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PPP1R6, a novel member of the family of glycogen-targetting subunits of protein phosphatase 1

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Abstract A complementary DNA encoding a novel human protein phosphatase 1 (PP1) glycogen-targetting subunit of molecular mass 33 kDa has been sequenced. PPP1R6 is 31% identical to the glycogen-targetting subunit (G_L) of PP1 from rat liver, 28% identical to the N-terminal region of the glycogen-targetting subunit (G_M) from human skeletal muscle and 27% identical to glycogen-targetting subunit PPP1R5. Unlike human PPP1R5 and its murine homologue PTG, whose mRNAs are most abundant in skeletal muscle, heart and liver, PPP1R6 is present at similar levels in a wide variety of tissues. The PPP1R6 is associated with glycogen in muscle but is not subject to the same modes of covalent and allosteric regulation as G_M and G_L.

Key words: Protein phosphatase 1; Targetting subunit; Glycogen metabolism; cDNA

1. Introduction

Protein phosphatase 1 (PP1) is known to form distinct complexes with more than 20 different proteins that allow it to carry out a wide variety of functions by targetting PP1 to various subcellular locations. For example, glycogen binding subunits target PP1 to glycogen particles, the myosin binding subunits target PP1 to the muscle contractile apparatus, while PP1 is associated with several different binding subunits in the nucleus. Interaction of PP1 with these targetting subunits frequently changes the substrate specificity of PP1 and may also allow PP1 complexes to be regulated by extracellular signals [1,2]. A short motif -(R/K)(V/I)xF- was recently shown to be sufficient for binding of these otherwise dissimilar subunits to PP1, and the presence of this motif in most of the known PP1 binding subunits explains why the interaction of these targetting subunits with PP1 is mutually exclusive [2,3].

PP1 plays a key role in the regulation of glycogen metabolism, through the dephosphorylation of glycogen synthase, phosphorylase and phosphorylase kinase. Three distinct glycogen-targetting subunits of PP1 have been identified. The skeletal muscle glycogen-targetting subunit G_M [4], which is also present in diaphragm and heart muscle, has been cloned from rabbit (termed R_{G1}) [5] and human tissues (termed PPP1R3 in the human genome nomenclature) [6]. Cloning of the 33 kDa liver specific PP1 glycogen-targetting subunit GL (PPP1R4) from rat [7], showed that although it was only 23% identical to the N-terminal third of the 126 kDa G_M subunit, conserved regions presumed to be involved in binding PP1 and glycogen could be identified. Determination of the crystal structure of PP1γ in complex with a 13 residue peptide from one of these regions, delineated the -(R/K)(V/I)xF- PP1 binding motif in G_M [3]. More recently, we identified a further 36 kDa glycogen-targetting subunit, termed human PPP1R5, which was most highly expressed in skeletal muscle and liver, but the mRNA was also present in a wide variety of other tissues. Comparison of the amino acid sequences of G_M, G_L and PPP1R5 with the *S. cerevisiae* glycogen binding subunit Gac1 [8] and *Rhizopus oryzae* glucoamylase [9] identified a putative glycogen/starch binding domain [10]. A murine glycogen-targetting subunit termed PTG [11] and chicken glycogen-targetting subunit U5 [12] appear to be homologues of human PPP1R5 (see Section 4). We describe here the identification of a fourth glycogen-targetting subunit which is expressed in many human tissues.

2. Materials and methods

2.1. Identification and characterisation of the cDNA encoding human protein phosphatase 1 binding subunit R6, PPP1R6

The National Centre for Biotechnology Information (NCBI) expressed sequence tag database was searched for sequences related to the putative glycogen binding region of the rat liver glycogen-targetting subunit (G_L) and PPP1R5, using the TBLASTN algorithm. Two overlapping cDNA clones in the plasmids Lafmid BA and pT7T3D were kindly provided by the IMAGE consortium, St. Louis. They were sequenced in their respective plasmids in both directions on an Applied Biosystems 373A automated DNA sequencer using Taq dye terminator cycle sequencing. One of the clones, 31199 in Lafmid BA, contained the full open reading frame of a protein with sequence similarity to rat G_L, hereafter termed PPP1R6.

2.2. Expression of PPP1R6 mRNA in human tissues

A Northern blot (Clontech, Palo Alto, CA) containing poly $(A)^+$ RNA from different human tissues was hybridised with an 850 bp *Bam*HI fragment encoding amino acids 15–394 of PPP1R6 labelled with [α-^{32}P]dATP, according to the manufacturer's instructions. The blot was washed at 60°C in 15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS. Following autoradiography, the blot was stripped by washing the membrane in 0.5% SDS at 100°C for 5 min. The blot was subsequently reprobed with either the full length coding region of PPP1R5 [10], the HIG 11 fragment of skeletal muscle G_M, encoding amino acids 2–687 [6], or a control β-actin probe (Clontech).

2.3. Heterologous expression of GST-PP1R6 in E. coli

IMAGE clone 31199 was used as a template in a polymerase chain reaction (PCR) with the oligonucleotide primers 5′-GCGCGAATTCATATGTCCAGAGGCCCGAGCTCC-3′ and 5′-GCGCAAGCTTCAGATGAAGTGGATCCAGCTC-3′. This produced a full length PPP1R6 coding region with an *Nde*I site 5′ of the initiating ATG and a *Hin*dIII site 3′ of the termination codon (sites underlined). The PCR product was subcloned into the TOPO 2.1 PCR cloning vector and transfected into TOP10 cells (Invitrogen, NV Leek, The Netherlands) for verification by sequencing. The PPP1R6 coding region was subsequently excised by restriction digestion with *Nde*I and *Hin*dIII and ligated into the pGEX-AH vector digested with the same restriction enzymes. The resultant construct encoded glutathione S-transferase (GST) fused to full length PPP1R6. Soluble GST-PPP1R6 was obtained by growing pGEX-AH/PPP1R6 transformed *E. coli* in LB

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medium containing 100 μg/ml ampicillin and inducing expression of GST-PPP1R6 in log phase at an $A_{600\ nm}$ of 0.5 with 0.2 mM isopropyl-thio-β-D-galactopyranoside. The cells were harvested after 16 h incubation at 28°C and soluble GST-PPP1R6 was purified as described [7].

2.4. Interaction of digoxygenin-PP1 with the PPP1R6

The PP1g isoform (40 μg) was labelled with the ester of digoxygenin-3-*O*-methylcarbonyl-ε-aminocaproic-acid-*N*-hydroxysuccinimide (Boehringer Mannheim, Germany) and separated from excess reagent according to the manufacturer's instructions.

Protein samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Non-specific binding to the membrane was blocked by incubation for 16 h at room temperature in 5% (w/v) Marvel dried milk powder in 25 mM Tris/HCl (pH 7.5), 250 mM NaCl. The membrane was then washed several times before incubation for 2 h with digoxygenin-PP1 diluted to 2.5 μg/ml in 25 mM Tris/HCl (pH 7.5), 250 mM NaCl, 1 mg/ml bovine serum albumin (BSA). The nitrocellulose membrane was washed extensively in several changes of 25 mM Tris/HCl (pH 7.5), 250 mM NaCl for 2 h and then probed for 1 h with anti-digoxygenin conjugated with horse radish peroxidase at a concentration of 150 mU peroxidase/ml (Boehringer) in 25 mM Tris/HCl (pH 7.5), 250 mM NaCl containing 5% (w/v) Marvel. The membrane was subsequently washed for 3 h in 25 mM Tris/HCl (pH 7.5), 250 mM NaCl and the proteins binding to digoxygenin labelled PP1 were visualised with the enhanced chemiluminescence system (Amersham International, Bucks, UK).

2.5. Production of PPP1R6 antibodies

The peptide RPIIQRRSRSLPTSPE corresponding to residues 48–63 of the rat sequence obtained from the NCBI expressed sequence tag database (corresponding to amino acids 65–80 in human PPP1R6) were conjugated to BSA and keyhole limpet haemocyanin and injected into a sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire, UK). The antibodies were affinity purified by chromatography of the anti-serum on peptide-CH-Sepharose columns. These anti-PPP1R6 antibodies recognised 1 ng of human GST-PPP1R6 but did not cross-react with 500 ng of GST-PPP1R5, GST-G_M (1–243) or GST-G_L (data not shown).

2.6. Isolation of the glycogen/sarcovesicle fraction from rabbit skeletal muscle

The skeletal muscle from one female New Zealand White rabbit was homogenised in 2 mM EGTA, 2 mM EDTA, pH 7.0, 0.1% 2-mercaptoethanol, 0.5 mM $PhMeSO_2F$, 1 mM benzamidine, 4 μg/ml leupeptin and a fraction containing glycogen particles and vesicular fragments of the sarcoplasmic reticulum was isolated as described in [13]. The pH 6.1 pellet was then resuspended in 30 ml of 0.1 M sodium glycerol-2-phosphate, 0.1 mM EGTA, 0.5 mM $PhMeSO_2F$, 1 mM benzamidine, 4 μg/ml leupeptin and centrifuged at $100000 \times g$ for 90 min. The supernatant was discarded and the glycogen/sarcovesicle pellet was resuspended in 100 ml of 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 5% glycerol, 0.5 mM $PhMeSO_2F$, 1 mM benzamidine, 4 μg/ml leupeptin, 0.1% 2-mercaptoethanol and recentrifuged. The resulting pellet was again resuspended in 30 ml of the same buffer.

3. Results

3.1. Human protein phosphatase 1 binding subunit R6 is related to the glycogen-targetting subunits of PP1

A search of the NCBI expressed sequence tag database with the putative glycogen binding region of rat G_L and human PPP1R5 proteins identified two partially sequenced cDNAs coding for proteins displaying significant similarity to these

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                                                                                taccgg  -270
gccccccctcgaggtcgacggtatcgataa gcttccactagagaaaaactccccactctt acaatttctttaaccgcaagaagcggagga  -180
cctggacaaggactcgaggagcaaggtggc gaaccaagggtagggcgcaccgggccgaga ggtcccccgcaggttgcagatacggtggac  - 90
tctctgcggcttctgagcacggagggagct gtcgcggggtcgggaggtcgtctctcatcc ctgcctcatcttccgacggccggcggggcc   -1

ATGTCCAGAGGCCCGAGCTCCGCGGTCCTG CCTAGCGCCCTGGGATCCCGGAAGCTCGGC CCCCGGAGCCTCAGCTGCCTGTCGGACCTG    90
 M  S  R  G  P  S  S  A  V  L   P  S  A  L  G  S  R  K  L  G   P  R  S  L  S  C  L  S  D  L    30

GACGGCGGCGTGGCCCTGGAGCCGCGGGCC TGTAGGCCCCCTGGGAGCCCGGGCCGCGCG CCGCCGCCAACGCCAGCGCCGTCGGGCTGC  180
 D  G  G  V  A  L  E  P  R  A   C  R  P  P  G  S  P  G  R  A   P  P  P  T  P  A  P  S  G  C    60

GACCCCCGCCTGCGGCCCATCATCCTGCGG CGGGCGCGCTCACTGCCCAGCTCCCCCGAA CGCCGCCAGAAGGCCGCGGGCGCCCCGGGC  270
 D  P  R  L  R  P  I  I  L  R   R  A  R  S  L  P  S  S  P  E   R  R  Q  K  A  A  G  A  P  G    90

GCTGCGTGTCGGCCGGGCTGCAGCCAGAAG CTCCGCGTGCGCTTCGCCGACGCCCTGGGC TTGGAGCTGGCACAGGTCAAGGTGTTCAAC  360
 A  A  C  R  P  G  C  S  Q  K   L  R  V  R  F  A  D  A  L  G   L  E  L  A  Q  V  K  V  F  N   120

GCGGGAGACGACCCGTCCGTGCCGCTGCAC GTGCTGTCGCGGCTCGCAATCAACTCGGAC CTGTGCTGCAGCAGCCAGGACCTGGAGTTC  450
 A  G  D  D  P  S  V  P  L  H   V  L  S  R  L  A  I  N  S  D   L  C  C  S  S  Q  D  L  E  F   150

ACCCTGCATTGCCTGGTGCCCGATTTCCCG CCGCCCGTCGAGGCCGCCGACTTTGGCGAG CGCCTGCAGCGGCAGCTCGTGTGCCTGGAG  540
 T  L  H  C  L  V  P  D  F  P   P  P  V  E  A  A  D  F  G  E   R  L  Q  R  Q  L  V  C  L  E   180

CGTGTCACTTGCTCGGACCTTGGCATCAGC GGTACGGTGCGCGTGTGCAACGTGGCCTTC GAGAAGCAGGTGGCTGTGCGCTACACTTTC  630
 R  V  T  C  S  D  L  G  I  S   G  T  V  R  V  C  N  V  A  F   E  K  Q  V  A  V  R  Y  T  F   210

TCGGGCTGGCGCAGTACCCACGAGGCGGTG GCGCGGTGGCGCGGGCCCGCAGGCCCCGAG GGCACGGAGGACGTTTTCACCTTCGGCTTT  720
 S  G  W  R  S  T  H  E  A  V   A  R  W  R  G  P  A  G  P  E   G  T  E  D  V  F  T  F  G  F   240

CCAGTACCGCCCTTCCTGCTGGAGCTCGGC TCCCGCGTGCACTTCGCGGTGCGCTACCAA GTGGCGGGTGCCGAGTACTGGGACAACAAC  810
 P  V  P  P  F  L  L  E  L  G   S  R  V  H  F  A  V  R  Y  Q   V  A  G  A  E  Y  W  D  N  N   270

GACCACCGAGACTACAGCCTCACATGTCGC AACCACGCGCTGCACATGCCTCGCGGGGAG TGCGAAGAGAGCTGGATCCACTTCATCtga  900
 D  H  R  D  Y  S  L  T  C  R   N  H  A  L  H  M  P  R  G  E   C  E  E  S  W  I  H  F  I      299

gccgcgcggggaccggccacctggagcctc cacacctaagctgcgcctcctgtcatttcc ctgctgggctctcacatctatctggttgtt  990
cttcaccaccctccaagtcctctgacctaa ttttctgctgcaaggtcccctggcagtggc ccatcctgtcttctacttgaaacgtctgag 1080
tcacttgtctaaaaagtagcctcaggtggc cagaaggccgagttgtgtaatgagttgggg cagggtggtggggtaggtgcatgggagggt 1170
gggccctctggggaggcccaagcagcttgt tttgcaaaggcccaagtcctcctgctagga aaagcttttgcatgtgtcctgaatgtgtct 1260
ctgtaaacatagctgttatttattattgtg atgttgggacctttagcccatagcggatgc ctcctcaggaatattctcgatttaataagc 1350
taaaaaaaaaaaaaaaaaaa                                                                       1370
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Fig. 1. Nucleotide and predicted amino-acid sequence of human protein phosphatase 1 binding subunit R6, PPP1R6.

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R6    MSRGPSSAVLPSALGSRK-LGPRSLSCLSDLDGGVALEPRACRPPGSPGR     49
R5        MSCTRMI------QVLDPRPLTSSV-MPVDVAMR-------LCLAH     32
G_L                                 MAVDIEY------------      7
G_M       MEPSEVPSQISKDNFLEVPNLSDSLCEDEEVTFQP---------GF     37

R6    APPPTPAPSGCDPRLRPIILRRA-----RSLPSSPERRQK-AAGAPGAAC     93
R5    SPPVKSFLGPYDEFQRRHFVNKL-----KPLKSCLNIKHK--AKSQNDWK     75
G_L   -----SYSSMAPSLRRERFTFKISPKLNKPLRPCIQLGSKDEAGRMVAPT     52
G_M   SPQPSRRGS---------------------------------DSSEDIYL     54
            ** *
R6    RPGCSQKLRVRFADALGLELAQVKVFNAG-DDPSVPLHV-LSRLA-INSD    140
R5    CSHNQAKKRVVFADSKGLSLTAIHVFSDLPEEPAWDLQFDLLDLNDISSA    125
G_L   VQEKKVKKRVSFADNQGLALTMVKVFSEF-DDP-LDIPFNITEL--LDNI     98
G_M   DTPSSGTRRVSFADSFGFNLVSVKEFDC------WELPSASTTF-DLGTD     97

R6    LCCSSQDLEFTLHCLVPDFPPPVEA-ADFGERLQRQLVCLERVTCSDLGI    189
R5    L---KHHEEKNL--IL-DFPQPSTDYLSFRSHFQKNFVCLENCSLQERTV    169
G_L   V---SLTTAESESFVL-DFPQPSADYLDFRNRLQTNHVCLENCVLKEKAI    144
G_M   I---FHTEEYVLAPLF-DLPSSKEDLMQ-QLQIQKAILESTESLLGSTSI    142

R6    SGTVRVCNVAFEKQVAVRYTFSGWRSTHEAVARWRGPAGPEGTEDVFTFG    239
R5    TGTVKVKNVSFEKKVQIRITFDSWKNYTDVDCVYMKNVYGGTDSDTFSFA    219
G_L   AGTVKVQNLAFEKVVKIRMTFDTWKSFTDFPCQYVKDTYAGSDRDTFSFD    194
G_M   KGIIRVLNVSFEKLVYVRMSLDDWQTHYDILAEYVPNSCDG-ETDQFSFK    191

R6    FPVPPFLLELGSRVHFAVRYQVAGAEYWDNNDHRDYSLTCRNH-------    282
R5    IDL-PPVIPTEQKIEFCISYHANGQVFWDNNDGQNYRIVHVQWKPDGVQT    268
G_L   ISL-PEKIQSYERMEFAVCYECNGQSYWDSNKGKNYRITRAELRSTQGMT    243
G_M   IVLVPPYQKDGSKVEFCIRYETSVGTFWSNNNGTNYTFICQKKEQEPEPV    241

R6    -------ALHM--PRGECE----------------------ESWIHFI    299
R5    QMAPQDCAFHQTSPKTELESTIFGSPRLASGLFPEWQSWGRMENLASYR    317
G_L   E------PYNG--PDFGISFDQFGSPRCSFGLFPEWPSYLGYEKLGPYY    284
G_M   K------PWKEVPNRQIKGCLKVKSSKEESSVTSEENNFENPKNTDTYI    284
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Fig. 2. Comparison of the amino-acid sequence of human PPP1R6 with rat G_L, the N-terminal 1–284 amino acids of human G_M and human PPP1R5. Identities are underlined. Residues corresponding to the -(R/K)(V/I)xF- PP1 binding motif are indicated by stars and the putative glycogen binding region by double underlining.

PP1 binding subunits. One of the cDNA clones, which was derived from a human infant brain library, contained a complete open reading frame encoding a putative protein of 299 amino acids with a predicted molecular mass of 32.5 kDa (Fig. 1). The 5′ untranslated region sequence preceding the putative initiating methionine conforms to the consensus sequence for eukaryotic translation initiation in 4 out of 6 nucleotides [14]. In addition, a search of the NCBI database with the N-terminal 100 amino acids of PPP1R6 identified a partial rat sequence encoding the N-terminal 70 amino acids of an approximately 70% identical protein, but which diverged from the human PPP1R6 sequence immediately upstream of the putative initiating methionine. Following the termination codon at nucleotide 898 there are also 6 additional in frame stop codons.

Comparison of the deduced amino acid sequence of human PPP1R6 with other glycogen-targetting subunits of PP1 (Fig. 2) shows that this novel protein is 31% identical to rat G_L, 28% to the N-terminal region of human G_M and 27% to human PPP1R5.

Analysis of the tissue distribution of PPP1R6 mRNA by Northern blotting shows that the PPP1R6 cDNA hybridises with two RNA species of 5.4 and 3.8 kb (Fig. 3A). These transcripts are detected in all tissues examined, with skeletal muscle and heart showing the highest amounts. In contrast, the other glycogen-targetting subunits have a more restricted expression pattern. The PPP1R5 transcript is expressed most abundantly in skeletal muscle, liver and heart (Fig. 3B), the G_M transcripts are only expressed in striated muscles (Fig. 3C and see [5]), while the G_L transcript is expressed exclusively in liver [7].

3.2. *PPP1R6 binds to PP1 and is associated with glycogen particles but not sarcovesicles of rabbit skeletal muscle*

The full length PPP1R6 cDNA sequence was expressed in *E. coli* as a GST-PPP1R6 fusion protein and purified to near homogeneity by affinity chromatography on reduced glutathione-agarose. The purified GST-PPP1R6 preparation consisted of the full length 58 kDa fusion protein and several smaller degradation products ranging in apparent molecular mass from 25–35 kDa (Fig. 4A). Only the 58 kDa GST-PPP1R6 protein bound to digoxygenin-PP1γ (Fig. 4B). In contrast, some degradation products of GST-G_M, GST-G_L and GST-R5 as well as the entire fusion proteins were detected by digoxygenin-PP1γ, presumably because they still contained the PP1 binding region. The interactions with PP1 were specific, since none of the molecular mass markers or the subunits of $PP2A_1$ bound to digoxygenin-PP1γ (Fig. 4B).

The PPP1R6 protein was found to be associated with a glycogen/sarcovesicle fraction from rabbit skeletal muscle as

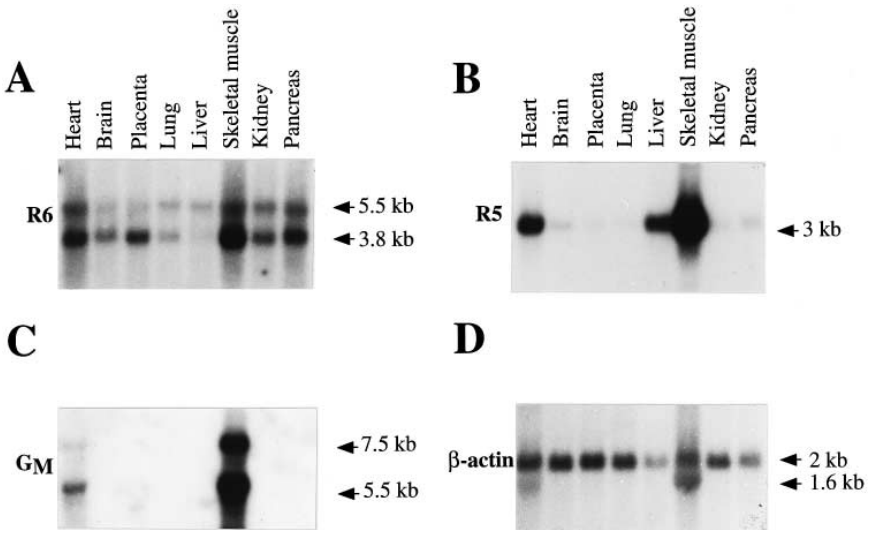

Fig. 3. Tissue distribution of the PPP1R6 mRNA. A human multiple tissue Northern blot (Clontech) containing approximately 2 μg of poly $(A)^+$ RNA per lane, was hybridised with cDNA probes to either human PPP1R6, designated R6 (A), PPP1R5, designated R5 (B), G_M (C) or a control probe, β-actin (D). Transcript sizes are indicated by arrows and calculated by their mobility relative to RNA standards.

judged by immunoblotting (Section 2.5, Fig. 5). In order to determine whether the protein was associated with glycogen or the sarcovesicles, the glycogen/sarcovesicle fraction was either incubated with α-amylase to degrade the glycogen, washed with Triton X-100 to solubilise the sarcovesicles, or subjected to both procedures. PPP1R6 immunoreactivity associated with the glycogen/sarcovesicle fractions was released by amylase treatment, but not by extraction with Triton X-100 (Fig. 5), demonstrating that PPP1R6 is specifically associated with glycogen particles.

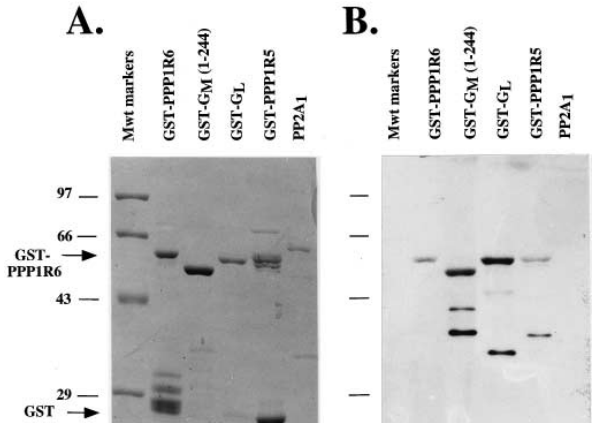

Fig. 4. The GST-PPP1R6 protein shows specific binding to digoxygenin-PP1. One μg of GST-PPP1R6, GST-G_M (1–243), GST-G_L, GST-PPP1R5 and 1.5 μg of $PP2A_1$ (comprising the catalytic subunit and regulatory A and B subunits) were separated by SDS-PAGE and either stained by Coomassie blue (A) or transferred to nitrocellulose membrane and probed with digoxygenin-PP1γ (B). Binding of digoxygenin-PP1 was visualised using with peroxidase conjugated anti-digoxygenin antibodies followed by enhanced chemiluminescence. The arrows show the position of full length GST-PPP1R6, GST and the molecular mass markers phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

4. Discussion

We have identified a fourth member of the family of glycogen-targetting subunits of PP1, termed PPP1R6. Although human PPP1R6 is most closely related to rat G_L, the low sequence identity to G_L (31%) and the distinct tissue distributions of these proteins (Fig. 3 and [7]) exclude the possibility that PPP1R6 is the human homologue of rat G_L. Moreover, an EST cDNA encoding part of the rat PPP1R6, which is approximately 70% identical to human PPP1R6 in the N-terminal region, is present in the NCBI database. Since PPP1R6 shows only low identity (< 28%) to human PPP1R5 and human G_M, PPP1R6 is clearly a novel PP1 glycogen-targetting subunit. A phylogenetic tree depicting the relationships between the known glycogen-targetting subunits in vertebrates is shown in Fig. 6. PPP1R5 [10] and murine PTG [11] appear to be species homologues since they show 85% identity and a similar tissue distribution. Although PTG was reported to be 23 amino acids shorter at the N-terminus [11] than PPP1R5, translation of the 52 nucleotides in the '5′ UTR' of PTG shows identity to PPP1R5 in 15 deduced amino acids preced-

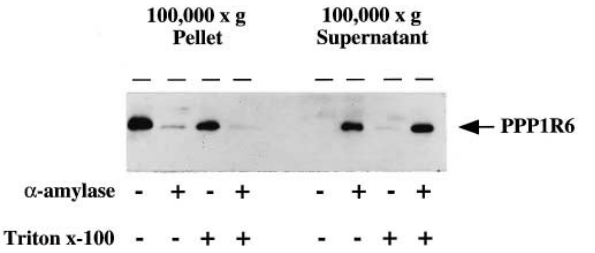

Fig. 5. Association of PPP1R6 with glycogen particles in rabbit skeletal muscle. A skeletal muscle glycogen particle/sarcoplasmic reticulum fraction was digested with 20 μg/ml human salivary a-amylase for 45 min at 30°C, washed with 1% Triton X-100 or subjected to both procedures before centrifugation for 90 min at 100 000 × *g*. The proteins in the resultant pellet and supernatant fractions were denatured in SDS, subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with anti-PPP1R6 antibodies.

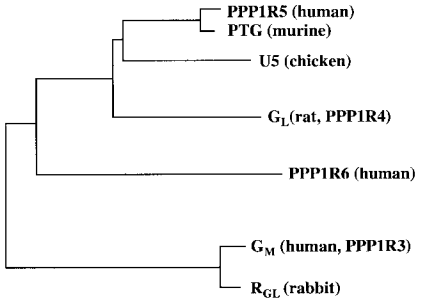

Fig. 6. Phylogenetic tree depicting the relationship between the vertebrate glycogen binding subunits. The sequences used are human PPP1R5 [10], murine PTG [11], chicken U5 [12], rat GL [7], human PPP1R6 (Fig. 1), the N-terminal regions of human G_M (amino acids 1–284) [6] and rabbit R_{Gl} (amino acids 1–286) [5]. The tree is derived from distance matrices after progressive multiple alignment [19] using the SEQNET facility at Daresbury, UK.

ing the residue that was presumed to be the initiating methionine, with divergence only occurring at the extreme 5′ end. This suggests that this region of the PTG mRNA is translated and that PPP1R5 and PTG have very similar, if not identical, N-terminal sequences. The protein U5 identified in smooth muscle may be the chicken homologue of PPP1R5, since it shows 47% identity to PPP1R5 and lower sequence identity to the other PP1 glycogen-targetting subunits ([12] and Fig. 6).

Unlike G_M, G_L and PPP1R5, which are predominantly expressed in the major glycogen metabolising tissues, PPP1R6 has a wide tissue distribution. The N-terminal half of PPP1R6 possesses the sequence -RVVF- which conforms to the minimum PP1 binding motif -(R/K)(V/I)xF-. Comparison of the amino acid sequence of PPP1R6 with G_M, G_L and PPP1R5 (Fig. 2) shows that the putative glycogen binding domain identified in these subunits [10] is present in the C-terminal half of PPP1R6. Immunoblotting studies established that PPP1R6 is specifically associated with glycogen particles and that, unlike G_M [1], PPP1R6 is not associated with the sarcoplasmic reticulum (Fig. 5). Therefore the function of PPP1R6 is likely to be restricted to the regulation of glycogen-bound PP1.

The PP1-G_M complex dissociates in response to adrenalin as a result of the phosphorylation of G_M at Ser46 and Ser65 by cAMP-dependent protein kinase (PKA). This leads to the release of PP1 from the glycogen particles thereby preventing it from dephosphorylating the enzymes of glycogen metabolism that remain associated with glycogen [1]. In contrast PPP1R6 lacks Ser65 and does not contain any other consensus sequence for phosphorylation by PKA. However, it is intriguing that the N-terminal region of PPP1R6 contains three consensus sequences -PX(S/T)P- for phosphorylation by mitogen-activated protein kinases (MAPKs). It also contains the sequence -LXRXXSL-, which is a consensus for phosphorylation by MAPK-activated protein kinase-2 (MAPKAP-K2) or calmodulin-dependent protein kinase-2 [15].

The PP1-G_L complex is regulated by the active form of phosphorylase (phosphorylase *a*). Phosphorylase *a* binds to PP1-G_L at nanomolar concentrations, preventing G_L from dephosphorylating and activating glycogen synthase. This is an important mechanism for inhibiting glycogen synthesis as glycogenolysis is activated and vice versa [16]. However, like PPP1R5 [10], PPP1R6 lacks a high affinity binding site for phosphorylase *a* ([7], M.J. Doherty and P.T.W. Cohen, unpublished data).

Further studies will be necessary to determine whether the complex of PPP1R6 with PP1 is subject to regulation by extracellular signals, such as insulin which stimulates the dephosphorylation of glycogen synthase in skeletal muscle. Since decreased insulin activation of glycogen synthase may contribute to insulin resistance in non-insulin-dependent diabetes mellitus [17,18], understanding the nature and regulation of the glycogen-associated forms of PP1 is of fundamental importance. The multiplicity of glycogen-targetting subunits revealed in this and other recent studies also implies that the relative contributions of all four glycogen-targetting subunits to the regulation of glycogen metabolism in skeletal muscle, liver and other tissues will need to be re-evaluated.

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