Human Skeletal Muscle Expresses a Glycogen-Targeting Subunit of PP1 That Is Identical to the Insulin-Sensitive Glycogen-Targeting Subunit G_L of Liver

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Insulin has been previously shown to regulate the expression of the hepatic glycogen-targeting subunit, G_L, of protein phosphatase 1 (PP1) and is believed to control the activity of the PP1-G_L complex by modulation of the level of phosphorylase *a*, which allosterically inhibits the activity of PP1-G_L. These mechanisms contribute to the ability of insulin to increase hepatic glycogen synthesis. Human G_L shows >88% amino acid identity to its rat and mouse homologs, with complete conservation of the phosphorylase *a* binding site. G_L is highly expressed in the liver and present at appreciable levels in heart tissue of all three species. Surprisingly, G_L is highly expressed in human skeletal muscle while only being detected at very low levels in rat, mouse, and rabbit skeletal muscle. The amino acid sequence of G_L predicted from the cDNA is identical in human liver and skeletal muscle and encoded by a gene on chromosome 8 at p23.1. The species-specific difference in the level of expression of G_L mRNA and protein in skeletal muscle has important implications for understanding the mechanisms by which insulin regulates glycogen synthesis in human skeletal muscle and for questions regarding whether rodents are appropriate models for this purpose. *Diabetes* 51:591–598, 2002

Insulin stimulates glycogen synthesis through the dephosphorylation and activation of the rate-determining enzyme glycogen synthase (1–3). Part of the action of insulin in this pathway in liver stems from the ability of insulin to activate the glycogen synthase phosphatase, which dephosphorylates sites on glycogen synthase that are phosphorylated by glycogen synthase kinase-3 (GSK3). Net dephosphorylation of these sites by either inhibition of GSK3 through the phosphatidylinositol-3 kinase (PI-3 kinase)/protein kinase B (PKB) pathway or activation of glycogen synthase phosphatase causes an activation of glycogen synthase (4–6).

Several forms of glycogen synthase phosphatase have been identified that are heterodimeric complexes of PP1 with different glycogen-targeting subunits. A 124- to 126-kDa protein, G_M, found in striatal muscles targets protein phosphatase 1 (PP1) catalytic subunit (PP1c) to glycogen as well as to the sarcoplasmic reticulum (7–10). A much smaller (33 kDa) protein, G_L, identified in rat liver is 23% identical to the NH_2-terminal region of G_M (11–13). G_L enhances PP1c activity toward glycogen synthase while suppressing its activity toward phosphorylase. R5/PTG (36 kDa) shows ~40% identity to G_L (14–16) and is expressed in a variety of tissues, with the highest mRNA levels being in liver, skeletal muscle, and heart muscle (17). R6 (33 kDa), with 31% identity to G_L, shows a wide tissue distribution (17). The four glycogen-targeting subunits bind to PP1c through a short highly conserved motif (RVXF). This motif is also responsible for the interaction of many other regulatory subunits with PP1, explaining why the binding of regulatory subunits to PP1c is mutually exclusive (18–20). The glycogen binding domain has been mapped to the central section of G_L, R5, and R6 and the corresponding region of G_M (13,14,17,21,22).

Hepatic glycogen synthase phosphatase activity is substantially decreased in streptozotocin-induced diabetes and is restored by insulin treatment (23). More recently, it has been shown that the levels of the G_L and R5 proteins and their associated phosphatase activities are decreased in the livers of streptozotocin diabetic rats by ~80 and 60%, respectively (24,25). Corresponding decreases are observed in G_L and R5 mRNA levels, and insulin treatment restores the levels and phosphatase activities to the control values. G_L and R5 protein and mRNA levels also decrease during fasting (24–26).

Shorter-term mechanisms for regulation of the glycogen-targeted forms of PP1 by hormones have been described for G_L and G_M. Hepatic glycogen synthesis is modulated by the active form of phosphorylase (phosphorylase *a*), which potently inhibits glycogen synthase phosphatase activity (27–29). Inhibition of glycogen synthase phosphatase activity catalyzed by a form of PP1 was found to occur by an allosteric mechanism at nanomolar concen-

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BAC, Bacterial Artificial Chromosome; GSK3, glycogen synthase kinase-3; HGMP, Human Genome Mapping Project; NCBI, National Center for Biotechnology Information; nt, nucleotide; PKA, protein kinase A; PKB, protein kinase B; PI-3 kinase, phosphatidylinositol-3 kinase; PP1, protein phosphatase 1; PP1c, PP1 catalytic subunit; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

trations in the presence of glycogen, without inhibition of the phosphorylase phosphatase activity (30–32). Phosphorylase *a* was shown to bind to a 16–amino acid section at the extreme COOH-terminus of G_L, a sequence that is absent from the other glycogen-targeting subunits, G_M, R5, and R6 (13). Thus, stimulation of hepatic glycogenolysis by glucagon (acting via cyclic AMP and protein kinase A [PKA]) and α-adrenergic agonists (acting via Ca^{2+}) leads to the activation of phosphorylase kinase, the increase of the level of phosphorylase *a*, and the consequent inhibition of glycogen synthase phosphatase activity. Insulin lowers hepatic cAMP levels, causing a reduction in the level of phosphorylase *a* that alleviates the phosphorylase *a*–mediated inhibition of the G_L-PP1c complex, causing an increase of glycogen synthesis. The binding of glucose to phosphorylase *a* enhances the rate at which phosphorylase is inactivated, contributing further to the stimulation of glycogen synthesis by insulin and high blood glucose (33).

G_M is phosphorylated by PKA in vivo in response to adrenaline at Ser 48 and Ser 67 (using the numbering for the rabbit G_M sequence) (34). Because Ser 67 lies within the PP1 binding site (19), phosphorylation causes dissociation of PP1c from G_M and inactivation of the G_M-PP1c complex (35). The mechanism by which insulin may regulate glycogen synthase phosphatase in skeletal muscle is unclear. Mice homozygous for a null allele of G_M show a similar insulin stimulation of glycogen synthase activity in skeletal muscle to that in control mice, indicating that G_M is not essential for insulin activation of glycogen synthase (36).

We report here that although the insulin-sensitive phosphatase G_L-PP1c is predominantly found in liver in rodents with only very low level expression in skeletal muscle, G_L is present at appreciable levels in both human liver and skeletal muscle.

RESEARCH DESIGN AND METHODS

Human biopsy samples. Vastus lateralis biopsies were obtained from three healthy, male volunteers (21 ± 0 years; 72.3 ± 3.3 kg; 176 ± 1 cm; BMI 23.3 ± 1.35 kg/m^2). The Tayside Ethics Committee approved the protocol, and the nature, purpose, and possible risks were explained to each subject before written consent was obtained. Subjects fasted overnight for 12 h. Under local anesthesia (1% lignocaine, no adrenaline), an incision of 10 mm length and depth was made in the skin and muscle fascia at ~20 cm above the knee, using an aseptic technique. Vastus lateralis biopsies (~100 mg) from the right quadriceps femoris muscle were obtained using Tilley-Henkel forceps. Samples were blotted dry and grossly dissected free of fat and connective tissue, frozen in liquid nitrogen and stored at −80°C before analysis. Soleus and gastrocnemius biopsies were taken from a patient in the fasted state under general anesthesia, with the patient's consent, during lower-limb surgery and were immediately frozen in liquid nitrogen. Liver biopsy samples were from control subjects or patients who had been previously studied for glycogen storage diseases, and the tissue was stored at −80°C (37).

Identification and sequence analysis of DNA encoding PP1 binding subunit G_L. The National Center for Biotechnology Information (NCBI) expressed sequence tag and cDNA databases were searched for sequences relating to rat G_L using the tBLASTN algorithm. A human testis cDNA clone in plasmid pT7T3D-Pac (accession no. AA954470) and a mouse embryonic cDNA in plasmid pME18S-FL*3* (accession no. AI115058) were provided by the IMAGE consortium. Sequence analysis of the plasmid inserts was performed in both directions on an Applied Biosystems 373A automated DNA sequencer using *Taq* dye terminator cycle sequencing by the University of Dundee DNA sequencing service managed by Dr. Nick Helps (www.dnaseq.co.uk). The human clone, which contained 200 bp that were highly similar to nucleotide (nt) 1–200 of rat G_L, was used in a hybridization screen of a human Bacterial Artificial Chromosome (BAC) library (performed by Genome Systems, St. Louis, MO). Three positive human genomic clones in pBELOBAC gave identical patterns after cleavage with *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, and *Ava*I; Southern blotting; and hybridization with the G_L coding region. Sequence analysis showed that the clones were identical from −800 to +852 of the G_L gene.

Amplification of G_L cDNA from human cDNA libraries. Human liver and testis Matchmaker cDNA libraries (Clontech, Palo Alto, CA) were used as templates to amplify nt 1–200 of G_L using the Hi-Fidelity PCR system (Roche Diagnostics, Lewes, Sussex, U.K.) according to the manufacturer's instructions with the forward primer 1 (nt 1–23, 5′ ATGGCTGTGGA CATCGAGTACAG 3′) and reverse primer 2 (complementary to nt 210–180, 5′ GTTGTCTGCGAA GGACACCCGC 3′). Marathon-Ready liver and skeletal muscle cDNA libraries (Clontech) were used as templates to amplify the full-length coding sequence for G_L by PCR using the forward primer 1 and primer 3 (complementary to nt 852–827, 5′ GTAGTAGGGCCCTAGCTTT TCATATC 3′). 5′ and 3′ rapid amplifications of cDNA ends [5′ and 3′ rapid amplification of cDNA ends (RACE), (38)] were performed using the same libraries. Gene-specific primers were primer 1 and primer 4 (complementary to nt 401–374, 5′-CCG ATT TCT AAA GTC TAA GTA ATC TGC-3′) for the 3′ and 5′ RACE respectively. PCR products were resolved on 1% (wt/vol) agarose gel, gel-purified, cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA), and sequenced in both directions using M13 forward and reverse primers.

Fluorescence in situ hybridization. Purified BAC or cosmid DNA was labeled with the fluorochrome Spectrum Red using Vysis Nick Translation Kit, as described in the manufacturer;s protocol (Vysis, Downers Grove, IL). The labeled probe was used for hybridization of metaphase chromosome spreads from lymphocyte cultures, and the slides were counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride.

Hybridization screening of Los Alamos National Library human single chromosome libraries. Chromosome 8 and 10 cosmid libraries, constructed at Los Alamos National Laboratory, were kindly provided by the U.K. Human Genome Mapping Project (HGMP) Resource Center in the form of high-density gridded filters. The libraries had an average insert size of 30–40 kb and contained ~20,000 clones providing a four- to fivefold coverage of each chromosome. The filters were hybridized with the G_L coding region (852 bp). Four chromosome 8 clones (LA0835p13, LA0848n12, LA0817a8, and LA0822f15 from the LA08NC01 library, kindly provided by the HGMP, were sequenced.

RNA analyses. Northern blots (Clontech) contained ~2 μg poly $(A)^+$ RNA from different tissues. Alternatively, total RNA was prepared using TRIzol Reagent (Life Technologies, Paisley, U.K.). Blots were hybridized according to Clontech's instructions with the last wash in 15 mmol/l NaCl:1.5 mmol/l sodium citrate:0.1% SDS at 55°C.

Immunological techniques. Rodents were killed by suffocation in CO_2, and tissues were excised, freeze clamped, and stored at −80°C. Heart and skeletal muscle were freeze-fractured –80°C and homogenized at 4°C in six volumes 50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 2 mmol/l EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% (vol/vol) 2-mercaptoethanol, and "Complete" protease inhibitor (Roche Diagnostics, Lewes, U.K). Homogenates were centrifuged at 16,000*g* for 10 min, and the supernatants were decanted, snap-frozen in liquid nitrogen, and stored at −80°C. Liver was homogenized in three volumes of 2 mmol/l EDTA, pH 7.0, 2 mmol/l EGTA, 250 mmol/l sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and "Complete" protease inhibitor and were processed similarly. Proteins in the 16,000*g* supernatants were separated by 4–12% SDS-PAGE, transferred to nitrocellulose, and probed with 0.2 μg/ml affinity purified antibodies raised in sheep to rat G_L (24), human PP1β peptide (amino acids 316–327), human G_M (amino acids 1–243), or a control antibody raised in mice to GAPDH (Research Diagnostics, Flanders, NJ). Antibody binding was determined using anti–sheep and anti–mouse IgG antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL), followed by enhanced chemiluminescence (Amersham International, Amersham, Bucks, U.K.).

RESULTS

The sequence of the hepatic glycogen binding subunit G_L of PP1 is highly conserved in mammals and encoded by a single gene in the human genome. Searches of the NCBI databases with the full-length sequence of rat hepatic glycogen-targeting subunit of PP1, G_L, did not uncover any human liver cDNA but did identify a cDNA derived from a human testis cDNA library that contained 200 bp encoding 66 amino acids that were highly similar to amino acids 1–66 of rat G_L. PCR with primers constructed to the ends of this cDNA fragment led to the amplification of identical 200-bp cDNA fragments from both human testis and liver cDNA libraries. Subsequent PCR using the liver library with the 5′ primer and a 3′ primer complementary to the extreme COOH-terminal coding region of rat G_L allowed the

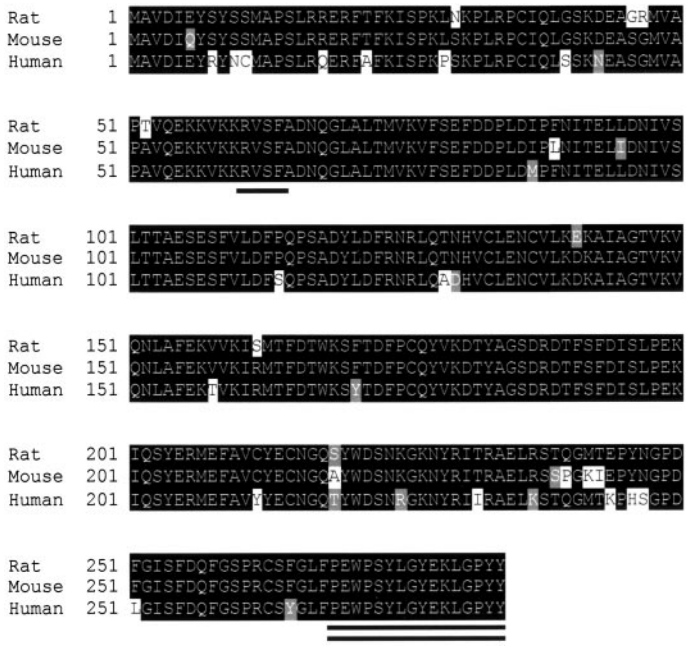

FIG. 1. Comparison of the amino acid sequences of rat, mouse, and human G_L. Identities are shaded in black and similarities are shaded in gray. The PP1 binding motif is indicated by a single underline, and the phosphorylase *a* binding region is indicated by a double underline.

isolation of cDNA for the human G_L coding region. The 5′ and 3′ untranslated regions (UTRs) and confirmation of the extreme NH_2- and COOH-terminal coding sequences was obtained by PCR using RACE methodology. Three genomic clones from a BAC library contained an identical region encoding G_L, and the sequence obtained for the G_L gene from −3,000 to +6,000 was identical to that reported in the human genome database recently. The liver G_L cDNA was colinear with the gene sequence (from −1,880 to +2,100). In contrast, a testis cDNA (accession no. AA 824377) was missing sequences from −3,600 to −147, while a retinoblastoma cDNA (accession no. AK024067) was missing sequences from −8,913 to −21, presumably as the result of alternative splicing.

Sequencing of a mouse embryonic cDNA clone identified the complete open reading frame of a putative G_L protein of 284 amino acids. Comparison of the amino acid sequence of rat G_L with the deduced amino acid sequence of human and mouse G_L reveals high levels of identity between the proteins from the three species and shows complete conservation of the PP1 and phosphorylase *a* binding regions. Human G_L is 89 and 88.5% identical to rat and mouse G_L respectively, and mouse G_L is 94% identical to rat G_L (Fig. 1).

Initial mapping of the human G_L gene using a human 120- to 150-kb BAC clone as a probe gave two locations at 8p23.1 and 10q25. Screening of chromosome 8 and 10 cosmid libraries with a G_L probe identified potential positive clones on the chromosome 8 filter but no positives on the chromosome 10 filter. Sequence analysis of four chromosome 8 clones hybridizing with a G_L probe showed that they all contained a region encoding G_L identical to that in the BAC genomic clones. Mapping using a smaller cosmid clone (30–50 kb) as a probe gave only the location 8p23 (Fig. 2), consistent with the map location cited in the HGMP database.

Restriction mapping, Southern blotting, and sequencing of three BAC genomic clones established that there was only one G_L gene within each clone, indicating that G_L is encoded by a single gene in the human genome.

Expression of G_L mRNA and protein in several tissues, including heart and skeletal muscle. Analysis of the tissue distribution of human G_L mRNA shows that G_L cDNA hybridizes to a single RNA species of 6.7 kb (Fig. 3*A*). However, surprisingly, highest levels are observed to be in skeletal muscle and heart and not in liver. In addition, G_L was present at low levels in placenta, lung, and kidney.

As this was an unexpected result, the rat mRNA expression was reanalyzed. The highest levels of 5-kb G_L mRNA were present in liver as reported previously (12), but a longer exposure time revealed that rat G_L mRNA shows appreciable expression in the heart and is evident at lower levels in other tissues, such as lung, kidney, and spleen (Fig. 3*B*). Mouse G_L mRNA (4.4 kb) shows a similar tissue distribution to rat G_L mRNA, with highest levels being present in liver and appreciable levels present in heart (Fig. 3*C*). Although there were no detectable levels of G_L mRNA in rat and mouse skeletal muscle on the Clontech blot (Fig. 3*B* and *C*), subsequent analyses of total RNA from skeletal muscle of mouse (Fig. 3*D*) and rat (data not shown) detected low levels of G_L mRNA.

Analyses of G_L at the protein level focused upon the insulin-sensitive tissues heart, liver, and skeletal muscle, as these tissues showed the highest levels of G_L expression at the mRNA level. Immunoblotting of rat and mouse tissue extracts with an anti-rat G_L antibody confirmed that liver exhibits the highest levels of G_L (Fig. 4*A* and *B*). Bands of the same size (33 kDa) were also present in rat kidney, heart, testis, spleen, and lung, and very low levels were detectable in skeletal muscle, indicating that G_L is not exclusively liver specific, as had been thought to be the case previously.

Analysis of human liver and skeletal muscle biopsy samples for the presence of G_L by immunoblotting with the anti-rat G_L antibody, revealed a protein migrating at the same size as rat liver G_L in both human liver and skeletal muscle extracts (Fig. 5). Although the mRNA analysis suggested that higher levels of human G_L mRNA are present in skeletal muscle than in liver, immunoblotting showed human G_L protein to be present at appreciable and fairly similar levels in these tissues.

G_L was examined for variation in the level of expression in different types of muscle. In mouse (Fig. 4*B*) and rabbit (data not shown), G_L levels were very low in the gastrocnemius, psoas, and soleus muscles and no variation was observed. The expression of G_L is appreciable in three different human muscles, with the level in soleus being slightly higher than those in the vastus lateralis and the gastrocnemius (Fig. 5*B*). Assessment of the G_L-to-G_M ratio was determined by specific immunoprecipitation assays in the presence of a peptide that dissociates the glycogen-targeting subunits from PP1c (25). In all three human muscles, the total PP1c activity bound to G_L was found to be ~40% of that bound to G_M (Fig. 5*B* and data not shown).

Although the sizes of the human G_L mRNA and protein were identical in liver and skeletal muscle, there remained a possibility that their sequences differed due to alterna-

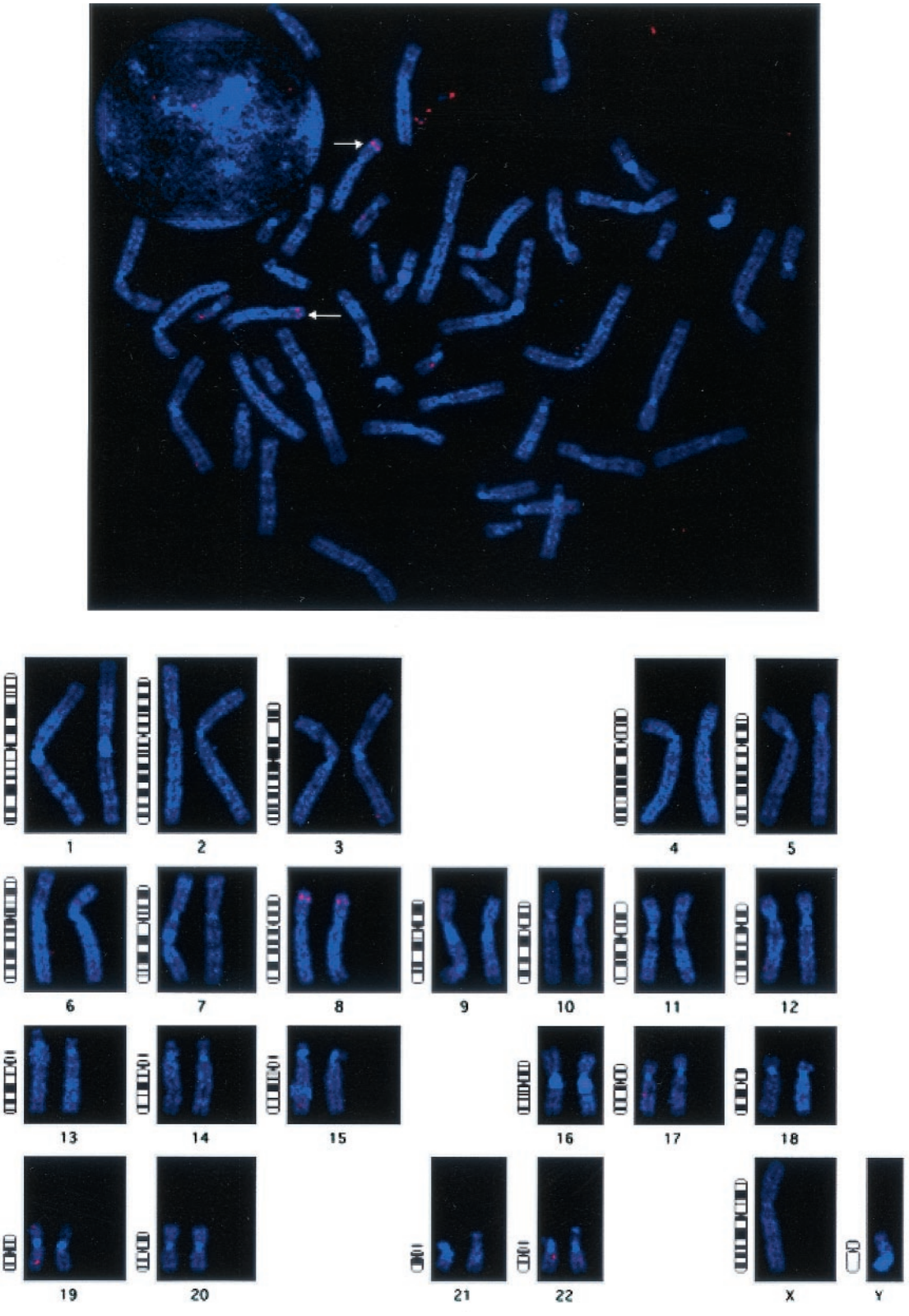

FIG. 2. Fluorescent in situ hybridization showing the location of the single G_L gene at 8p23. The upper panel shows the hybridization of a human lymphocyte metaphase chromosome spread with a cosmid probe (containing 30–50 kb genomic DNA) encoding G_L and labeled with the fluorochrome Spectrum red. Chromosomes were viewed under an Olympus BX60 fluorescent microscope and images were captured using Vysis Smartcapture software. The lower panel identifies the chromosomes. Arrows point to the position of the G_L gene on both chromatids of both copies of chromosome 8.

tive splicing of the mRNA in the two tissues. G_L cDNA was therefore amplified by PCR from a human skeletal muscle cDNA library and compared with that amplified by PCR from the liver cDNA library. Amplification of the coding region gave fragments of identical size and sequence in skeletal muscle and liver (Fig. 6).

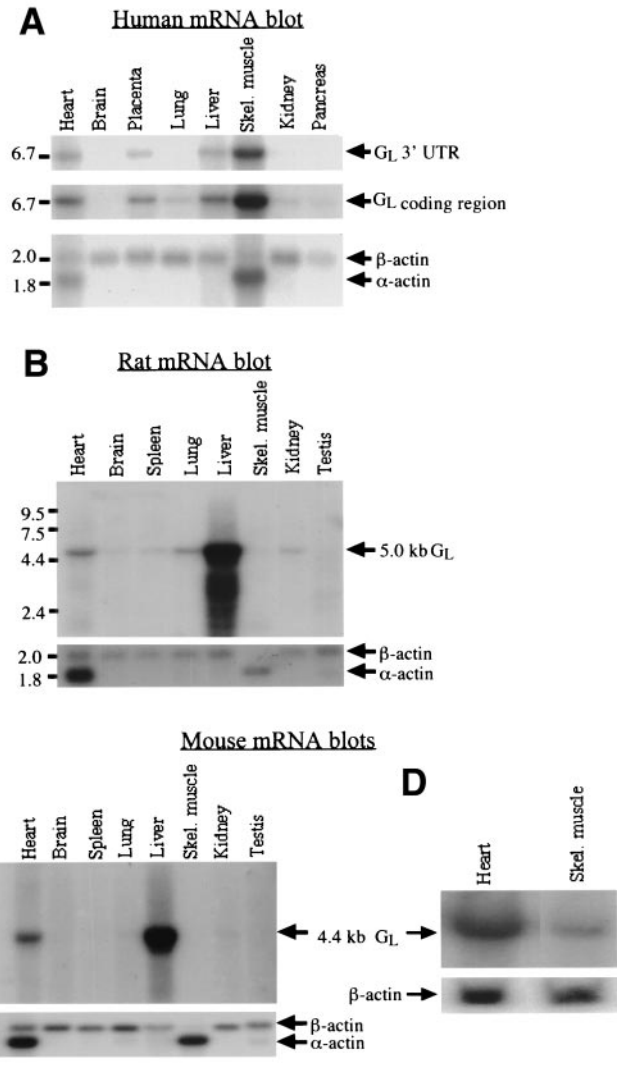

FIG. 3. Tissue distribution of the G_L mRNA. Human (*A*), rat (*B*), and mouse (*C*) multiple tissue poly $(A)^+$ RNA blots (Clontech) and a blot of mouse total RNA (*D*) were hybridized with the G_L coding region cDNA (852 bp) of human (*A*, middle panel), rat (*B*, upper panel), and mouse (*C* and *D*, upper panels), respectively. The upper panel in *A* was hybridized with G_L probe corresponding to the 3′ UTR of human G_L mRNA. The human skeletal muscle poly $(A)^+$ RNA was from a mixture of the quadriceps, gluteus maximus, pectoralis major, and ileopsoas muscles. Rat and mouse skeletal muscle poly $(A)^+$ RNA was from the thigh and back, and mouse skeletal muscle total RNA was prepared from hind limb. After autoradiography, membranes were stripped in 0.5% SDS at 100°C for 5 min and subsequently reprobed with a β-actin probe. Lower panels show β-actin mRNA that was used as a control to assess the variation in sample loading. Transcripts are indicated by arrows, and their sizes are calculated by their mobility relative to RNA standards.

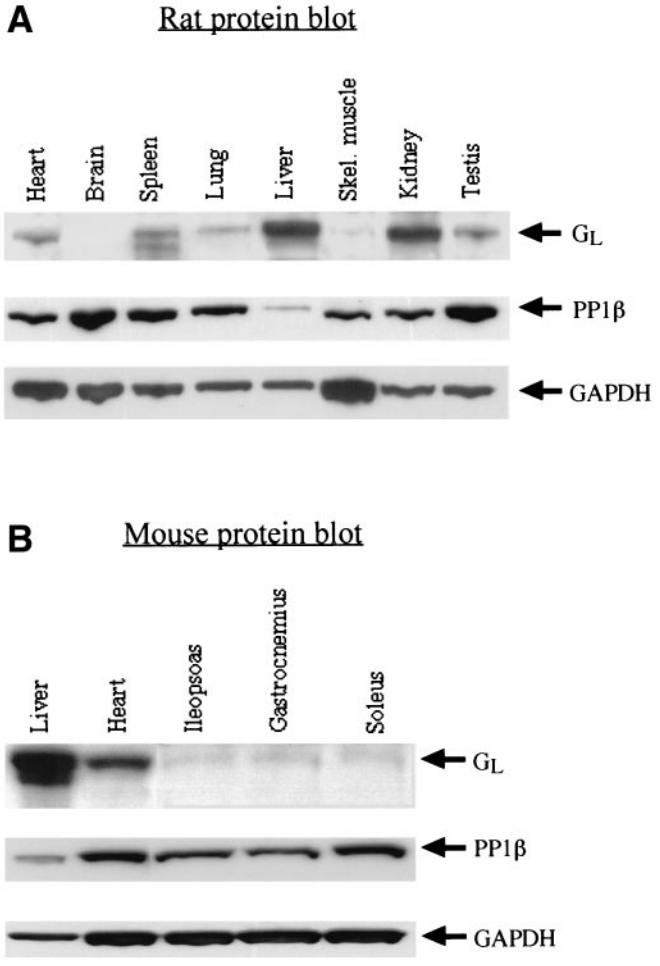

FIG. 4. Detection of G_L in rat (*A*) and mouse (*B*) tissues by immunoblotting. Protein (20 μg) from 16,000*g* supernatants was subjected to gel electrophoresis, transferred to nitrocellulose, and probed with anti–rat G_L antibody. In *B*, heart muscle and three different types of skeletal muscle from mouse were examined. Middle and lower panels show the blots stripped and probed with anti–PP1-β antibody and anti-GAPDH antibody, respectively.

RACE methodology shows that the 5′ UTRs from both skeletal muscle and liver cDNA libraries commenced at −1,800 with a sequence identical to the genomic sequence. Amplification of the 3′ UTRs gave fragments that terminated at +2,100 with the liver cDNA library and terminated at +2,100 and +5,380 with the skeletal muscle cDNA library, the sequence of all fragments being identical to the genomic G_L sequence. The larger skeletal muscle cDNA amplified (−1,880 to +5,380) is comparable in size to the mRNA (6.7 kb) detected in both liver and muscle by blotting (Fig. 3*A*).

DISCUSSION

We show here that the glycogen-targeting subunit, G_L, of PP1 is very highly conserved, with >88% identity in amino acid sequence between mammalian species. In comparison, G_M shows 73% identity between rabbits and humans (10) and R5/PTG shows 84% identity between mouse and humans (14,15). The regulatory phosphorylase *a* binding site at the extreme COOH-terminus of G_L is invariant between species, suggesting that allosteric regulation of G_L-PP1c activity by phosphorylase *a* is crucially important in mammals. Although G_L was believed to be specific to liver (3,11,12), analyses of human, rat, and mouse tissues show that the G_L mRNA is present at significant levels in heart and at low levels in several rat tissues, including kidney and lung. More surprising is that G_L mRNA is more abundant in human skeletal muscle than in liver. Immunological detection of G_L protein at high levels in liver and low levels in heart and skeletal muscle of rat and mouse correlates with the mRNA results for these species. Human G_L protein was detected immunologically in biopsy samples at fairly similar levels in liver and skeletal muscle. The disparity between the human mRNA and protein analyses did not appear to arise from different levels of

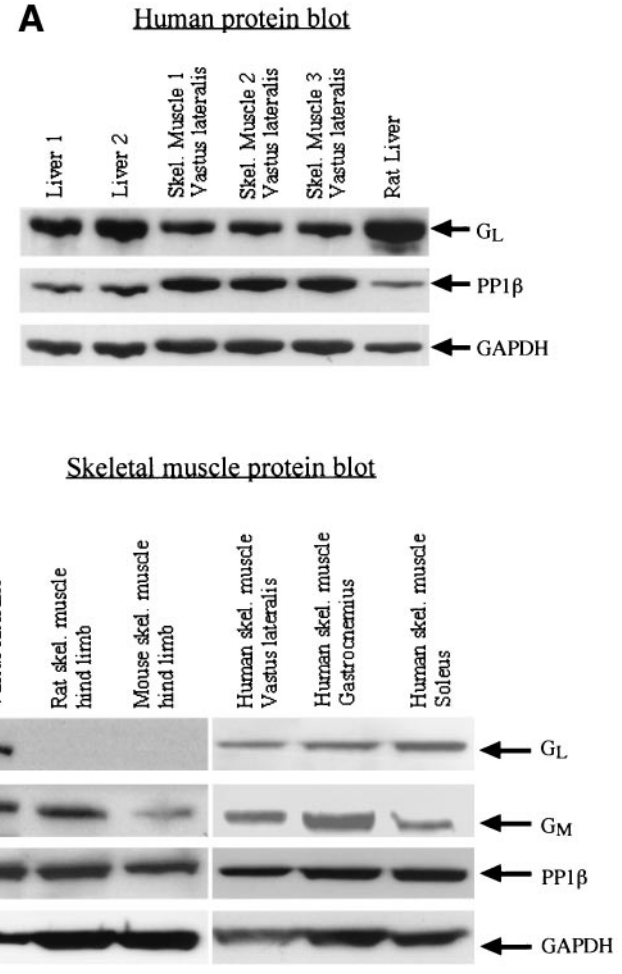

FIG. 5. Detection of G_L in human skeletal muscle. *A*: Comparison of the levels of G_L in skeletal muscle biopsies (from three different individuals) and liver biopsies (from two different individuals). *B*: Comparison of the level of G_L in human, rat, and mouse skeletal muscles and in three different human skeletal mucles. Protein (20 μg) from 16,000*g* supernatants was subjected to gel electrophoresis, transferred to nitrocellulose, and probed with anti–rat G_L antibody (top panel). Other panels show the blots stripped in Reblot Plus (Chemicon International) and reprobed with anti–human G_M antibody, anti–PP1-β antibody, and anti-GAPDH antibody. Immunoblotting suggests more variability in the G_L-to-G_M ratio in different human muscles than that estimated by assays (see RESULTS) probably because G_M is extremely sensitive to proteolysis and undergoes rapid partial degradation from the 160-kDa band (shown above) to smaller fragments that still bind PP1c and glycogen.

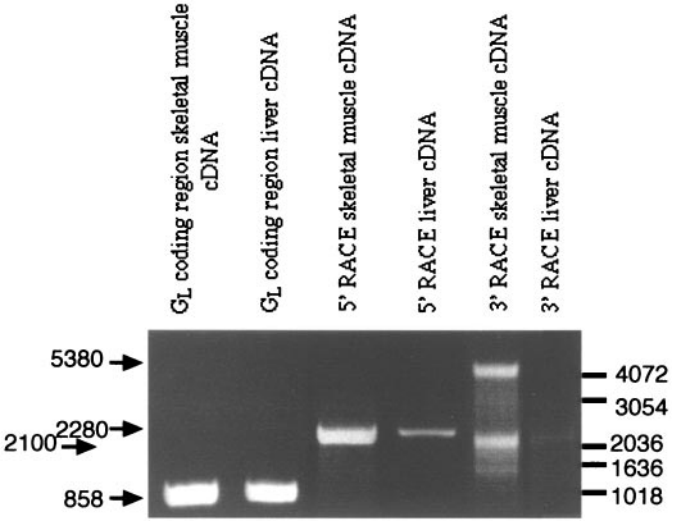

FIG. 6. Amplification of G_L cDNA coding region, 5′ UTR and 3′ UTR, by PCR using human skeletal muscle and liver cDNA libraries as templates. The coding region was amplified with primers 1 and 3 (852 bp). The 5′ UTR and half of the coding region were amplified with primer 4 and a vector primer (total size ~2,200 bp), and the 3′ UTR and coding region were amplified with primer 1 and a vector primer (total sizes ~2,100 and ~5,380 bp in skeletal muscle and ~2,100 bp in liver). The precise termination points of the 5′ UTR (−1,800 nt) and the 3′ UTR (+2,100 nt and +5,380 nt in skeletal muscle and +2,100 nt in liver) were determined by sequencing the entire 5′ and 3′ UTR regions.

expression of G_L in different types of muscle, since the level in soleus, comprising slow fatigue-resistant fibers, was only slightly higher than those in the gastrocnemius and vastus lateralis muscles that are composed of both fast and slow fibers in similar proportions. In situ hybridization and sequence analysis of genomic clones indicates that there is only a single human gene encoding G_L at 8p23.1. There was no evidence for alternative splicing of the liver and skeletal muscle mRNA since the cDNA sequences from these tissues were identical and only a single mRNA species was detected by Northern blotting of several tissues in each species. A possible explanation is that the translation of that mRNA may be controlled differentially in liver and skeletal muscle by tissue-specific factors. Although three further human genes (PPP1R3E, PPP1R3F, and PPP1R3G) encoding related glycogen-targeting subunits of PP1 have recently been reported (39), the encoded proteins show <30% identity to G_L. PPP1R3F and PPP1R3G encode proteins larger than G_L (79 and 38 kDa, respectively). The sizes of the PPP1R3E mRNA transcripts (5.9 and 7.2 kb) encoding a 30.6-kDa protein are different from that for G_L (data not shown), and PPP1R3E 3′ UTR contains no significant sequence similarities to G_L 3′ UTR.

The fact that G_L appears to be present at much higher levels in human skeletal muscle than in mouse, rat, and rabbit skeletal muscle is unusual but not without precedent. Other proteins have been shown to exhibit species-specific differences in expression. For example, inhibitor-1, which is expressed in a wide variety of tissues, is found in rabbit, pig, sheep, and guinea pig liver, but is absent from mouse and rat liver, despite being present in other tissues in these rodents (40). The abundance of G_L in human skeletal muscle compared with that in rodents and rabbits indicates that the latter animals may not be appropriate models from which to gain an understanding of hormonal regulation of human muscle glycogen synthase phosphatase.

The high level of G_L protein in human skeletal muscle is also of considerable importance for understanding the mechanisms by which insulin regulates glycogen synthesis in this tissue. Increasing evidence indicates that insulin acts via the PI-3 kinase/PKB pathway to inhibit GSK3 and elicit an activation of glycogen synthase. However, the mechanisms by which insulin may mediate activation of glycogen-targeted PP1, thereby causing dephosphorylation and activation of glycogen synthase, are unclear. The most abundant glycogen-targeted form of PP1 in skeletal muscle is $PP1G_M$, but recent studies indicate that this glycogen-targeted form of PP1 does not appear to be regulated by insulin. Although Ser48 and Ser67 of rabbit G_M become phosphorylated in response to adrenaline, these residues are not phosphorylated significantly in

response to insulin by MAPKAP kinase 1 or any other insulin-stimulated protein kinases (34). Overexpression of G_M/PPP1R3 in L6 myotubes resulted in increased basal and insulin-stimulated glycogen synthesis but did not increase the approximately twofold stimulation of glycogen synthesis by insulin, suggesting that G_M-PP1c may not be the major insulin-stimulated protein phosphatase in skeletal muscle (41). In G_M null mice, insulin stimulation of glycogen synthase in skeletal muscle does not differ from that in wild-type mice (36). In addition, in these G_M "knockout" mice, an insulin-stimulated glycogen synthase phosphatase, which was not thought to be the more widely distributed R5 and R6 complexes with PP1, was still detected. The studies we report here raise the possibility that G_L-PP1c may contribute to this insulin-stimulated phosphatase activity.

Previous studies have shown that the hepatic levels of G_L are reduced in streptozotocin-induced diabetes and restored by insulin treatment (24,25), but the low levels of G_L in rodent muscle make similar investigations difficult in this tissue. Modulation of G_L-PP1c interaction with the allosteric inhibitor phosphorylase *a* is more likely to underlie the short-term action of insulin, although the pathway by which insulin may decrease the levels of phosphorylase *a* in skeletal muscle is less clear. Unlike the studies with liver, where changes in cAMP levels in response to insulin are well documented, it is thought that in most situations changes in cAMP levels may not underlie the ability of insulin to activate glycogen synthase in skeletal muscle (1). Nevertheless, pretreatment of isolated rat diaphragm with insulin lowered the amount of cAMP produced in response to adrenaline (42,43). Another report shows that while insulin had no effect on basal cAMP release from perfused rat hind limb, it inhibited by 50% the elevated cAMP release produced in response to adrenaline (44). In adrenalectomized animals, isoproterenol, a β-adrenergic agonist, produced a twofold rise in inhibitor-1 phosphorylation, an effect that was completely prevented by the addition of insulin, and the antagonism of isoproterenol by insulin was shown to correlate with a decrease in the muscle content of cAMP (45). Overall, it appeared likely that for insulin to modulate cAMP levels, adrenaline concentrations should be >10 nmol/l in normal physiological range. These effects of insulin may be mediated by its ability to activate cAMP phosphodiesterase in skeletal muscle (43,46,47). It will be important to demonstrate whether insulin acting via this or another as yet unknown pathway can enhance the activity of the PP1c-G_L complex in human skeletal muscle.

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REFERENCES

1. Craig JW, Larner J: Influence of epinephrine and insulin on uridine diphosphate glucose-α-glucan transferase and phosphorylase in muscle. *Nature* 202:971–973, 1964
2. Cohen P: The structure and regulation of protein phosphatases. *Annu Rev Biochem* 58:453–508, 1989
3. Bollen M, Stalmans W: The structure, role and regulation of type I protein phosphatases. *Crit Rev Biochem Mol Biol* 27:227–281, 1992
4. Parker PJ, Caudwell FB, Cohen P: Glycogen synthase from rabbit skeletal muscle; effect of insulin on the state of phosphorylation of the seven phosphoserine residues *in vivo*. *Eur J Biochem* 130:227–234, 1983
5. Welsh GI, Proud CG: Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochemical J* 294:625–629, 1993
6. Shepherd PR, Nave BT, Siddle K: Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3–L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p70 ribosomal protein-S6 kinase. *Biochemical J* 305:25–28, 1995
7. Strålfors P, Hiraga A, Cohen P: The protein phosphatases involved in cellular regulation: purification and characterisation of the glycogen bound form of protein phosphatase-1 from rabbit skeletal muscle. *Eur J Biochem* 149:295–303, 1985
8. Tang PM, Bondor JA, Swiderek KM, DePoali-Roach P: Molecular cloning and expression of the regulatory (R_{Gl}) Subunit of the glycogen associated protein phosphatase. *J Biol Chem* 266:15782–15789, 1991
9. Hubbard MJ, Cohen P: On target with a new mechanism for the regulation of protein phosphorylation. *Trends Biochem Sci* 18:172–177, 1993
10. Chen YH, Hansen L, Chen MX, Bjørbæk C, Vestergaard H, Hansen T, Cohen PTW, Pedersen O: Sequence of the human glycogen associated regulatory subunit of type 1 protein phosphatase and analysis of its coding region and mRNA level in muscle from patients with non-insulin-dependent diabetes mellitus. *Diabetes* 43:1234–1241, 1994
11. Moorhead G, MacKintosh C, Morrice N, Cohen P: Purification of the hepatic glycogen-associated form of protein phosphatase-1 by microcystin-Sepharose affinity chromatography. *FEBS Lett* 362:101–105, 1995
12. Doherty MJ, Moorhead G, Morrice N, Cohen P, Cohen PTW: Amino acid sequence and expression of the hepatic glycogen binding (G_L) subunit of protein phosphatase-1. *FEBS Lett* 375:284–298, 1995
13. Armstrong CG, Doherty MJ, Cohen PTW: Identification of the separate domains in the hepatic glycogen-targeting subunit of protein phosphatase 1 that interact with phosphorylase *a*, glycogen and protein phosphatase 1. *Biochem J* 336:699–704, 1998
14. Doherty MJ, Young PR, Cohen PTW: Amino acid sequence of a novel protein phosphatase 1 binding protein (R5) which is related to the liver and muscle specific glycogen binding subunits of protein phosphatase 1. *FEBS Lett* 399:339–343, 1996
15. Printen JA, Brady MJ, Saltiel AR: PTG, a protein phosphatase 1-binding protein with a role in glycogen metabolism. *Science* 275:1475–1478, 1997
16. Newgard CB, Brady MJ, O'Doherty RM, Saltiel AR: Organizing glucose disposal: emerging roles of the glycogen targeting subunits of protein phosphatase-1. *Diabetes* 49:1967–1977, 2000
17. Armstrong CG, Browne GJ, Cohen P, Cohen PTW: PPP1R6, a novel member of the family of glycogen-targetting subunits of protein phosphatase 1. *FEBS Lett* 418:210–214, 1997
18. Johnson DF, Moorhead G, Caudwell FB, Cohen P, Chen YH, Chen MX, Cohen PTW: Identification of the protein phosphatase-1-binding domains on the glycogen and myofibrillar targetting subunits. *Eur J Biochem* 239:317–325, 1996
19. Egloff M-P, Johnson F, Moorhead G, Cohen PTW, Cohen P, Barford D: Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J* 16:1876–1887, 1997
20. Zhao S, Lee EYC: A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J Biol Chem* 272:28368–28372, 1997
21. Wu J, Kleiner U, Brautigan DL: Protein phosphatase type-1 and glycogen bind to a domain in the skeletal muscle regulatory subunit containing conserved hydrophobic sequence motifs. *Biochemistry* 35:13858–13864, 1996
22. Wu J, Liu J, Thompson I, Oliver CJ, Shenolikar S, Brautigan DL: A conserved domain for glycogen binding in protein phosphatase-1 targeting subunits. *FEBS Lett* 439:185–191, 1998
23. Bollen M, Stalmans W: The hepatic defect in glycogen synthesis in chronic diabetes involves the G-component of synthase phosphatase. *Biochem J* 217:427–434, 1984
24. Doherty MJ, Cadefau J, Stalmans W, Bollen M, Cohen PTW: Loss of the

hepatic glycogen-binding subunit (G_L) of protein phosphatase 1 underlies deficient glycogen synthesis in insulin-dependent diabetic rats and in adrenalectomized starved rats. *Biochem J* 333:253–257, 1998
25. Browne GJ, Delibegovic M, Keppens S, Stalmans W, Cohen PTW: 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. *Biochem J* 360:449–459, 2001
26. O'Doherty RM, Jensen PB, Anderson P, Jones JG, Berman HK, Kearney DCBN: Activation of direct and indirect pathways of glycogen synthesis by hepatic overexpression of protein targeting to glycogen. *J Clin Invest* 105:479–488, 2000
27. Stalmans W, de Wulf H, Hers HG: The control of liver glycogen synthase phosphatase by phosphorylase. *Eur J Biochem* 18:582–587, 1971
28. Miller TB, Garnache A, Vicalvi JJ: Hormonal regulation of hepatic glycogen synthase phosphatase. *J Biol Chem* 256:2851–2855, 1981
29. Mvumbi L, Doperé F, Stalmans W: The inhibitory effect of phosphorylase *a* on the activation of glycogen synthase depends on the type of synthase phosphatase. *Biochem J* 212:407–416, 1983
30. Alemany S, Cohen P: Phosphorylase a is an allosteric inhibitor of the glycogen and microsomal forms of rat hepatic protein phosphatase-1. *FEBS Lett* 198:194–202, 1986
31. Alemany S, Pelech S, Brierley CH, Cohen P: The protein phosphatases involved in cellular regulation: evidence that dephosphorylation of glycogen phosphorylase and glycogen synthase in the glycogen and microsomal fractions of rat liver are catalysed by the same enzyme: protein phosphatase-1. *Eur J Biochem* 156:101–110, 1986
32. Mvumbi L, Stalmans W: High affinity binding of glycogen synthase phosphatase to glycogen particles in liver: role of glycogen in the inhibition of synthase phosphatase by phosphorylase. *a Biochem J* 246:367–374, 1987
33. Bollen M, Keppens S, Stalmans W: Specific features of glycogen metabolism in the liver. *Biochem J* 336:19–31, 1998
34. Walker KS, Watt PW, Cohen P: Phosphorylation of the skeletal muscle glycogen-targetting subunit of protein phosphatase 1 in response to adrenaline in vivo. *FEBS Lett* 466:121–124, 2000
35. Hubbard MJ, Cohen P: Regulation of protein phosphatase-1G from rabbit skeletal muscle. 2. Catalytic subunit translocation is a mechanism for reversible inhibition of activity toward glycogen-bound substrates. *Eur J Biochem* 186:711–716, 1989
36. Suzuki Y, Lanner C, Kim J-H, Vilardo PG, Zhang H, Yang J, Cooper LD, Steele M, Kennedy A, Bock CB, Scrimgeour A, Lawrence JC, Roach AA: Insulin control of glycogen metabolism in knockout mice lacking the muscle-specific protein phosphatase PP1G/R_{GL}. *Mol Cell Biol* 21:2683–2694, 2001
37. Besley GTN, Cohen PTW, Faed MJW, Wolstenholme J: Amylo-1,6-glucosidase activity in cultured cells: a deficiency in type III glycogenosis with prenatal studies. *Prenatal Diagnosis* 3:13–19, 1983
38. Chenchik A, Diachenko L, Moqadam F, Tarabykin S, Lukyanov, Siebert PD: Full-length cDNA cloning and determination of mRNA 5′ and 3′ ends by amplification of adaptor-ligated DNA. *BioTechniques* 21:526–534, 1996
39. Ceulemans H, Stalmans W, Bollen M: Regulator-driven Functional diversification of protein phosphatase-1 in eukaryotic evolution. *Bioessays.* In press
40. MacDougall LK, Campbell DG, Hubbard MJ, Cohen P: Partial structure and hormonal regulation of rabbit liver inhibitor-1; distribution of inhibitor-1 and inhibitor-2 in rabbit and rat tisues. *Biochim Biophys Acta* 1010:218–226, 1989
41. Rasmussen SK, Hansen L, Frevert EU, Cohen PTW, Kahn BB, Pedersen O: Adenovirus-mediated expression of a naturally occurring Asp905Tyr variant of the glycogen-associated regulatory subunit of protein phosphatase 1 in L6 myotubes. *Diabetologia* 43:718–722, 2000
42. Craig JW, Rall TW, Larner J: The influence insulin and epinephrine on adenosine 3′,5′-phosphate and glycogen transferase in muscle. *Biochim Biohys Acta* 177:213–219, 1969
43. Eden S, Albertsson-Wikland K, Rosberg S, Isaksson O: Effect of insulin and adrenaline on cyclic AMP in the diaphragm of normal and diabetic rats. *Acta Endocrinol* 85:806–817, 1977
44. Rushakoff RJ, Lewis SB, Schultz TA, Kaplan RA, Davis JL, Pilkis SJ, Ho RJ, Wallin JD: Epinephrine-induced cyclic AMP production and vasoconstriction in the noncyclically perfused rat hindlimb: a possible role for insulin. *Life Sci* 22:61–65, 1978
45. Foulkes JG, Cohen P, Strada S, Everson WV, Jefferson LS: Antagonistic effects of insulin and β-adrenergic agonists on the activity of protein phosphatase inhibitor-1 in skeletal muscle of the perfused rat hemicorpus. *J Biol Chem* 257:12493–12496, 1982
46. Gain KR, Appleman MM: Distribution and regulation of the phosphodiesterases of muscle tissues. *Adv Cyclic Nucleotide Res* 9:221–231, 1978
47. Enoksson S, Degerman E, Hagstrom-Toft E, Large V, Arner P: Various phosphodiesterase subtypes mediate the in vivo antilipolytic effect of insulin on adipose tissue and skeletal muscle in man. *Diabetologia* 41:560–568, 1998