The level of the glycogen targetting regulatory subunit R5 of protein phosphatase 1 is decreased in the livers of insulin-dependent diabetic rats and starved rats

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Hepatic glycogen synthesis is impaired in insulin-dependent diabetic rats owing to defective activation of glycogen synthase by glycogen-bound protein phosphatase 1 (PP1). The identification of three glycogen-targetting subunits in liver, G_L, R5/PTG and R6, which form complexes with the catalytic subunit of PP1 (PP1c), raises the question of whether some or all of these PP1c complexes are subject to regulation by insulin. In liver lysates of control rats, R5 and R6 complexes with PP1c were found to contribute significantly (16 and 21 % respectively) to the phosphorylase phosphatase activity associated with the glycogen-targetting subunits, G_L–PP1c accounting for the remainder (63 %). In liver lysates of insulin-dependent diabetic and of starved rats, the phosphorylase phosphatase activities of the R5 and G_L complexes with PP1c were shown by specific immunoadsorption assays to be substantially decreased, and the levels of R5 and G_L were shown by immunoblotting to be much lower than those in control extracts. The phosphorylase phosphatase activity of R6–PP1c and the concentration of R6 protein were unaffected by these treatments. Insulin administration to diabetic rats restored the levels of R5 and G_L and their associated activities. The regulation of R5 protein levels by insulin was shown to correspond to changes in the level of the mRNA, as has been found for G_L. The *in vitro* glycogen synthase phosphatase/phosphorylase phosphatase activity ratio of R5-PP1c was lower than that of G_L–PP1c, suggesting that R5–PP1c may function as a hepatic phosphorylase phosphatase, whereas G_L–PP1c may be the major hepatic glycogen synthase phosphatase. In hepatic lysates, more than half the R6 was present in the glycogen-free supernatant, suggesting that R6 may have lower affinity for glycogen than R5 and G_L

Key words: diabetes, glycogen metabolism, insulin, PP1, starvation.

INTRODUCTION

Protein phosphatase 1 (PP1) is a major phosphatase in eukaryotic cells that dephosphorylates serine and threonine residues in proteins. The enzyme regulates numerous distinct cellular processes by interaction of the catalytic subunit of PP1 (PP1c) with a diverse range of targetting subunits that localize PP1c to specific sites within the cell, modulate its activity towards particular substrates and allow the heterodimers to respond differentially to cellular signals. Interaction of most targetting subunits with PP1c occurs via a short highly conserved motif (-RVXF- in the glycogen-targetting subunits), which explains why the binding of otherwise dissimilar subunits is mutually exclusive [1–4]. The hormonal regulation of glycogen metabolism encompasses key roles for PP1 through the dephosphorylation of glycogen synthase, phosphorylase and phosphorylase kinase. Four distinct glycogen-targetting subunits of PP1 have been identified. A skeletal-muscle protein, G_M, present also in diaphragm and heart muscle, is a 124–126 kDa protein that targets PP1c to membranes of the sarcoplasmic reticulum as well as to glycogen [5–7]. The binding of G_M to PP1c modulates the specificity of PP1c, enhancing activity towards phosphorylase, phosphorylase kinase and glycogen synthase, but not towards other substrates such as myosin P-light chains [1]. A 33 kDa hepatic protein, G_L, shows 23 % identity to the N-terminal quarter of G_M, and was demonstrated to possess a glycogen-binding domain and PP1c interaction motif [8–10]. G_L binds to PP1c, enhancing the dephosphorylation of glycogen synthase and suppressing the dephosphorylation of phosphorylase. A database search identified a 36 kDa protein, R5, that showed 42 % identity with G_L and 28 % identity with G_M respectively, and modulated the specificity of PP1c towards phosphorylase [11]. R5 mRNA is expressed in a variety of tissues, with the highest levels being in liver, skeletal muscle and heart muscle [11]. Yeast two-hybrid screens with PP1c as bait identified PTG, a murine homologue of human R5 [12] and U5, a probable homologue of R5 in chicken [13]. Overexpression of PTG in Chinese-hamster ovary cells expressing the insulin receptor raised basal glycogen synthesis and glycogen synthesis in insulin-stimulated cells, but did not increase the ratio of insulin-stimulated/basal glycogen synthesis [12]. A 33 kDa human protein, R6, identified in a database search, showed 31 % identity with G_L and 27 % identity with G_M respectively, and it was shown to bind to both glycogen and PP1c [14]. R6 mRNA shows a wide tissue distribution, with similar levels in different tissues.

A major mechanism by which insulin increases glycogen synthesis in the liver is through dephosphorylation and activation of the rate-determining enzyme, glycogen synthase. The reaction

Abbreviations used: PP1, protein phosphatase 1; PP1c, protein phosphatase 1 catalytic subunit; G_M (also termed R_{GL}), skeletal-muscle glycogen targetting subunit of PP1 encoded by the gene *PPP1R3(3A)*; G_L, hepatic glycogen targetting subunit of PP1 encoded by the gene *PPP1R4(3B)*; R5 (also termed PTG), regulatory subunit of PP1 encoded by the gene *PPP1R5(3C)*; R6, regulatory subunit of PP1 encoded by the gene *PPP1R6(3D)*; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK'); MBP, maltose-binding protein; GST, glutathione S-transferase; ECL® (Amersham), enhanced chemiluminescence.

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is catalysed by glycogen-targetted PP1c. Insulin is believed to regulate one form of glycogen-bound phosphatase, G_L–PP1c, by two mechanisms: in the short term through the modulation of G_L–PP1c activity allosterically by phosphorylase *a* [15–17,10] and in the longer term by modulation of the level of G_L protein [18]. Stimulation of hepatic glycogenolysis by glucagon (acting via cyclic AMP and protein kinase A) and α-adrenergic agonists (acting via Ca^{2+}) results in the activation of phosphorylase kinase and an increase in the level of the active phosphorylated form of glycogen phosphorylase (phosphorylase *a*). Phosphorylase *a* binds to G_L [8,9] and potently inhibits its glycogen synthase phosphatase activity, thereby inhibiting glycogen synthesis. Insulin lowers hepatic cAMP levels, causing a decrease in the level of phosphorylase *a* and alleviation of the phosphorylase *a*-mediated inhibition of the G_L–PP1c complex, while the binding of glucose to phosphorylase *a* increases the rate at which phosphorylase is inactivated. These processes contribute to the stimulation of glycogen synthesis by insulin and high blood glucose [19]. The inhibition of glycogen synthase phosphatase (but not phosphorylase phosphatase) activity of the G_L–PP1c complex by phosphorylase *a* occurs at nanomolar concentrations in the presence of glycogen [20–22]. The site of interaction of phosphorylase *a* with G_L was mapped to 16 amino acids at the extreme C-terminus of G_L, a sequence that is absent from the other glycogen-targetting subunits, G_M, R5 and R6 [10].

Regulation of the level of G_L protein by insulin was demonstrated in rats in which insulin-dependent diabetes had been induced by destruction of the pancreatic β-cells with streptozotocin. This system provides an animal model for human Type I (insulin-dependent) diabetes mellitus, in which loss of insulin production results from autoimmune destruction of the pancreatic β-cells. In streptozotocin-diabetic animals the activation of hepatic glycogen synthase in response to raised glucose levels is severely impaired, owing to a specific loss of the glycogen-bound glycogen synthase phosphatase activity [23–25] that was shown to be a form of PP1 [20,26]. Treatment with insulin restored the glycogen synthase phosphatase activity [24,25,17]. Analysis of G_L protein by immunoblotting showed that the level was severely decreased in streptozotocin-diabetic rats and that it could be restored to control levels by insulin treatment [18]. Starvation also caused a decrease in the level of the G_L protein, which was restored by refeeding. The regulation of the G_L protein levels by insulin and starvation/feeding was shown to correlate with changes in the level of the G_L mRNA [18].

The recognition that other PP1 glycogen-targetting subunits, R5/PTG and R6, are found in the liver raises the question of whether complexes of these regulatory subunits with PP1c contribute significantly to the hepatic glycogen synthase phosphatase and phosphorylase phosphatase activities and, if so, whether their activities are modulated by insulin. Although R5 and R6 do not possess a phosphorylase *a* binding sequence similar to that in G_L, we show here that R5 and R6 contribute approx. 37% of the total phosphorylase phosphatase activity associated with the glycogen-targetting subunits and that the level of hepatic R5, but not R6, is decreased in diabetes and can be restored by insulin treatment.

MATERIALS AND METHODS

Treatment of animals and subcellular fractionation

Experiments were performed with male Wistar rats weighing about 300 g and fed *ad libitum* unless otherwise stated. The animals were killed by decapitation without prior anaesthesia between 09.00 and 11.00 h. Diabetes was induced by an intravenous injection of streptozotocin (55 mg/kg), and the animals were killed 4 days later, or they received at that time one subcutaneous injection of insulin (Insulin Lente; Novo-Nordisk A/S, Bagsværd, Denmark; 5 units) and were killed 24 h later, or they received daily injections of Insulin Lente (5 units) for 4 days and were killed 96 h after the first insulin injection. Blood glucose levels, measured as described in [24], ranged from 4.7 to 6.3 mg/ml in diabetic animals prior to insulin treatment. For investigation of the effect of withholding food, rats were fed *ad libitum* or starved for 19–48 h. The animals were killed by suffocation in CO_2, followed by cervical dislocation. Liver was freeze-clamped and stored at −80 °C. The liver was homogenized in 3 vol./g of ice-cold 2 mM EDTA, pH 7.0, 2 mM EGTA, 250 mM sucrose, 0.1% (v/v) 2-mercaptoethanol, 4 μg/ml leupeptin, 1 mM benzamidine, 0.1 mM PMSF and 0.5 mM tosylphenylalaninylchloromethane (Tos-Phe-CH_2Cl; 'TPCK'), and lysates were prepared by centrifugation of the homogenates at 16000 ***g*** for 15 min. For preparation of the glycogen fractions, livers of rats fed *ad libitum* were excised without freezing and homogenized at 0–4 °C as described above. The 16000 ***g*** lysate was subjected to centrifugation at 100000 ***g*** for 90 min to sediment the glycogen and microsomes. The supernatant was decanted and retained. The upper microsomal/glycogen layer was resuspended in one volume/g liver of buffer A [50 mM Tris/HCl, pH 7.0, 0.1 mM EGTA, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 0.5 mM PMSF, 0.5 mM Tos-Phe-CH_2Cl, 1 mM benzamidine and 4 μg/ml leupeptin]. The glycogen pellet was resuspended in 1 vol. of buffer A plus 300 mM NaCl and re-centrifuged at 100000 ***g*** for 90 min. The supernatant was discarded and the purified glycogen protein particles resuspended in 0.5 vol. of buffer A. All fractions were frozen in aliquots and stored at −80 °C.

Antibodies and immunological methods

Affinity-purified antibodies to bacterially expressed human PP1γ were coupled to Protein G–Sepharose as described in [27]. Antibodies to rat G_L protein fused to glutathione S-transferase (GST) and affinity-purified against maltose-binding protein (MBP)–G_L were described in [18]. Antibodies to human R5 protein fused to GST were raised [11] and affinity-purified against MBP–R5. Peptides corresponding to amino acids 11–24 of rat G_L (SMAPSLRRERFTFK), 36–49 of mouse R5 (GPYNGFQRRNFVNK) and of 45–60 of rat PPP1R6 (RPIIQRRSRSLPTSPE) [14] (synthesized by Dr Graham Bloomberg, Department of Biochemistry, University of Bristol, Bristol, U.K.) were conjugated to a mixture of BSA and keyhole-limpet (*Diodora aspera*) haemocyanin and injected into separate sheep by Diagnostics Scotland (Carluke, Lanarkshire, Scotland, U.K). The anti-peptide antibodies were purified by affinity chromatography of the antisera against their respective peptides coupled to Sepharose-4B and then coupled to Protein G–Sepharose [27] and resuspended in an equal volume of 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100 and 0.1% (v/v) 2-mercaptoethanol.

To prepare immunoblots, proteins in liver extracts and subcellular fractions were separated by SDS/PAGE, transferred to nitrocellulose membranes and probed with 0.1 μg/ml affinity-purified antibodies. Staining was detected using anti-(sheep IgG) antibodies conjugated to horseradish peroxidase (Pierce), followed by enhanced chemiluminescence (ECL®; Amersham).

Immunoadsorption from cell lysates or subcellular fractions was performed as follows. The lysate or extract was diluted to 1 mg/ml in buffer B [50 mM Tris/HCl, pH 7.5, 150 mM NaCl,

0.1 mM EGTA, 5% (v/v) glycerol, 0.5% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 0.5 mM PMSF, 0.5 mM Tos-Phe-CH_2Cl, 1.0 mM benzamidine and 4 μg/ml leupeptin] and centrifuged for 5 min at 16000 ***g*** at 4 °C to sediment any insoluble material. A 100 μl portion of the supernatant was added to 20 μl of the antibody-coupled Protein G–Sepharose beads and shaken gently for 1 h at 4 °C. The antibody-coupled Protein G–Sepharose beads were sedimented by centrifugation for 30 s at 16000 ***g***, the supernatant decanted and the pellet washed four times with 1 ml of buffer B. The immunoprecipitated protein was analysed by SDS/PAGE directly and/or assayed for phosphatase activity after washing the beads in 1 ml of 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 0.01% (v/v) Brij-35.

Protein phosphatase assays

Glycogen phosphorylase and phosphorylase kinase were prepared from rabbit skeletal muscle by Miss F. Douglas and Dr N. Morrice [28], and human glycogen synthase kinase 3β was expressed in the baculovirus/insect-cell system by Dr A. Patterson [29]. ^{32}P-labelled glycogen phosphorylase was prepared by phosphorylation with phosphorylase kinase to a stoichiometry of 1 mol of phosphate/mol of subunit [28]. ^{32}P-labelled rabbit skeletal muscle glycogen synthase [30] was phosphorylated in the site-3 region with glycogen synthase kinase-3β to a stoichiometry of 1.5 mol of phosphate/mol of subunit [29,31]. Phosphorylase phosphatase and glycogen synthase phosphatase assays were carried out in the absence of bivalent cations using standard procedures [28,32], except that for the assay of immunoprecipitated phosphatase activity, the washed immune pellet was resuspended in an equal volume of a solution containing 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol and 1 mg/ml BSA, and the assay was performed on a shaking platform (1200 rev./min) at 30 °C. The substrate concentrations in the assays were 10 μM ^{32}P-labelled phosphorylase or 1 μM ^{32}P-labelled glycogen synthase. One unit of activity is the amount of enzyme that catalyses the release of 1 μmol of [^{32}P]phosphate/min. The phosphorylase phosphatase and glycogen synthase phosphatase activities were assayed before ('spontaneous' activity) or after preincubation with 0.1 mg/ml 'dissociating' peptide (GKRTNLRKTGSERIAMGMRVKFNPLALLLDSC) for 5 min at 30 °C ('total' activity), that causes the release of free PP1c from the glycogen-targetting subunit [33].

Measurement of R5 mRNA levels by multiplex RT-PCR

Total RNA was extracted from liver, stored, and ground at −80 °C using TRIzol Reagent (Gibco BRL, Gaithersburg, MD, U.S.A.) according to manufacturer's instructions. For preparation of first-strand cDNA, total RNA was diluted in diethyl pyrocarbonate-treated water to 0.2 μg/μl, denatured at 85 °C for 3 min and chilled on ice. A 5 μl portion of the total RNA was mixed with 20 μl of RT-mix [50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 units of Moloney-murine-leukaemia-virus reverse transcriptase (Promega Corp., Madison, WI, U.S.A.), 40 units of RNAsin (Promega), 3 μg of random hexamers (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), and 0.9 mM dNTPs (Amersham Pharmacia Biotech)]. This was left at room temperature for 10 min and subsequently incubated at 37 °C for 1 h, after which the reaction mixture was diluted with 50 μl of water. Multiplex PCR was set up in a 50 μl total reaction volume using 3 μl of the diluted cDNA reaction mixture and 47 μl of PCR-mix {*Taq* DNA polymerase (2.5 units) and buffer (Promega Corp.), dNTP mix (final concns. of 40 μM dTTP, dGTP and dCTP; 20 μM dATP), 2.5 μCi of 3000 Ci/mmol [α-^{32}P]dATP (Amersham) and 10 pmol of each primer}. One primer pair was specific for rat R5, amplifying a 334-bp PCR fragment (forward primer 5′ ATG GCC ATG AGG GTT TGC 3′ and a reverse primer 5′ CCA GAT TTT TCT CCT CGT 3′) and a second set specific for rat β-actin control, amplifying a 133-bp fragment (forward primer 5′ GGT TCC GCT GCC CTG AGG CAC 3′ and reverse primer 5′ CAC TGT GTT GGC GTA GAG GTC 3′). Mineral oil was added to each tube. A single denaturing step at 94 °C/1 min was followed by 30 cycles of: 94 °C/30 s; 52 °C/1 min; 72 °C/1 min 30 s, and an elongation step at 72 °C/10 min. The final PCR reaction mixtures were mixed with Novex TBE/Urea sample buffer (2×) and run on Novex 6% TBE/Urea gels (Invitrogen Corp., Carlsbad, CA, U.S.A.) in Novex TBE running buffer (5×) for 50 min at 180 V. The gels were then dried and exposed to a PhosphoImager screen (Fuji Photo Film Co., Tokyo, Japan) and the bands quantified using Aida 200 analysis software (Raytest version 2).

RESULTS

Characterization and relative levels of PP1c complexes with R5, R6 and G_L

The R5 regulatory subunit of PP1 in rat and rabbit was detected as an approx. 36 kDa protein on blots of skeletal muscle and liver glycogen fractions probed with anti-(human R5) antibody (results not shown). The size is consistent with the predicted molecular mass of 36 kDa for the human R5 protein [11]. However, as antibodies raised to the full-length R5 protein could potentially cross-react with related glycogen-targetting subunits, further antibodies were raised to short (14–16 amino acid) sections of the N-termini of rat R5, R6 and G_L that exhibited minimal identity with the other glycogen-targetting subunits. Each affinity-purified peptide antibody was judged to be specific for its corresponding bacterially expressed glycogen-targetting subunit (Figure 1). Liver lysates were prepared from adult male Wistar rats and immunoadsorption was carried out with the appropriate peptide antibodies coupled to Protein G–Sepharose beads. The immune pellets were assayed for phosphorylase phosphatase activity (Figure 2). Immunoadsorption of the R5-associated phosphorylase phosphatase activity by the anti-(R5

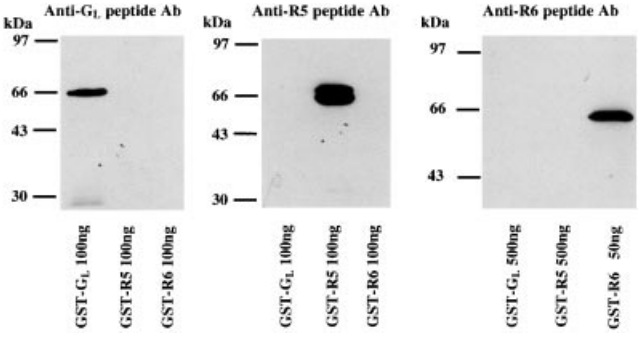


Figure 1 Specificity of the antibodies raised against G_L, R5 and R6 peptides

The indicated amounts of G_L, R5 and R6 proteins fused to GST were denatured in SDS buffer, subjected to electrophoresis on an SDS/10% polyacrylamide gel, transfered to nitrocellulose and probed with 1 μg/ml of anti-(R6 peptide) antibody (Ab), 1 μg/ml of anti-(G_L peptide) antibody and 1 μg/ml of anti-(R5 peptide) antibody. Immunoreactive bands were detected by ECL®. Note that GST–R5 appears as a doublet owing to proteolytic cleavage during bacterial expression and purification. Molecular mass markers in kDa are indicated.

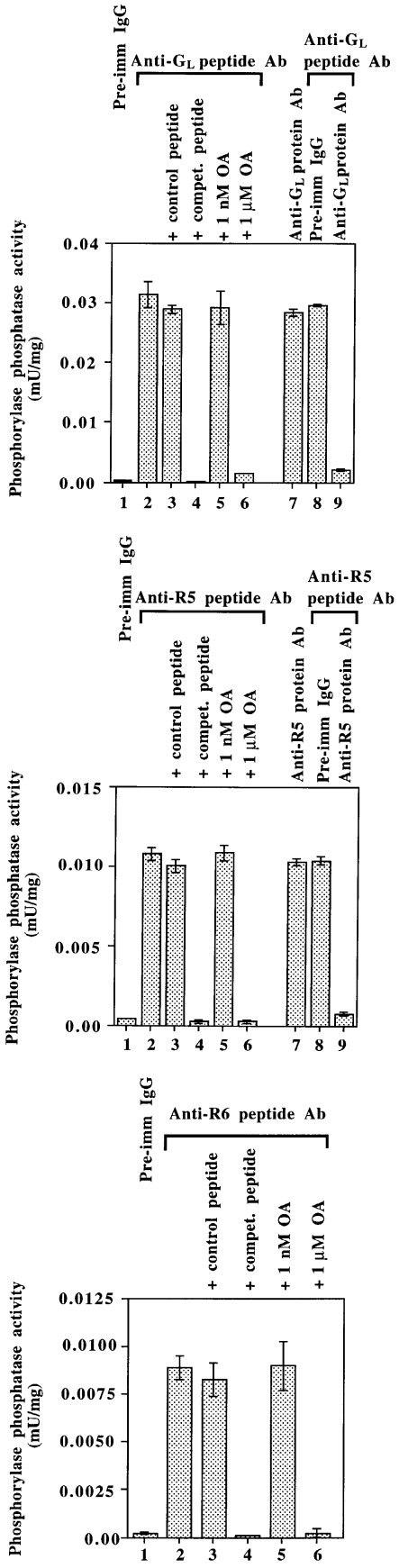


Figure 2 Characterization of the G_L-, R5- and R6-associated phosphorylase phosphatase activities

PP1c complexes with G_L, R5 and R6 were immunoadsorbed from 100 μl of a 1 mg/ml rat liver lysate in the presence or absence of added control or competitive peptide for the anti-peptide antibodies (Abs), or in the presence of 1 nM or 1 μM okadaic acid (OA) or with antibodies raised to G_L or R5 proteins. The immunoadsorbed phosphorylase phosphatase activity is expressed as munits (mU)/mg of total protein in the rat liver lysate. Results are expressed as the mean $\pm$ S.E.M. for triplicate immunoadsorption assays. Upper panel: lanes are: (1) pre-immune IgG; (2) anti-(G_L peptide antibody); (3) anti-(G_L peptide antibody) + 50 μM control RPCIQLGSKDEAGR peptide; (4) anti-(G_L peptide) antibody + 50 μM competitive SMAPSLRRERFTFK peptide; (5) anti-(G_L peptide) antibody + 1 nM OA; (6) anti-(G_L peptide) antibody + 1 μM OA; (7) anti-(G_L peptide) antibody; (8) anti-(G_L peptide) antibody after pre-clearing with pre-immune IgG; (9) anti-(G_L peptide) antibody after pre-clearing with anti-G_L antibody. Middle panel: lanes are: (1) pre-immune IgG; (2) anti-(R5 peptide) antibody; (3) anti-(R5 peptide) antibody + 50 μM control KPCLSVKQEAKSQS peptide; (4) anti-(R5 peptide) antibody + 50 μM competitive GPYNGFQRRNFVNK peptide; (5) anti-(R5 peptide) antibody + 1 nM OA; (6) anti-(R5 peptide) antibody + 1 μM OA; (7) anti-R5 antibody; (8) anti-(R5 peptide) antibody after pre-clearing with pre-immune IgG; (9) anti-(R5 peptide) antibody after pre-clearing with anti-R5 antibody. Lower panel: lanes are: (1) pre-immune IgG; (2) anti-(R6 peptide) antibody; (3) anti-(R6 peptide) antibody + 50 μM control KGPDSALLPSTPGPRK peptide; (4) anti-(R6 peptide) antibody + 50 μM competitive RPIIQRRSRSLPTSPE peptide; (5) anti-(R6 peptide) antibody + 1 nM OA; (6) anti-(R6 peptide) antibody + 1 μM OA.

peptide) antibody was completely blocked by inclusion of the immune peptide, but not by a control peptide (Figure 2, middle panel). The observation that the R5-associated phosphorylase phosphatase activity was inhibited by 1 μM, but not 1 nM, okadaic acid indicates that the activity is due to PP1 and not the related PP2A-like phosphatases. Sequential immunoadsorption with anti-R5 antibody or pre-immune IgG followed by anti-(R5 peptide) antibody showed that both R5 antibodies precipitated the same activity. Experiments with anti-(G_L peptide) antibody and an antibody to the full-length G_L gave similar results for the specificity of the G_L antibodies and characterization of the associated phosphorylase phosphatase activity (Figure 2, upper panel). In the case of R6, only a peptide antibody was available for characterization of the associated phosphorylase phosphatase activity (Figure 2, lower panel).

The relative proportions of G_L, R5 and R6 complexes with PP1 were initially determined in the purified glycogen fraction from rat liver using glycogen-targetting subunit-specific immunoadsorption, followed by assay of the associated phosphorylase phosphatase activity (Figure 3, upper panel). Immunoblotting of the supernatants after immunoadsorption demonstrated that the correct glycogen-targetting subunit had been specifically and completely removed from the glycogen fraction (Figure 3, lower panel). Assay of the immune pellets showed that the G_L–PP1 accounted for over 90% of the phosphorylase phosphatase activity present, with R5–PP1 accounting for most of the remainder. Further assay of the immune pellets demonstrated that the G_L–PP1 also accounted for over 90% of the glycogen synthase phosphatase activity in the purified hepatic glycogen fraction (Figure 3, upper panel). Assay of the supernatant after the immunoadsorption of G_L confirmed that $> 85\%$ of the glycogen synthase phosphatase activity was recovered by anti-G_L antibodies (results not shown).

However, investigations of the relative proportions of G_L, R5 and R6 complexes with PP1 in the rat liver lysate indicated that the contributions of R5–PP1 and R6–PP1 activities were significantly higher than those observed in the purified glycogen fraction. Therefore analysis of the activities of the different glycogen-targetted forms of PP1 in the lysate and various subcellular fractions was performed. Table 1 shows that only 12% of the total G_L-associated phosphorylase phosphatase activity in the lysate was present in the purified glycogen fraction, 71% being located in the crude microsomal/glycogen fraction that formed a distinct layer above the purified glycogen pellet. In the case of R5, 81% of the associated activity was present in the

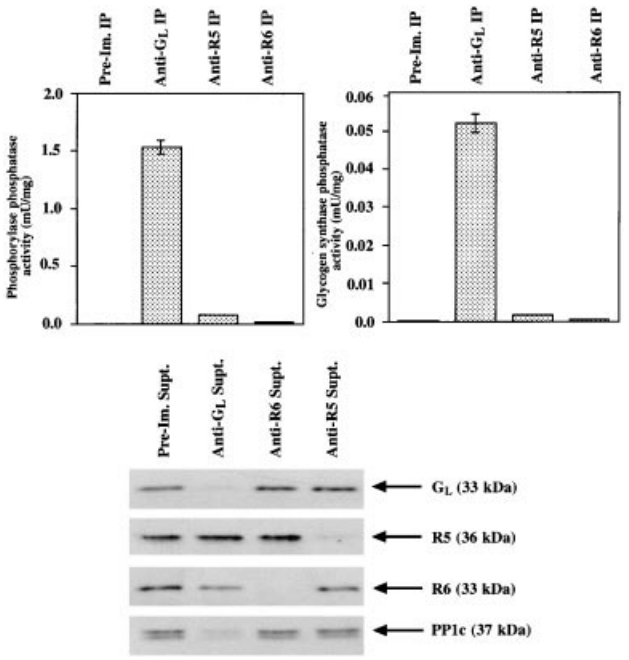


Figure 3 Quantification of the phosphorylase phosphatase and glycogen synthase phosphatase activities immunoadsorbed from a rat liver glycogen fraction using antibodies (Abs) specific for each of the PP1 glycogen targetting subunits

PP1c complexes with G_L, R5 and R6 were immunoadsorbed from a rat liver glycogen fraction by anti-peptide antibodies coupled to Protein G–Sepharose. Upper panel: immune pellets assayed for phosphorylase phosphatase and glycogen synthase phosphatase activities. The immunoadsorbed activities are expressed as munits (mU)/mg of total protein in the rat liver glycogen-protein fraction. Results are expressed as the mean ± S.E.M. for triplicate immunoadsorption assays. Samples are pre-immune IgG pellet ('Pre-Im. IP'), anti-(G_L peptide) antibody pellet ('Anti-G_L IP'), anti-(R5 peptide) antibody pellet ('Anti-R5 IP') and anti-(R6 peptide) antibody pellet ('Anti-R6 IP'). Lower panel: the immune supernatants were denatured in SDS buffer and 2 μg of protein per lane subjected to electrophoresis on SDS/10% polyacrylamide gels, transferred to nitrocellulose and probed with 1 μg/ml of either anti-G_L, anti-R5, anti-R6-peptide or anti-PP1 antibody. Immunoreactive bands were detected by ECL®. Lanes are pre-immune IgG supernatant ('Pre-Im. Supt.'), anti-(G_L peptide) antibody supernatant ('Anti-G_L Supt.'), anti-(R6 peptide) antibody supernatant ('Anti-R6 Supt.') and anti-(R5 peptide) antibody supernatant ('Anti-R5 Supt.').

Table 2 Relative proportions of the three glycogen-targetted forms of PP1 in rat liver lysate and subcellular fractions

The 'spontaneous' phosphorylase phosphatase activities of the PP1 complexes in each fraction are given in Table 1. The percentage activity of each PP1 complex relative to the total activity of the three PP1 complexes in the same fraction is presented in this Table.

	Phosphorylase phosphatase activity (% relative to total)			
	Liver lysate	100 000 ***g*** supernatant	Microsomal/ glycogen fraction	Purified glycogen fraction
G_L–PP1c	63	46	74	92
R5–PP1c	16	8	16	7
R6–PP1c	21	46	10	1

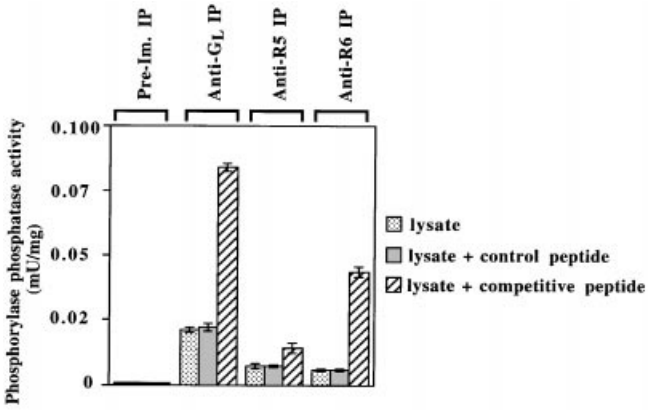


Figure 4 Effect of addition of a dissociating peptide on the phosphorylase phosphatase activity associated with each of the glycogen-targetting subunits of PP1 immunoadsorbed from a rat liver lysate

PP1c complexes with G_L, R5 and R6 were immunoadsorbed from 100 μl of 1 mg/ml rat liver lysate. The immune pellets were assayed for spontaneous phosphorylase phosphatase activity and also in the presence of dissociating peptide for determination of the total phosphorylase phosphatase activity. Dissociating GKRTNLRKTGSERIAMGMRVKFNPLALLLDSC peptide (2 μM) and control GKRTNLRKTGSERIAMGMRVKANPLALLLDSC peptide (2 μM) or buffer alone was included in each assay. The immunoadsorbed phosphorylase phosphatase activity is expressed as munits (mU)/mg of total protein in the rat liver lysate. Error bars indicate the S.E.M. for triplicate immunoadsorption assays. Samples were pre-immune IgG pellet ('Pre-Im. IP'), anti-(G_L peptide) antibody pellet ('Anti-G_L IP'), anti-(R5 peptide) antibody pellet ('Anti-R5 IP') and anti-(R6 peptide) antibody pellet ('Anti-R6 IP').

crude microsomal/glycogen fraction, with only 3% in the purified glycogen pellet. More remarkable was the observation that most of the R6 (53%) was present in the supernatant fraction, whereas only 0.3% was found in the purified glycogen fraction. Endogenous R6 has previously been demonstrated to bind specifically to glycogen [14], and endogenous R5 was similarly demonstrated to bind to glycogen, being released from glycogen particles by α-amylase treatment and not by Triton X-100 (results not shown). Therefore the differential distribution of

Table 1 Distribution of the three glycogen-targetted forms of PP1 in various rat liver fractions

Each fraction was immunoassayed in triplicate. The 'spontaneous' phosphorylase phosphatase activity of each PP1 complex, expressed as munits/mg of protein, is the mean ± S.E.M for samples from four rats. The percentage of each PP1 complex in each subcellular fraction is indicated in parentheses, the values in the lysate being assumed to be 100% for each complex.

	Activity (munits/mg) (% of each complex)			
	Liver lysate	100 000 ***g*** supernatant	Microsomal/glycogen fraction	Purified glycogen fraction
G_L–PP1c	0.024 ± 0.006 (100)	0.006 ± 0.002 (17)	0.079 ± 0.02 (71)	1.313 ± 0.4 (12)
R5–PP1c	0.006 ± 0.0006 (100)	0.001 ± 0.0005 (16)	0.017 ± 0.001 (81)	0.095 ± 0.02 (3)
R6–PP1c	0.008 ± 0.002 (100)	0.006 ± 0.0001 (53)	0.011 ± 0.002 (46)	0.009 ± 0.004 (0.3)

Table 3 'Spontaneous' phosphorylase phosphatase activity and 'total' phosphorylase phosphatase activity (measured in the presence of dissociating peptide) of the three glycogen-targetted forms of PP1 in the rat liver lysate and purified glycogen fraction

Each fraction was assayed in triplicate. The phosphorylase phosphatase activity of each PP1 complex, expressed as munits/mg of protein, is the mean ± S.E.M for samples from four rats. For the 'total' activities, the percentage activity of each PP1 complex relative to the combined activities of the three PP1 complexes in that fraction is indicated in parentheses. The corresponding percentages for the spontaneous phosphorylase phosphatase activities are given in Table 2. The percentage inhibition of PP1c by the glycogen-targetting subunits is an average of the inhibition seen in the lysate and glycogen fraction. For R6–PP1c, the low activities allow only an approximation of the percentage inhibition of this complex by its targetting subunit.

	Phosphorylase phosphatase activity (munits/mg) (% activity of each complex)				
	Liver lysate		Glycogen fraction		
	Spontaneous	Total	Spontaneous	Total	PP1c inhibited by targetting subunit (%)
G_L–PP1c	0.025 ± 0.005	0.078 ± 0.006 (55)	1.247 ± 0.3	5.465 ± 0.7 (95)	73
R5–PP1c	0.007 ± 0.0002	0.018 ± 0.005 (13)	0.110 ± 0.02	0.225 ± 0.004 (4)	57
R6–PP1c	0.008 ± 0.003	0.046 ± 0.01 (32)	0.009 ± 0.003	0.085 ± 0.001 (1)	≈ 86

Table 4 'Spontaneous' glycogen synthase phosphatase (GSP) activity and 'total' GSP activity (measured in the presence of dissociating peptide) of the three glycogen-targetted forms of PP1 in the rat liver glycogen fraction

The GSP activity of each PP1 complex is expressed as munits/mg of protein in each fraction ± S.E.M. for triplicate immunoassays. The percentage inhibition of the GSP activity of PP1c by each glycogen-targetting subunit is given. The spontaneous GSP activity of each complex as a percentage of the total GSP activity was calculated. The spontaneous phosphorylase phosphatase (PhP) activity of each complex as a percentage of the total PhP activity was calculated from the data in Table 3. The ratio of these two values is the GSP/PhP activity ratio.

	Glycogen fraction GSP activity (munits/mg)		PP1c inhibited by targetting subunit (%)	GSP/PhP activity ratio
	Spontaneous	Total		
G_L-PP1c	0.052 ± 0.017	0.104 ± 0.003	50	1.9
R5–PP1c	0.0016 ± 0.000048	0.0032 ± 0.00012	50	0.9
R6–PP1c	0.0004 ± 0.000025	0.0016 ± 0.000027	75	≈ 2

the glycogen-targetted complexes of PP1 suggests that these proteins may bind to glycogen with different affinities, G_L binding the most tightly and R6 having the lowest affinity and therefore dissociating during the isolation of the glycogen pellet. Calculation of the relative proportions of G_L, R5 and R6 complexes with PP1 in the rat liver lysate showed that G_L–PP1 accounted for 63% of the total phosphorylase phosphatase activity present, R5–PP1 contributing 16% and R6–PP1 21% (Table 2).

Table 5 Plasma glucose levels in control rats, streptozotocin-induced diabetic rats and 24 h- or 96 h-insulin-treated streptozotocin-induced diabetic rats

Column headings A and B refer to rats A and B, as described in the legend for Figure 5.

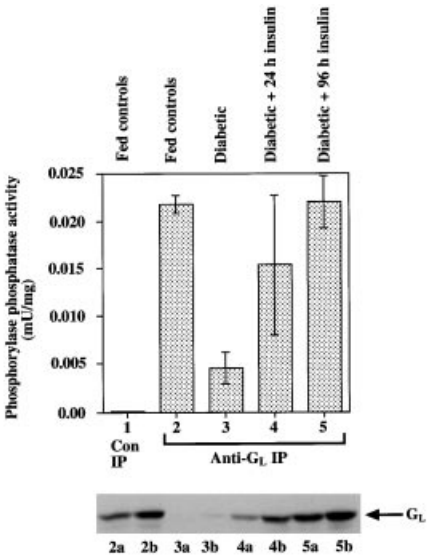
	Plasma glucose (mg/ml) Individual values		
Rats	A	B	Average
Control	1.3	1.5	1.4
Streptozotocin-induced diabetic	5.9	5.1	5.5
Diabetic + 24 h-insulin-treated	6.3	4.7	5.5
Diabetic + 96 h-insulin-treated	1.6	3.0	2.3

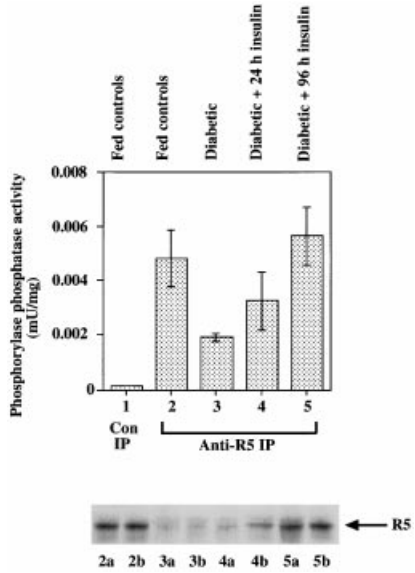
Glycogen synthase/phosphorylase phosphatase ratios of the PP1c complexes with R5, R6 and G_L

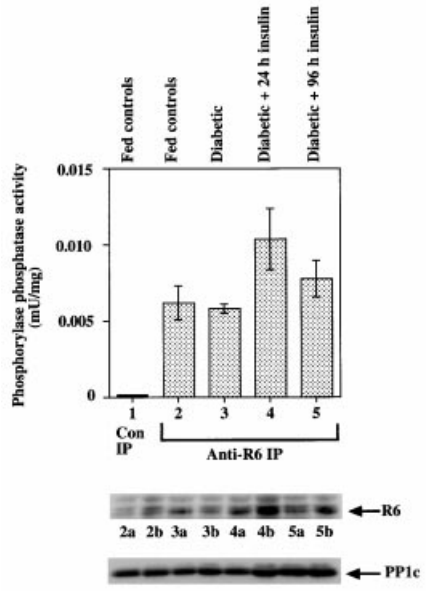
The interaction of regulatory subunits with PP1c may modify substrate specificity, decreasing the activity of PP1c against some substrates while increasing it against others. In order to investigate whether interaction of the glycogen-targetting subunits modified the activity of PP1c towards phosphorylase and glycogen synthase, activities of the glycogen-targetting subunit–PP1 complexes were measured in the presence and absence of a peptide corresponding to residues 780–810 of the PP1- and p53-binding protein, termed 53BP2. This peptide contains the PP1-binding motif -RVKF-, which has been shown to dissociate regulatory subunits, including G_M, from PP1 [3,33]. Addition of dissociating peptide relieved the inhibition of phosphorylase phosphatase activity exerted by G_L, R5 and R6 in immune pellets from rat liver lysate (Figure 4) and glycogen fraction (Table 3). A control peptide, in which the phenylalanine residue in the -RVXF- motif was replaced by alanine, had no effect on the activities of the G_L, R5 and R6 complexes with PP1c (Figure 4) or on free PP1c (results not shown). Assays in the presence of dissociating peptide measure the PP1c bound to each targetting subunit and this is highest for G_L (55%), followed by R6 (32%) and R5 (13%). Inhibition by the different glycogen-targetting subunits varied, R5 showing the least inhibition (57%), while G_L showed 73% inhibition and R6 approx. 86% inhibition (Table 3). Dissociating peptide also relieved inhibition of the glycogen synthase phosphatase activity by G_L, R5 and R6 in immunoprecipitates from a rat liver microsomal/glycogen fraction (Table 4). The different relative inhibition by the glycogen-targetting subunits of glycogen synthase phosphatase activity (G_L, 50%; R5, 50%; and R6, 75%) leads to different glycogen synthase phosphatase/phosphorylase phosphatase activity ratios for the three glycogen-targetted PP1-complexes (Table 4).

Levels of R5 and G_L are substantially decreased in the livers of streptozotocin-diabetic rats and starved rats

The activities of G_L, R5 and R6 complexes with PP1c were measured in liver lysates of streptozotocin diabetic and control rats (Table 5) following specific immunoadsorption of each complex. Figure 5 upper and middle panels show that the phosphorylase phosphatase activities associated with G_L and R5 are substantially decreased in streptozotocin-diabetic rats compared to fed control animals. This corresponded to decreases in the G_L and R5 protein levels detected by immunoblotting. In







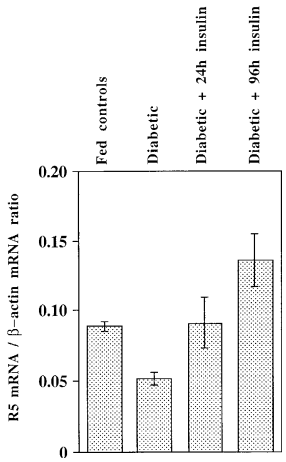


Figure 6 Effect of streptozotocin diabetes and insulin treatment on the expression of R5 mRNA

Total liver RNA was analysed by multiplex RT-PCR to evaluate the levels of R5 mRNA compared with those of β-actin mRNA. The PCR products were separated by gel electrophoresis and quantified by PhosphoImager analysis. The data are presented as the R5/β-actin signal ratio. The data show the means $\pm$ S.E.M. for three control, five diabetic, two diabetic + 24 h-insulin-treated and four diabetic + 96 h-insulin-treated rats.

contrast, the phosphorylase phosphatase activity of R6 remained essentially constant (Figure 5, lower panel) and no variation in levels of R6 was detected by immunoblotting (Figure 5, lower panel). Treatment of the streptozotocin-diabetic rats with 5 units of insulin for 24 h led to a partial restoration of G_L and R5 protein levels, while administration of 4×5 units of insulin for 96 h led to an increase in the levels of the G_L and R5 proteins to the same or slightly above those seen in control samples. In contrast, R6–PP1 activity showed an increase with insulin at 24 h, but this returned to near normal levels at 96 h. As also shown in Figure 5, lower panel, diabetes and insulin treatment did not affect the total level of the PP1c subunit in the liver lysate. Analysis of R5 mRNA levels by RT-PCR revealed an approx. 40 % decrease in the livers of streptozotocin-diabetic rats, which

Figure 5 Effect of streptozotocin-diabetes and insulin treatment on the expression of G_L, R5 and R6 proteins and their associated phosphorylase phosphatase activity

PP1c complexes with G_L, R5 and R6 were immunoadsorbed from liver lysates of control, streptozotocin-diabetic and insulin-treated rats and the immune pellets assayed for phosphorylase phosphatase activity. Immunoadsorption assays were performed with anti-(G_L peptide) antibody (upper panel), anti-(R5 peptide) antibody (middle panel), anti-(R6 peptide) antibody (lower panels). Upper sections of each panel: the immunoadsorbed phosphorylase phosphatase activity is expressed as munits (mU)/mg of total protein in the rat liver lysate. Immunoadsorption assays were performed in triplicate. The average levels were calculated for two liver samples (rat A and rat B for each condition) in most cases. The panels show the mean levels ($\pm$ individual values) from the two rats, except for anti-R5 immunoprecipitate from control and diabetc rats, where the levels are the mean ($\pm$ S.E.M) for four and five animals respectively. (1) Pre-immune pellet from the liver lysate of a normal control rat; (2) anti-peptide antibody pellet from the liver lysate of a normal control rat; (3) anti-peptide antibody pellet from the liver lysate of a diabetic rat; (4) anti-peptide antibody pellet from the liver lysate of a diabetic + 24 h-insulin-treated rat; (5) anti-peptide antibody pellet from the liver lysate of a diabetic + 96 h-insulin-treated rat. Lower sections of panels: 20 μg of protein from rat liver lysates were separated by electrophoresis on SDS/10 %-polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose membranes were probed with 1 μg/ml anti-G_L antibody, 1 μg/ml anti-R5 antibody, 1 μg/ml anti-(R6 peptide) or anti-PP1 antibody. Immunoreactive bands were detected by ECL®. Lanes are: (2a) normal control rat A; (2b) normal control rat B; (3a) diabetic rat A; (3b) diabetic rat B; (4a) diabetic + 24 h-insulin-treated rat A; (4b) diabetic + 24 h-insulin-treated rat B; (5a) diabetic + 96 h-insulin-treated rat A; (5b) diabetic + 96 h-insulin-treated rat B.

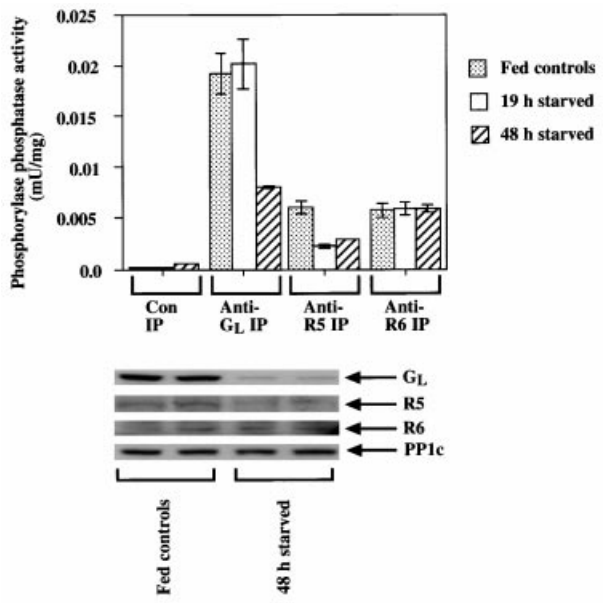


Figure 7 Effect of withholding food for 19 or 48 h on the expression of G_L, R5 and R6 proteins and their associated phosphorylase phosphatase activity

PP1c complexes with G_L, R5 and R6 were immunoadsorbed from liver lysates of fed control, 19 h-starved and 48 h-starved male Wistar rats. Immune pellets were assayed for phosphorylase phosphatase activity. The immunoadsorbed phosphorylase phosphatase activity is expressed as munits (mU)/mg of total protein in the extract. Immunoadsorption assays were performed in triplicate, and the average levels calculated for two liver samples (rat A and rat B for each condition). The panels show the mean levels (±individual values) from the two rats. Upper panel: samples are pre-immune IgG pellet ('Con IP'), anti-(G_L peptide) antibody pellet ('Anti-G_L IP'), anti-(R5 peptide) antibody pellet ('Anti-R5 IP') and anti-(R6 peptide) antibody pellet ('Anti-R6 IP'). Lower panel: 20 μg of protein from rat liver lysates were separated by electrophoresis on SDS/10%-polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose membrane was probed with either 1 μg/ml anti-(G_L peptide) ('G_L'), anti-(R5 peptide) ('R5'), anti-(R6 peptide) ('R6') or anti-PP1 antibody ('PP1c') as indicated and immunoreactive bands were detected by ECL®. Lanes are, from left to right: normal fed rat A, normal fed rat B, 48 h-starved rat A and 48 h-starved rat B.

returned to control or higher levels on insulin treatment (Figure 6).

Withholding food for 48 h substantially decreased the activities of the G_L and R5 complexes with PP1c compared with fed controls, while the activity of R6–PP1c did not vary significantly (Figure 7). The decreased G_L and R5 activities corresponded with lower levels of the G_L and R5 protein detected by immunoblotting at 48 h. However, withholding food for 19 h caused a decrease in the activity of R5–PP1c without affecting the activity of G_L–PP1c, indicating that these two glycogen-targetted forms of PP1c are differentially regulated by withdrawal of food. At both 19 and 48 h starvation, hepatic glycogen appeared to be nearly totally depleted as judged from the redistribution of the glycogen targetted complexes of PP1c from the 100000 ***g*** glycogen and microsomal pellet into the cytosolic supernatant (results not shown). Starvation for 19 or 48 h did not affect the total level of the PP1c subunit in the liver lysate.

DISCUSSION

We have developed specific immunoadsorption assays to characterize the activities of the different glycogen-targetted forms of PP1 and assess whether their levels are regulated differentially by insulin and nutrition. Three glycogen-targetting subunits of PP1 are found in liver, G_L, R5/PTG and R6, all of which possess a section with significant sequence similarity to a domain shown to bind starch in a fungal glucoamylase [11]. GST-fusion proteins have been used to map the glycogen-binding domain to the central region experimentally [10,34]. The endogenous forms of R5 and R6 have been demonstrated to interact specifically with glycogen ([14]; the present study). However, their distribution among subcellular fractions reported here suggests that they may have different binding affinities for glycogen, with G_L having the highest affinity and R6 the weakest, resulting in 53% of R6 being recovered in the supernatant collected after sedimentation of glycogen particles. In addition, the glycogen-targetting subunits may have varying affinities for glycogen particles of different sizes, with G_L associating better than R5 and R6 with the larger more rapidly sedimenting glycogen particles found in the pure glycogen fraction and R5 associating preferentially with the smaller glycogen particles sedimenting in the crude microsomal/glycogen fraction. The differential hepatic subcellular distribution of G_L, R5 and R6 leads to estimates of their relative abundance and importance that depend on the fraction considered. The G_L–PP1c complex accounts for over 90% of the phosphorylase phosphatase activity, and 95% of the glycogen synthase phosphatase activity present in the purified glycogen fraction with R5–PP1c accounting for most of the remainder, whereas in the lysate the contribution of G_L–PP1c is only 63% of the total phosphorylase phosphatase activity bound to G_L, R5 and R6. The phosphorylase phosphatase activity attributable to R5–PP1c (16%) and R6–PP1c (21%) in the liver lysates of control animals indicate that these forms of PP1 may contribute significantly to the regulation of hepatic glycogen metabolism and that the lysate rather than the glycogen fraction would be appropriate to assess changes in the levels of R5 and R6 glycogen-targetting subunits under different conditions.

Previous immunoblotting analyses showed that the level of G_L was decreased in the liver lysates of streptozotocin-diabetic animals, could be restored on insulin treatment, and corresponded to a 70–90% decrease in the glycogen synthase phosphatase activity associated with the glycogen fraction [18]. The targetting subunit specific immunoadsorption assays reported here show that not only does the phosphorylase phosphatase activity of G_L–PP1c decrease by approx. 75%, as expected in streptozotocin-diabetic-rat liver lysates, but that the phosphorylase phosphatase activity of R5–PP1c also decreases by approx. 60% and that both activities can be restored by insulin treatment. Corresponding decreases in the levels of R5 and G_L proteins in liver lysates of diabetic rats and restoration of their levels with insulin treatment was observed, confirming the previous results for G_L and indicating that R5 levels are also under the control of insulin. In contrast, similar studies with R6 showed that neither its level nor the associated phosphorylase phosphatase activity changed significantly in the livers of diabetic animals or with prolonged insulin treatment.

Withholding food for 48 h resulted in an approx. 60% decrease in the phosphorylase phosphatase activities of both G_L–PP1c and R5–PP1c in liver lysates, but no changes in that of R6–PP1c. Corresponding decreases in the level of G_L and R5 proteins were detected by immunoblotting. These studies are in agreement with decreases in the levels of mRNA encoding G_L [18] and R5/PTG [35] observed on fasting. Interestingly, in the studies reported here, there was a difference in the time at which the phosphorylase phosphatase activities of G_L–PP1c and R5–PP1c were observed to decrease, with R5–PP1c showing an approx. 55% decrease after only 19 h starvation, while G_L–PP1c was unaffected by this shorter time of starvation. A possible explanation is that R5–PP1c may need to be more tightly regulated than G_L–PP1c at the level of expression, because its glycogen synthase phosphatase activity

cannot be inhibited by phosphorylase *a* as is believed to occur for G_L–PP1c in response to glucagon signalling upon fasting. Alternatively, since R5–PP1c has a lower glycogen synthase phosphatase/phosphorylase phosphatase activity ratio than G_L–PP1c and therefore may predominantly function as a phosphorylase phosphatase *in vivo* (see below), a decrease in its level early during fasting would allow the active *a* form of phosphorylase to increase. The latter would then inhibit the glycogen synthase phosphatase activity of G_L–PP1, shutting down glycogen synthesis, while allowing sufficient up-regulation of glycogenolysis to prevent peripheral hypoglycaemia.

The decrease in R5 mRNA levels in diabetes and the increase on insulin treatment determined here, and similar changes for G_L mRNA levels observed previously 18], indicates that G_L and R5 may be regulated co-ordinately by insulin through alterations in mRNA levels. In contrast, studies on fasting animals indicate that G_L and R5 proteins are regulated differentially, suggesting that, in addition to changes at the mRNA level [18,35], a further mechanism may be operating. One possibility is that G_L and R5 may have different susceptibilities to proteolytic degradation and that these may be increased by low glucose or insulin levels.

In order to analyse how each glycogen-targetting subunit affects the activity of PP1c, a peptide containing the consensus PP1-binding sequence -RVXF- was used to dissociate PP1c from its regulatory subunits [3,33]. This allowed the activity, and therefore the amount, of the free catalytic subunit to be assessed. The peptide does not affect the activity of the free PP1 catalytic subunit ([33]; results not shown). Previous approaches have used trypsin to determine the total PP1c bound to the glycogen-targetted PP1 [15,6], but this has the major drawback that incomplete digestion of a particular PP1-targetting subunit may occur and/or digestion of the free PP1c, resulting in variable measurements of the intrinsic phosphatase activity of PP1c. The method employing a dissociating peptide circumvents these problems and allows more accurate measurement of total PP1c bound to each targetting subunit. Use of this method coupled to immunoadsorption assays allows us to estimate that G_L accounts for 55 %, R5 13 % and R6 32 % of the total PP1 bound to the hepatic glycogen-targetting subunits (Table 3). These values differ significantly from the relative proportions of G_L and R5/PTG mRNA quantified in rat liver, where R5/PTG mRNA was reported to be 2.6-fold higher than G_L [35], but mRNA and protein levels are not necessarily correlated, owing to differential rates of translation and protein degradation.

The immunoadsorption assays reported here show that each targetting subunit inhibits the phosphorylase phosphatase and glycogen synthase phosphatase activities of PP1c to different extents, resulting in the G_L–PP1c complex having a higher glycogen synthase phosphorylase/phosphorylase phosphatase ratio than R5–PP1c (Tables 3 and 4). The different glycogen synthase phosphatase/phosphorylase phosphatase ratios suggest that these PP1c complexes may function to dephosphorylate distinct substrate proteins in glycogen metabolism. However, it should be borne in mind that the binding of antibodies that distinguish between the different glycogen-targetting subunits in the immunoadsorption assay may perturb the targetting subunit–PP1c complexes. Digestion of hepatic glycogen-bound PP1 with trypsin results in an increase in phosphorylase phosphatase activity but a decrease in glycogen synthase phosphatase activity [15,36]. Nevertheless, these previous studies using trypsin support different glycogen synthase phosphorylase/phosphorylase phosphatase ratios for G_L and R5, because the purified glycogen fraction, here found to contain 92 % G_L and 7 % R5 (Table 2) exhibited higher glycogen synthase phosphorylase/phosphorylase phosphatase ratios [15,36] than the microsomal fraction, here found to contain 74 % G_L and 16 % R5 (Table 2). Overall these results suggest that G_L–PP1c has a higher glycogen synthase phosphorylase/phosphorylase phosphatase ratio than R5–PP1c and favour the view that G_L–PP1c may be the major glycogen synthase phosphatase and R5–PP1c may function predominantly as a phosphorylase phosphatase in liver *in vivo*.

Studies on the activation of *in vivo*-phosphorylated liver glycogen synthase noted that the glycogen synthase phosphatase activity in a liver lysate as well as in the glycogen fraction was completely inhibited by low concentrations of phosphorylase *a* [15], identifying G_L–PP1c as the glycogen synthase phosphatase. Overexpression of the different glycogen-targetting subunits in hepatocytes by others can also be interpreted to support this conclusion. Berman et al. [37] and Gasa et al. [38] found that adenoviral-mediated overexpression of either PTG or G_L in primary rat hepatocytes resulted in a very high activation of glycogen synthesis. Glycogenolytic agents such as forskolin could stimulate glycogen breakdown in G_L-overexpressing cells, but were ineffective or poor at activating glycogen breakdown in PTG-overexpressing cells, despite the fact that these treatments robustly elevated cAMP levels. These results can be explained if R5/PTG functions predominantly *in vivo* as a hepatic phosphorylase phosphatase, while G_L–PP1c is the major glycogen synthase phosphatase and the regulatory mechanisms involving the phosphorylase *a* inhibition of G_L–PP1c operate *in vivo*. Overexpression of R5/PTG would dephosphorylate phosphorylase *a*, relieving the phosphorylase *a* inhibition of G_L–PP1c glycogen synthase activity with a consequent increase in glycogen synthesis. Agonists increasing cAMP level would be ineffective or poor in enhancing glycogenolysis, because they would be unable to properly overcome the high level of phosphorylase phosphatase activity resulting from overexpression of R5/PTG. In G_L-overexpressing cells, high levels of G_L–PP1c would increase glycogen synthesis, but cAMP agonists would be expected to enhance glycogenolysis by counteracting the endogenous R5/PTG phosphorylase phosphatase activity. In accordance with this hypothesis, activation of phosphorylase phosphatase activity by forskolin in PTG-overexpressing cells was very much decreased compared with that in control and G_L-overexpressing cells [37,38]. Insulin increased glycogen synthesis in PTG-overexpressing cells, but not in G_L-overexpressing cells, probably because G_L overexpression causes complete activation of glycogen synthase such that no further activation can be observed [38]. In PTG-overexpressing cells, glycogen synthase was not fully activated (as judged by its $\pm$glucose 6-phosphate activity ratio) [38], and therefore it is possible that insulin could cause a further activation of glycogen synthase by inhibition of glycogen synthase kinase-3 via the protein kinase B pathway. In addition, the observation that inclusion of GST–PTG in 3T3-L1 adipocyte extracts decreased the K_m of PP1c for phosphorylase 5-fold without affecting PP1c activity against another substrate, hormone-sensitive lipase [39], would also support the model in which R5/PTG functions predominantly as a phosphorylase phosphatase. An analogous situation does not appear to exist in muscle, since in mice lacking G_M, the activity ratios of both glycogen synthase and phosphorylase phosphatase are both substantially altered [40].

Printen et al. [12] suggested that PTG may act as a molecular scaffold assembling glycogen synthase, phosphorylase *a*, phosphorylase kinase and PP1c on to the glycogen particle, generating a module for reception of intracellular signals. More recent studies report that glycogen synthase, phosphorylase *a* and phosphorylase kinase interact with R5/PTG at the same site between the glycogen-binding domain and the C-terminus, so that

they are not all able to bind to R5/PTG at the same time [41]. This site therefore resembles a substrate docking site. A homologous binding site for glycogen synthase, together with a secondary interaction site near the N-terminus of G_M, was identified in some studies [42], but not others [43]. However, the substrate docking site is distinct from the allosteric phosphorylase *a* binding site identified at the extreme C-terminus of G_L [10], which is absent from G_M, R5/PTG and R6.

Currently there is no evidence for a short-term regulatory mechanism operating on R5 and R6. G_M is phosphorylated by protein kinase A *in vivo* in response to adrenalin at Ser^{48} and Ser^{67} (using the numbering for the rabbit G_M sequence) [44]. Since Ser^{67} lies within the PP1 binding site [3], phosphorylation causes dissociation of PP1c from G_M and inactivation of the G_M–PP1c complex [45]. Although G_L contains a homologous serine residue, the G_L–PP1c complex is not regulated in the same manner, but is subject to allosteric regulation by phosphorylase *a* [10,19]. R5/PTG and R6 do not possess a serine residue within the PP1c binding motif [11,14,41], and no evidence for phosphorylation of R5/PTG in response to forskolin was found [39]. Long-term regulation at the mRNA level in response to cellular signals has only been reported for G_L and R5/PTG ([18,35]; the present study). The regulation and functions of R6 are unknown, but given its wide tissue distribution, it may regulate the low level of glycogen turnover that occurs in many mammalian tissues. Alternatively, the abundance of R6–PP1c complex in the glycogen-free supernatant fraction, and the potent inhibition of both glycogen synthase and phosphorylase phosphatase activities of PP1c by R6, raise the possibility that R6 has additional or different protein substrates to those found on glycogen particles.

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