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 NH2-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).

Short-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 concent-
trations in the presence of glycogen, without inhibition of the phosphorylase phosphatase activity (30–32). Phosphorylase α was shown to bind to a 16–αmin 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 α, and the consequent inhibition of glycogen synthase phosphatase activity. Insulin lowers hepatic cAMP levels, causing a reduction in the level of phosphorylase α that alleviates the phosphorylase α–mediated inhibition of the G_L-PP1c complex, causing an increase of glycogen synthesis. The binding of glucose to phosphorylase α 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 adrenaline at Ser 48 and Ser 67 (using the numbering for the rabbit G_M sequence) (34). Because Ser 67 lies within identical patterns after cleavage with

Human liver and testis Matchmaker cDNA libraries (Clontech, Palo Alto, CA) were used as templates to amplify the full-length coding sequence for G_M by using the forward primer 1 and primer 3 (complementary to nt 852–827, 5′ GATGTTAGGCCCCGACAGTTTTTCATATGC 3′), 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′ rapid amplification of cDNA ends (RACE), (35) were performed using the same degenerate primers and specific primers were generated (complementary to nt 401–374, 5′-CCG ATT TCT AAA GTC TAA GTA ATC TTCG-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.

Fluorescent in situ hybridization. Purified BAC or cosmid DNA was labeled with the fluorescein 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 Library, 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 five-fold coverage of each chromosome. The filters were hybridized with the G_L coding region (852 bp). Four chromosome 8 clones (LA0835p13, LA0849a12, LA0817a5, and LA0822215 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 CO₂, and tissues were excised, freeze clamped, and stored at ~80°C. Heart and skeletal muscle were freeze-fractured ~80°C and homogenated at 4°C in six volumes 50 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 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 16,000g for 10 min, and the supernatants were decanted, snap-frozen in liquid nitrogen, and stored at ~80°C. Livers were homogenized in three volumes of 2 mmol/l EDTA, pH 7.0, 2 mmol/l EDTA, 250 mmol/l sucrose, 0.1% (vol/vol) 2-mercaptoethanol, and “Complete” protease inhibitor and were processed similarly. Proteins in the 16,000g 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 1–240), 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
isolation of cDNA for the human GL coding region. The 5’ and 3’ untranslated regions (UTRs) and confirmation of the extreme NH2- and COOH-terminal coding sequences was obtained by PCR using RACE methodology. Three genomic clones from a BAC library contained an identical region encoding GL, and the sequence obtained for the GL gene from −3,000 to +6,000 was identical to that reported in the human genome database recently. The liver GL 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 GL protein of 284 amino acids. Comparison of the amino acid sequence of rat GL with the deduced amino acid sequence of human and mouse GL 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 GL is 89 and 88.5% identical to rat GL and mouse GL respectively, and mouse GL is 94% identical with the gene sequence (from skeletal muscle to rat GL (Fig. 1).)

Analyses of GL at the protein level focused upon the insulin-sensitive tissues heart, liver, and skeletal muscle, as these tissues showed the highest levels of GL expression at the mRNA level. Immunoblotting of rat and mouse tissue extracts with an anti-rat GL antibody confirmed that liver exhibits the highest levels of GL mRNA (Fig. 4A 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 GL 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 GL by immunoblotting with the anti-rat GL antibody, revealed a protein migrating at the same size as rat liver GL in both human liver and skeletal muscle extracts (Fig. 5). Although the mRNA analysis suggested that higher levels of human GL mRNA are present in skeletal muscle than in liver, immunoblotting showed human GL protein to be present at appreciable and fairly similar levels in these tissues.

GL was examined for variation in the level of expression in different types of muscle. In mouse (Fig. 4B) and rabbit (data not shown), GL levels were very low in the gastrocnemius, psoas, and soleus muscles and no variation was observed. The expression of GL 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. 5B). Assessment of the GL-to-GM 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 GL was found to be −40% of that bound to GM (Fig. 5D and data not shown).

Although the sizes of the human GL mRNA and protein were identical in liver and skeletal muscle, there remained a possibility that their sequences differed due to alterna-
GL 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).
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. 3A).

**DISCUSSION**

We show here that the glycogen-targeting subunit, G_L, of PPI 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 α 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 α 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 GL mRNA is more abundant in human skeletal muscle than in liver. Immunological detection of GL protein at high levels in liver and low levels in heart and skeletal muscle of rat and mouse correlate with the mRNA results for these species. Human GL 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

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**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 iliopectosus 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 16,000g 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.
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 $c$DNA sequences from these tissues were identical and only a single $m$RNA species was detected by Northern blotting of these residues are not phosphorylated significantly in 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 \( \delta \) 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 \( G_{M}/PPP1c \) 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 \( G_{L}/PPP1c \) 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 \( \alpha \) is more likely to underlie the short-term action of insulin, although the pathway by which insulin may decrease the levels of phosphorylase \( \alpha \) 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 \( \beta \)-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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