ. (B) Glycogen–protein particles isolated from the liver of a fed rabbit (lane 1), a fasted rat (lane 2) and a fed rat (lane 3), and Pool 1 from MC-Sepharose (lane 4) were probed with digoxygenin–PP1γ. (C) Pool 1 from chromatography of rat hepatic glycogen–protein particles on MC-Sepharose was probed with ^{32}P-labelled phosphorylase *a*. The molecular mass standards are as in Fig. 1 and the positions of the G_L subunit of hepatic PP1G and the M subunit of smooth muscle PP1M are marked.

subunit (i.e. PP1 itself) or the 20 kDa subunit that are also present in this enzyme [16]. The 33 kDa protein in Pool 1 was also recognised specifically by a second probe, digoxygenin–PP1γ (Fig. 5B, lane 4), previously used to identify the PP1-binding subunit of smooth muscle PP1M [16]. Digoxygenin–PP1γ not only recognised the 33 kDa protein specifically in Pool 1 (Fig. 5B, lane 4), but also in glycogen–protein particles from rabbit (Fig. 5B, lane 1) and rat (Fig. 5B, lanes 2 and 3) liver. Moreover, the 33 kDa band in hepatic PP1G was recognised specifically with a ^{32}P-labelled phosphorylase *a* probe (Fig. 5C), indicating that it is a high affinity phosphorylase *a*-binding protein. The specificity of the phosphorylase *a* probe was established by the finding that it did not label the PP1 catalytic subunit, the M subunit of smooth muscle PP1M, the major glycogen-metabolising enzymes present in the glycogen-protein particles, or the molecular mass standards run on the same SDS-PAGE gel (data not shown).

The 33 kDa band was digested with Lys-C endoproteinase and the resulting peptides were purified and sequenced. Two Lys-C peptides RVSFADNQGLALTMVK and VQNLAFEK were very similar to residues 65–80 (RVSFADNFGFNLVS-VK) and residues 150–157 (VLNISFEK), respectively, of the G_M subunit of skeletal muscle $PP1_G$ [30].

The later eluting fractions (4–7) from MC-Sepharose (Pool 2) had a 7- to 10-fold higher PhP/GSP activity ratio than Pool 1 (Fig. 2), and the GSP activity of Pool 2 was only inhibited by phosphorylase *a* very weakly (Fig. 3). Pool 2 was largely the 38/37 kDa doublet and contained only traces of the 33 kDa protein (Fig. 1, lane 3). The phosphatase activity in Pool 2 was eluted from Superose 12 as a major peak of apparent molecular mass 35 kDa (data not shown), indicating that it is largely the free catalytic subunit of PP1.

4. Discussion

Our results establish that PP1G purified from rat liver is a heterodimer composed of the α or β isoform of PP1 complexed to a 33 kDa (G_L) subunit. These components were greatly enriched in Pool 1 from MC-Sepharose (Fig. 1), had a PhP/GSP activity ratio and sensitivity to phosphorylase *a* indistinguishable from hepatic glycogen–protein particles, and continued to co-purify through gel-filtration on Superose 12. The G_L subunit was recognised by two PP1 probes and by phosphorylase *a* (Fig. 5) and remained entirely bound to glycogen after incubation of hepatic glycogen–protein particles for 10 min with 1 M LiBr, a treatment which released 90% of the glycogen synthase phosphatase activity (data not shown). This demonstrates that the G_L subunit is the glycogen binding component.

Two peptides from G_L were strikingly similar to sequences found in the glycogen-binding subunit from skeletal muscle. The sequence homologous to residues 150–157 of G_M lies within the region speculated to correspond to the glycogen-binding site (see legend of Fig. 2 in [1]), while the sequence highly homologous to residues 65–80 of G_M contains the serine residue whose phosphorylation of which by cyclic AMP-dependent protein kinase triggers the dissociation of PP1 from the G_M subunit [12]. The latter finding is intriguing because no evidence currently exists for a role of phosphorylation in the regulation of hepatic PP1G.

The small size of the G_L subunit was surprising since the molecular mass of G_M is 124 kDa [30] and the G subunit homologue in *S. cerevisiae* (GAC-1) is an 88 kDa protein [31]. However, the 33 kDa protein does not seem to be a proteolytic fragment of a larger precursor, because it retains all the known properties of the putative G_L subunit, and no other PP1-bind-

ing protein was detected, even in rat or rabbit glycogen–protein particles isolated rapidly at 0–4°C in the presence of several proteinase inhibitors (Fig. 5B). The 33 kDa protein was also present in hepatic glycogen–protein particles isolated according to [26] (Fig. 5B, lane 2) which are free of microsomes. This eliminates the possibility that the 33 kDa protein is a microsomal PP1-targetting subunit present as a contaminant. It is particularly noteworthy that no PP1-binding protein with an apparent molecular mass of 160 kDa was detected in glycogen–protein particles. The 160 kDa protein present in a purified preparation of PP1G from rat liver and suggested to be the G_L subunit in [22] may, therefore, be an impurity (perhaps glycogen debranching enzyme), while the minor 32 kDa band in this preparation may be the G_L subunit and not a proteolytic fragment of PP1. The G_L subunit is homologous to the N-terminal domain of G_M, which is known to contain the binding sites for PP1 and for glycogen [32]. It may well be that the G_L subunit is much smaller than its muscle counterpart because no C-terminal SR-binding domain is required (see section 1). Detailed analysis of the structure of G_L is in progress.

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