

# A novel 50 kDa protein forms complexes with protein phosphatase 4 and is located at centrosomal microtubule organizing centres

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Protein phosphatase 4 (PPP4) is a protein serine/threonine phosphatase that has been implicated in microtubule organization at centrosomes. Complexes of PPP4 with high apparent molecular masses (450 and 600 kDa) were purified from mammalian skeletal muscle and testis to near homogeneity. Amino acid sequences derived from a protein component present in both complexes were utilized to identify a human cDNA. The encoded putative PPP4 regulatory subunit (termed PPP4R2), comprising 453 amino acids, had a molecular mass of 50.4 kDa. The interaction of PPP4R2 with PPP4 catalytic subunit (PPP4c) was confirmed by co-sedimentation of PPP4c with PPP4R2

expressed in bacteria and human cells. PPP4c formed a complex of 450 kDa with baculovirus expressed His<sub>6</sub>-tagged PPP4R2. Immunocytological detection of PPP4R2 at centrosomes suggests that it may target PPP4c to this location. Native 450 kDa and 600 kDa PPP4 complexes are inactive, but can be activated by basic proteins, suggesting that PPP4R2 may also regulate the activity of PPP4c at centrosomal microtubule organising centres.

**Key words:** centrosome, fostriecin, microtubule nucleation, okadaic acid, protein phosphatase.

## INTRODUCTION

Centrosomes are the main organizing centres of microtubules, which are involved in a wide range of cellular processes, including formation of the mitotic spindle during mitosis and transport of intracellular organelles, cell polarity and directed cell movement during interphase. Although microtubules can form spontaneously *in vitro*, centrosomes lower the critical concentration of tubulin required for polymerization, as well as determining the number, direction and polarity of microtubules. However, the mechanisms by which centrosomes nucleate microtubule growth and co-ordinate microtubule rearrangements to cell cycle events have remained elusive [1,2]. Many of the proteins that have been identified at centrosomes are likely to be structural proteins.  $\gamma$ -Tubulin, a low abundance isoform of the  $\alpha$ - and  $\beta$ -tubulins that form the microtubules, is concentrated at centrosomes in higher eukaryotes, and their counterparts (spindle pole bodies) in yeasts. Gene disruption in several organisms and immunodepletion studies have demonstrated that  $\gamma$ -tubulin is essential for microtubule growth at centrosomes/spindle pole bodies [3]. A ring-like structure isolated from centrosomes, termed the  $\gamma$ -tubulin ring complex, was able to nucleate microtubule growth [4,5] and was essential for this process to occur *in vitro* from salt-stripped centrosomes [6]. The ring complex contains  $\alpha$ - and  $\beta$ -tubulins and several unidentified proteins, as well as  $\gamma$ -tubulin. Spc98p is an essential spindle pole body component in *Saccharomyces cerevisiae* that has been shown to bind to  $\gamma$ -tubulin [7]. Antibodies to the recently identified human homologue of Spc98p have been shown to inhibit microtubule nucleation *in vitro* [8]. Pericentrin, an integral component of the pericentriolar material, has

been reported to interact with  $\gamma$ -tubulin to form a centrosome lattice [9].

There is increasing evidence that phosphorylation plays a role in the regulation of microtubule growth and organization at centrosomes. A monoclonal antibody, named MPM2, which recognizes an epitope phosphorylated at M phase, was shown to block microtubule nucleation [10]. Several protein kinases and phosphatases have been located at centrosomes, although evidence for a functional role in microtubule growth or organization has been reported in only a few cases. p34<sup>cdc2</sup>-cyclin A, which has major roles in mitosis, has been shown to increase the nucleating activity of microtubules at centrosomes *in vitro* [11]. Polo-like kinases, implicated in the activation of the Cdc25 phosphatase at the G2–M transition [12], may also play a role in the recruitment to centrosomes of other proteins that are recognized by the MPM2 antibody [13]. More recently, polo-like kinases have been found in complexes associated with  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tubulins [14] and reported to phosphorylate tubulins *in vitro* [14,15]. LK6, a protein serine/threonine kinase identified in *Drosophila*, was found to associate with centrosomes and microtubules, while overexpression of LK6 caused defects in microtubule growth and organization at centrosomes in early embryos [16].

Protein phosphatase 4 (PPP4; previously named PP4, PPX), a protein serine/threonine phosphatase, showed a predominant localization at centrosomes in mammalian cells [17] and in *Drosophila* embryos [18]. P-element mutagenesis produced a *Drosophila* semi-lethal *emm* mutant (centrosomes minus microtubules), with 25% of normal PPP4 catalytic subunit (PPP4c) protein levels in the early embryo, nuclei arrested in mitosis and centrosomes without attached radiating microtubules [18].

Abbreviations used: PPP4, protein phosphatase 4; PPP4c, PPP4 catalytic subunit; PPP4R1 and PPP4R2, protein phosphatase 4 regulatory subunits; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A, PP2Ac, PP2A catalytic subunit; Tos-Phe-CH<sub>2</sub>Cl, tosylphenylalanylchloromethane; GST, glutathione S-transferase.

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The human and *D. melanogaster* sequences reported have been deposited in the GenBank® Nucleotide Sequence Database under accession numbers AJ 271448 and AJ 271449 respectively.

Reduced immunostaining of  $\gamma$ -tubulin at centrosomes, coupled with normal total  $\gamma$ -tubulin levels in *cmn* mutants, implicated PPP4c in the regulation of microtubule growth or organization at centrosomes, and was consistent with a change in conformation or reduced recruitment of  $\gamma$ -tubulin to centrosomes. In contrast, *Drosophila* early embryos deficient in protein phosphatase 2A catalytic subunit (PP2Ac), a closely related protein serine/threonine phosphatase, exhibited a lethal phenotype in which elongated microtubules radiated from centrosomes but were unconnected to the chromosomes [19]. PP2A (or a PP2A related phosphatase) has also been shown to dephosphorylate stathmin/Op18 and thereby maintain a shorter steady-state length of microtubules *in vitro* [20].

Both PPP4 and PP2A are members of the PPP family of protein serine/threonine phosphatases, which includes protein phosphatase 1 (PP1), PP2A, PP2B (calcineurin) and protein phosphatase 5 [21]. (The human genome nomenclature is used for the recently discovered protein phosphatases [21a], while the older nomenclature is retained for PP1 and PP2A.) We have recently purified native PPP4c and shown that, like PP2Ac, it is potently inhibited by tumour promoters such as okadaic acid, and by the anti-tumour drug, fostriecin [22]. Although most closely related to PP2Ac, with 65% amino acid sequence identity, PPP4c does not bind the 65 kDa regulatory subunit [23] of PP2Ac [17]. It therefore appears likely that PPP4c will form complexes with distinct regulatory subunits. PPP4c has recently been identified in a yeast two-hybrid screen as a protein that interacts with the transcription factor c-Rel [24]. Overexpressed PPP4c was reported to stimulate the DNA binding activity of c-Rel and activate transcription of the related transcription factor, NF- $\kappa$ B, when both were overexpressed in 293 cells. PPP4c, PP2Ac and PPP6c have also been identified in a yeast two-hybrid screen as proteins that interact with  $\alpha$ 4, a mammalian protein related to Tap42p in *S. cerevisiae* which appears to be controlled by TOR protein kinase, the target of an immunosuppressant drug, rapamycin [25]. More recently, Kloeker and Wadzinski [26] purified a 270–300 kDa form of PPP4 from bovine testis that comprised PPP4c bound to a 105 kDa putative regulatory subunit (PPP4R1), but the subcellular location of PPP4R1 has not been reported. Here we purify PPP4 complexes with apparent molecular masses of 450 and 600 kDa and identify a regulatory subunit of PPP4c which influences its activity and localizes to centrosomes.

## EXPERIMENTAL

### Materials

PP2Ac [27] was purified from bovine heart. PPP4c [22] was purified from pig testis. Human PP1 $\gamma_1$  [28] was expressed in *Escherichia coli* and purified [29]. Buffer A comprised 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, 0.03% (v/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine and 0.1 mM PMSF, 0.2 mM tosylphenylalanylchloromethane (Tos-Phe-CH<sub>2</sub>Cl) and 5  $\mu$ g/ml leupeptin. Buffer B contained 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1 mM EGTA, 0.03% (v/v) Brij 35, 0.1% (v/v) 2-mercaptoethanol, and 5% (v/v) glycerol. Buffer C was the same as buffer A with the addition of 5% (v/v) glycerol. Buffer D comprised 50 mM Mops (pH 7), 1 mM EDTA, 0.1 mM EGTA, 0.03% (v/v) Brij 35, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF, 0.2 mM Tos-Phe-CH<sub>2</sub>Cl and 5  $\mu$ g/ml leupeptin. SDS buffer contained 60 mM Tris/HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.02% (w/v) Bromophenol Blue.

### Purification of high-molecular-mass forms of PPP4 from pig testis

Four freshly excised pig testes (1 kg) were placed on ice at the slaughterhouse. All subsequent steps were performed at 0–4 °C, except for the FPLC separations at room temperature. The testes were decapsulated, minced and homogenized within 20 min in a Waring blender (30 s at low speed) in 1 vol. of buffer A. The homogenate was centrifuged at 4200 g for 30 min and the supernatant was filtered through glass wool and recentrifuged at 100000 g for 1 h. The supernatant was poured into a sintered glass funnel containing 300 ml of fast flow Q-Sepharose resin equilibrated in buffer A. The column was washed with 1 litre of buffer A, containing 0.1 M NaCl, and PPP4 was eluted with 2 litres of buffer A, containing 0.5 M NaCl. The eluate was subjected to a 30–55% ammonium sulphate fractionation and the pellet was resuspended in 100 ml of buffer C, dialysed against this buffer overnight and the dialysate centrifuged for 30 min at 100000 g. The supernatant was applied to a HiLoad 16/10 Q-Sepharose column equilibrated in buffer C and chromatographed with a 300 ml linear gradient of NaCl from 0.1 M to 0.5 M at a flow rate of 5 ml/min, with 5 ml fractions being collected. Fractions eluting at 340–375 (PPP4<sub>2</sub>) and 375–420 mM (PPP4<sub>3</sub>) NaCl were pooled and further purified separately. After dialysis into buffer C, each PPP4 pool was applied to a 5 ml HiTrap Q-Sepharose column equilibrated in buffer C, and concentrated by elution with 8 ml of 0.5 M NaCl. The eluate was dialysed against buffer D and applied to a 5 ml HiTrap heparin-Sepharose column equilibrated in buffer D. The flowthrough containing PPP4 was applied to a 5 ml HiTrap SP-Sepharose column equilibrated in buffer D. Flowthrough from the latter was dialysed against buffer C and applied to a 5 ml HiTrap Blue-Sepharose column equilibrated in buffer C. The subsequent flowthrough containing PPP4 was chromatographed on a HR 5/5 Mono-Q column equilibrated in buffer C. The column was developed with a 20 ml 0.1–0.5 M NaCl linear gradient at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. Fractions containing PPP4 eluting at 340–370 mM (PPP4<sub>2</sub>) and 380–410 mM (PPP4<sub>3</sub>) NaCl were separately pooled, concentrated to 200  $\mu$ l in a Centricon-30 microconcentrator and subjected to gel filtration on a HR 10/30 Superose 6 column equilibrated in buffer C containing 0.4 M NaCl. Fractions containing PPP4 were snap frozen in liquid nitrogen and stored at –70 °C.

Microcystin-Sepharose-affinity analysis was performed by incubation of PPP4 complexes eluting from the Superose 6 column (20  $\mu$ l) with 10  $\mu$ l of microcystin-Sepharose beads [30] in buffer B for 1 h at 4 °C. After washing the beads 6 times in buffer B containing 0.4 M NaCl, the bound proteins were eluted in SDS buffer and analysed by SDS/PAGE and immunoblotting.

### Purification of high-molecular-mass forms of PPP4 from rabbit skeletal muscle and preparation of rat liver extracts

Rabbit skeletal muscle extracts were prepared as in Cohen et al. [27]. After removal of the glycogen pellet at pH 6.1, the supernatant was readjusted to pH 7.2 with 10 M ammonium hydroxide and applied to QAE-Sephadex. Purification then followed that for pig testis PPP4 from the fast flow Q-Sepharose (which replaced the QAE-Sephadex). The Blue-Sepharose column was omitted and only one pool of PPP4 was collected at the Hi-Load Q-Sepharose step, eluting between 330 and 415 mM NaCl.

Liver from Wistar rats was homogenized in 3 vol. of 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF, 0.2 mM Tos-Phe-CH<sub>2</sub>Cl, 5  $\mu$ g/ml leupeptin and centrifuged at 16000 g for 15 min at 4 °C. The supernatant was recentrifuged at 100000 g for

90 min at 4 °C, and the final supernatant filtered through a 0.45- $\mu$ m filter and chromatographed on Mono-Q, as described above.

### Determination of peptide amino acid sequences

Protein samples were fractionated by SDS/PAGE and stained with Coomassie Blue. PPP4c and candidate regulatory subunits were excised from the gel and digested *in situ* with trypsin [31]. Tryptic peptides were separated on a C<sub>18</sub> column (150 mm  $\times$  0.5 mm; Perkin–Elmer Applied Biosystems Inc., Warrington, Cheshire, U.K.) using Applied Biosystems 173A HPLC system equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear increase in acetonitrile concentration of 0.5% per min. Peptides detected by absorbance at 214 nm were collected on a miniblitter fitted with two sheets of Problot (Perkin–Elmer Applied Biosystem Inc.) and sequenced by Edman degradation on an Applied Biosystems 476A sequencer. Alternatively the proteins were excised from the gel, digested *in situ* with trypsin as described in [32]. Tryptic peptide masses were analysed using a thin film matrix of 4-hydroxy- $\alpha$ -cyanocinnamic acid/nitrocellulose (2:1) in an Elite STR mass spectrometer (PerSeptive Biosystems, Foster City, CA, U.S.A.) in reflectron mode. Spectra were internally calibrated with matrix and trypsin autolysis ions, and the peptide masses were then used to search databases within the UCSF Protein Prospector program, using MS-FIT analysis software with a mass error set at 50 p.p.m.

### PPP4 regulatory subunit 2 (PPP4R2) cDNA identification and sequence analysis

The National Center for Biotechnology Information (NCBI) expressed-sequence-tag database was searched for cDNA sequences encoding peptides isolated from the candidate PPP4R2 using the BLASTp algorithm. Clones H56614 and aa564356 were obtained from IMAGE consortium and sequenced on an Applied Biosystems 373A DNA sequencer using Taq dye terminator cycle sequencing. Clone H56614 contained 417 nt, including a Kozak consensus start sequence 5' of an open reading frame encoding all of the identified peptide sequences, but lacking C-terminal coding sequence and a stop codon. Clone aa564356 lacked extreme N-terminal coding sequence and a section of 60 nt encoding the amino acids 243–263, but contained 273 nt following the stop codon and a polyadenylated tail. DNA encoding the entire open reading frame was constructed by PCR using these clones as templates and engineered to contain a *Nde*I site at the initiating methionine codon and a *Xho*I site just 3' of the termination codon. The *Nde*I–*Xho*I fragment containing the 1359 nt open reading frame encoding 453 amino acids was cloned into pCR 2.1-TOPO, a topoisomerase-activated plasmid, and transfected into TOP10 competent cells (Invitrogen, Leek, The Netherlands) for verification by sequencing.

### Preparation of expression constructs

The *Nde*I–*Xho*I fragment encoding PPP4R2 was inserted into pGEX-AH [33] and expressed in *E. coli*. Glutathione S-transferase (GST)-PPP4R2 was affinity purified on GSH–Sephacrose. His<sub>6</sub>-PPP4R2 was expressed in insect cells, following insertion of the *Nde*I–*Xho*I fragment into the FASTBAC1 vector and production of recombinant baculovirus using the Bac to Bac system (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.). *Spodoptera frugiperda* 21 cells (1.5  $\times$  10<sup>6</sup>/ml) were infected with recombinant virus at a multiplicity of infection of 5 and harvested 72 h post infection. His<sub>6</sub>-PPP4R2 was purified on Ni<sup>2+</sup>-nitrilo-

triacetate agarose, dialysed into buffer B containing 1 mM benzamidine, 0.1 mM PMSF, 150 mM NaCl, 50% glycerol and stored at –20 °C. FLAG-tagged PPP4R2 was prepared by PCR using GST-PPP4R2 as a template and the 5' oligonucleotide 5'-GGGAATTCGCCACCATGGACTACAAGGACGACGATGACAAGTGCCAGGCGCCATGTTGGAGG-3', which incorporated an *Eco*RI site (underlined) and encoded the FLAG tag (DYKDDDDK), and the 3' oligonucleotide 5'-GCATCGATCAGAAGAACCCTACTTTCAGG-3' which incorporated a *Cl*aI site (underlined). The resulting PCR product was ligated into the pCR 2.1-TOPO vector, verified by sequencing and the *Eco*RI–*Cl*aI fragment subcloned into pCMV5 for expression in human cells.

### Sedimentation and gel filtration of PPP4R2 and PPP4c

PPP4c (2 nM) and a 2-fold molar excess of GST–PPP4R2 were incubated in 20  $\mu$ l of buffer B, containing 150 mM NaCl and 0.1 mg/ml BSA, for 1 h at 4 °C, before addition of 10  $\mu$ l of GSH–Sephacrose beads, which had been pre-equilibrated in buffer B containing 150 mM NaCl. After incubation for a further hour, the beads were sedimented at 14000 *g* for 2 min and the supernatant retained. The beads were washed four times with 0.5 ml of buffer B containing 150 mM NaCl and resuspended in SDS buffer. The resuspended beads and supernatant fractions were boiled for 5 min in SDS buffer, briefly centrifuged at 14000 *g*, and the solubilized proteins were subjected to SDS/PAGE, transferred to nitrocellulose and probed with anti-PPP4 antibodies. Gel filtration on Superose 6 was performed before and after incubation of His<sub>6</sub>-PPP4R2 with PPP4c or PP2Ac for 1 h at 4 °C in buffer B containing 200 mM NaCl. Density gradient sedimentation of PPP4R2 was performed by layering the protein on the top of 16 ml 2–20% and 10–30% glycerol gradients in buffer B containing 200 mM NaCl, prepared using Pharmacia FPLC gradient maker. After centrifugation at 100000 *g* for 36 h at 4 °C, the position of PPP4R2 was determined by immunoblotting of the 0.5 ml fractions collected from the top of the gradient using Nyegaard Diagnostica apparatus. Marker proteins were BSA (67 kDa) and ferritin (450 kDa).

### Cell culture, transfection of 293 cells and immunoprecipitation of FLAG-tagged PPP4R2

Human embryonic kidney 293 cells, human epidermal carcinoma A431 cells and HeLa cells were cultured at 37 °C in an atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin and 100  $\mu$ g/ml streptomycin. Transfection of 293 cells was performed in 10 cm diameter dishes using a modified calcium phosphate method [34] and 10  $\mu$ g of a vector capable of expressing FLAG–PPP4R2 or a control protein (amino acids 908–984 of protein kinase C-related kinase-2) with a FLAG epitope tag. Following transfection, each dish of cells was cultured for 24 h and lysed in 0.5 ml of ice-cold 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.03% Brij 35, 2 mM EDTA, 0.1 mM EGTA, and 5% glycerol by passage through a 0.8 mm gauge needle ten times. After centrifugation for 10 min at 13000 *g*, the lysates were collected. For immunoprecipitation of FLAG–PPP4R2 and control protein, lysates containing 1 mg of total cell protein were precleared by incubation at 4 °C for 50 min on a shaking platform with sheep preimmune IgG covalently coupled to Protein G–Sephacrose [35]. Following centrifugation for 1 min at 13000 *g*, the supernatant was removed and incubated for 1 h as above, with 10  $\mu$ g of anti-FLAG antibodies (Sigma) covalently coupled to 10  $\mu$ l of Protein G–Sephacrose. The immunoprecipitates were

washed five times with 1 ml of lysis buffer and resuspended in SDS buffer for analysis by SDS/PAGE and immunoblotting.

### Immunological techniques

Immunoblotting was performed following fractionation of proteins by SDS/PAGE and transference of the proteins to nitrocellulose membranes (Schleicher und Schüll, Dassel, Germany). The blots were probed with affinity-purified antibodies, and antibody binding was detected using anti-sheep IgG antibodies conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (Amersham International). For immunocytological analyses, A431 or HeLa cells were plated on to 12 mm glass cover slips and allowed to grow for a further 24–48 h but not to confluency. The cells were fixed in freshly prepared 2% (w/v) paraformaldehyde in PBS for 5–10 min or 90% methanol in PBS for 5 min. Cells were permeabilized using 1% Nonidet P40 in PBS, then washed in PBS and incubated in the presence of 3% BSA and 1% donkey serum in PBS for 10 min, before being exposed to primary and secondary antibodies as described by Helps et al. [18]. Anti-PPP4 antibodies were raised against the N-terminal 57 amino acids of human PPP4 [18] and against full-length human PPP4, expressed from the pT7.7 vector in *E. coli* and affinity purified against their respective antigens. Anti-PPP4R2 antibodies were produced against GST-PPP4R2 and affinity purified. Anti-PPP4R2 peptide antibodies were made against the peptide ESFMTSREM-IPERK coupled to keyhole-limpet haemocyanin and affinity purified. All the above antibodies were raised in sheep at the Scottish Antibody Production Unit, Carluke, Scotland, U.K. Anti-PP2A antibodies were purchased from Promega. Mouse anti- $\gamma$ -tubulin antibodies and anti-sheep and anti-mouse secondary antibodies are described in Helps et al. [18].

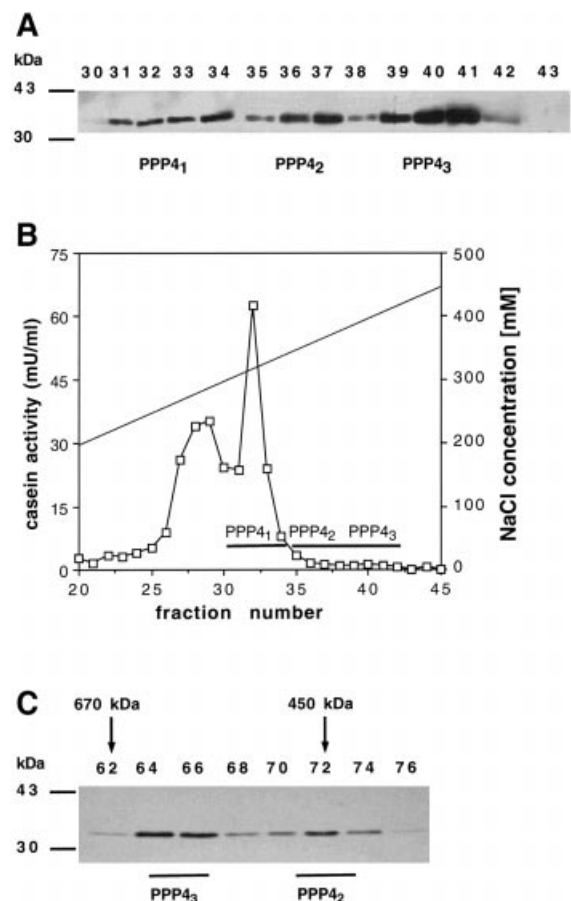
### Protein phosphatase assays

Protein phosphatase assays were performed in the absence of divalent cations using phosphorylase (phosphorylated by phosphorylase kinase) and casein (phosphorylated by protein kinase A) as substrates [22,27]. One unit of activity is the amount of enzyme which catalyses the release of 1  $\mu$ mol of [ $^{32}$ P]phosphate per minute.

## RESULTS

### High-molecular-mass complexes of PPP4 exist in tissue extracts

Elution profiles of PPP4 from different tissue extracts chromatographed on anion exchange columns led to the identification of three pools of PPP4 immunoreactive material, termed PPP4<sub>1</sub>, PPP4<sub>2</sub> and PPP4<sub>3</sub>, eluting at 300–340 mM NaCl, 350–380 mM NaCl and 380–420 mM NaCl respectively. Figure 1(A) shows the separation of PPP4 species from liver extracts by Mono-Q chromatography, but essentially identical profiles were obtained from skeletal muscle and testis. PP2A, detected with anti-PP2A antibodies (results not shown), eluted earlier than PPP4<sub>2</sub> and PPP4<sub>3</sub> and coincided with the two peaks of casein protein phosphatase activity eluting at 290 mM and 320 mM NaCl (Figure 1B). The phosphatase activity was inhibited by 2 nM okadaic acid but not by the PP1 specific inhibitor 2. PP1, which elutes earlier than PP2A, was not detected since it has low phosphatase activity towards casein [27]. From the elution profiles (Figures 1A and 1B) it is clear that there was no casein phosphatase activity associated with PPP4<sub>2</sub> and PPP4<sub>3</sub>, and these PPP4 species also failed to exhibit any phosphatase activity when assayed using phosphorylase *a* as substrate (results not shown).



**Figure 1** Anion-exchange analysis of PPP4 species from rat liver

The 100000 *g* supernatant from rat liver was fractionated by Mono-Q ion exchange chromatography. (A) Fractions were analysed for PPP4 by SDS/PAGE and immunoblotting. The PPP4 N-terminal antibody used for the immunoblotting was specific for PPP4 and did not cross-react with 100 ng of PP2A or with PP6 in cell extracts. The positions of the three PPP4 pools, PPP4<sub>1</sub>, PPP4<sub>2</sub> and PPP4<sub>3</sub>, are indicated. The fraction number is given above each lane and the NaCl gradient is indicated. Molecular mass markers are ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). (B) Casein phosphatase activity present in each fraction. The elution positions of PPP4<sub>1</sub>, PPP4<sub>2</sub> and PPP4<sub>3</sub> (determined by immunoblotting) are indicated. (C) Gel filtration of fractions containing PPP4<sub>2</sub> and PPP4<sub>3</sub> on Superose 6. Fractions eluting from the Superose 6 column were analysed for PPP4 by immunoblotting. The fraction number is given above each lane and the elution positions of the molecular mass markers, thyroglobulin (670 kDa) and ferritin (450 kDa), are indicated by arrows.

It could not be ascertained whether any phosphatase activity was associated with PPP4<sub>1</sub>, since this PPP4 species co-eluted with PP2A activity. Pools PPP4<sub>2</sub> and PPP4<sub>3</sub> comprised approx. 25% and 50% of the total PPP4 in cell extracts respectively.

In order to determine the molecular sizes of PPP4<sub>2</sub> and PPP4<sub>3</sub>, fractions containing these species from skeletal muscle and testis were subjected to gel filtration on Superose 6 together and separately. Immunoblotting of the eluted fractions revealed that PPP4<sub>2</sub> and PPP4<sub>3</sub> possessed apparent molecular masses of 450 kDa and 600 kDa respectively (Figure 1C). Since the native bovine PPP4c [22] and baculovirus-expressed rabbit PPP4c [17] elute at 35 kDa on gel filtration, these high-molecular-mass species are likely to contain PPP4c in association with regulatory subunit(s). During successive purification steps the 600 kDa PPP4<sub>3</sub> species was sometimes partially or completely converted into the 450 kDa PPP4<sub>2</sub> species.

**Table 1** Tryptic peptides sequenced from rabbit skeletal muscle PPP4R2

Position of first residue	Amino acid sequence*	Position of last residue
84	LQEALKDFEKR	94
113	TGETMIQWSQFK	124
147	GPPNPVVEYIPFDEMK	162
206	NVMVV	210
228	MNGVMFP	234
246	INGPGTPRPLNRP	258
281	NLQQNEEKNH	290
310	HPDE	313

\*Note that amino acids L225 and N289 are R225 and T289 respectively in the human sequence.

### Purification of the 600 and 450 kDa complexes of PPP4 from rabbit skeletal muscle and pig testis

Although PPP4 is expressed in all tissues examined, the highest levels of PPP4c mRNA and protein are present in testis [17,26], which was therefore chosen as one tissue for the purification of PPP4. We also purified PPP4 from skeletal muscle, which has a much lower level of PPP4. Initial subcellular fractionation was not employed, since analysis of both nuclear and cytoplasmic extracts from liver by anion exchange chromatography revealed identical profiles to unfractionated liver extracts, and investigation of a crude preparation of centrosomes gave insufficient levels of PPP4 for purification. Since affinity purification on microcystin–Sepharose was unsuccessful (see below), PPP4<sub>2</sub> and PPP4<sub>3</sub> were purified by classical biochemical procedures, being detected by anti-PPP4 specific antibodies throughout the purifications.

Purification of PPP4 complexes from rabbit skeletal muscle which eluted from the Q-Sepharose column at 330–415 mM NaCl, and subsequently from Mono-Q at 335–370 mM NaCl, yielded an 450 kDa PPP4 immunoreactive species by gel filtration on Superose 6. The PPP4-containing fractions from the Superose 6 column were examined by SDS/PAGE followed by staining with Coomassie Blue. Digestion of the 35 kDa immunoreactive band with trypsin and examination of the tryptic peptides by Edman sequencing confirmed the presence of PPP4c. Although several other bands were present, only one protein eluted from Superose 6 in an identical manner with PPP4c. This protein migrated with an apparent molecular mass between 53 and 67 kDa on the SDS/polyacrylamide gel (results not shown). Tryptic peptides generated from this protein and analysed by Edman sequencing revealed it was a novel protein with no similarities to any previously identified protein of known function (Table 1). A search of the NCBI expressed-sequence-tag database for cDNA sequences encoding peptides isolated from the candidate regulatory subunit identified two cDNA clones, H56614 and aa564356. The complete open reading frame (1359 nt) derived from the DNA sequence of these clones encoded a protein of 453 amino acids with a predicted molecular mass of 50.4 kDa and a low isoelectric point of 4.7. Peptides from this protein, designated PPP4R2, were used to raise antibodies.

During purification of PPP4 complexes from pig testis, two pools of PPP4 eluting from Q-Sepharose at 340–380 mM NaCl (PPP4<sub>2</sub>) and 380–420 mM NaCl (PPP4<sub>3</sub>) were isolated and purified separately after this step. Neither PPP4<sub>2</sub> nor PPP4<sub>3</sub> bound to heparin–Sepharose, SP-Sepharose or Blue-Sepharose, but together these columns bound and eliminated > 80% of the total protein from the PPP4 preparations. Immunoblotting with

anti-PPP4c antibodies after gel filtration on Superose 6 showed that PPP4<sub>2</sub> eluted with a molecular mass of 450 kDa, while PPP4<sub>3</sub> had a molecular mass of 600 kDa. Immunoblotting with anti-PPP4R2 peptide antibodies demonstrated that PPP4R2 was present in PPP4<sub>3</sub> eluting from Superose 6 in an identical manner to the PPP4c (Figure 2A). The Superose 6 fractions of PPP4<sub>3</sub> were examined by SDS/PAGE followed by staining with Coomassie Blue (Figure 2B). The 35 kDa band cross-reacting with anti-PPP4c antibodies, and a protein cross-reacting with anti-PPP4R2 peptide antibodies and migrating between 53 and 67 kDa, were digested with trypsin. Analysis of the peptides by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry confirmed the presence of PPP4c and PPP4R2 respectively. It was not possible to identify any further protein bands in the PPP4<sub>3</sub> preparation that followed the elution profiles of PPP4c and PPP4R2. Analysis of the Superose 6 fractions of PPP4<sub>2</sub> from pig testis by immunoblotting revealed co-elution of PPP4R2 and PPP4c (Figure 3), as observed for the skeletal muscle PPP4<sub>2</sub> preparation. The yields of PPP4<sub>2</sub> and PPP4<sub>3</sub> from testis preparations after the Mono-Q chromatography were each approx. 0.5% of the PPP4<sub>2</sub> and PPP4<sub>3</sub> pools present in crude 100000 g extracts respectively.

### Co-isolation of PPP4R2 and PPP4c on microcystin–Sepharose

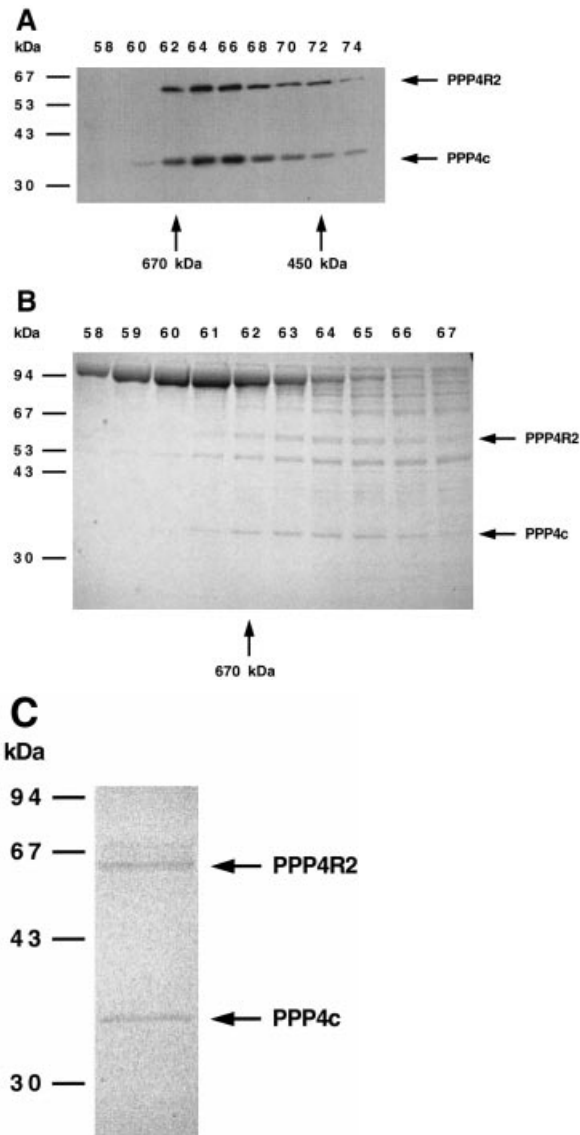
Although microcystin–Sepharose has been successfully used for isolating PP1 and PP2A complexes [30,36], this affinity resin was ineffective for obtaining good yields of highly purified PPP4, since only a small fraction of the total PPP4 in an extract bound to the column. A possible explanation, given the lack of phosphatase activity associated with PPP4 complexes, is that the regulatory subunit(s) may partially or completely block the active site so that the phosphatase inhibitor, microcystin, cannot gain proper entry to its normal binding site. Nevertheless, incubation of the Superose 6 fractions containing the highly purified pig testis 600 kDa PPP4<sub>3</sub> with microcystin–Sepharose resulted in binding of approx. 10% of the PPP4c. Elution of the bound proteins with SDS buffer revealed that PPP4R2 was the only protein present after this procedure in approximately stoichiometric proportions with PPP4c (Figure 2C). Other protein bands were present in some, but absent from other, Superose 6 fractions of PPP4<sub>3</sub>, and always present in less than stoichiometric amounts compared with PPP4R2 and PPP4c.

### Activation of the phosphorylase phosphatase activity of PPP4 complexes by basic proteins

Assay of the virtually pure PPP4<sub>2</sub> and PPP4<sub>3</sub> species using either casein or phosphorylase *a* as substrates revealed very little phosphatase activity. Since basic proteins are known to activate PP2A heterotrimers [37], their influence on the activity of the purified PPP4 complexes was examined. Protamine, at a concentration of 10 µg/ml, caused a 20-fold activation of the 600 kDa PPP4<sub>3</sub> species from testis, and a 10-fold activation of the 450 kDa PPP4<sub>2</sub> species from muscle and testis, using phosphorylase *a* as substrate. Polylysine at 10 µg/ml was less effective, causing an 8-fold activation of 600 kDa PPP4<sub>3</sub> species. Protamine also caused a similar activation of the 600 kDa PPP4<sub>3</sub> species when casein was used at substrate. In contrast, no further activation of native (active) PPP4c [22] was observed with either substrate in the presence of protamine or polylysine.

### Interaction of the PPP4 catalytic subunit with PPP4R2

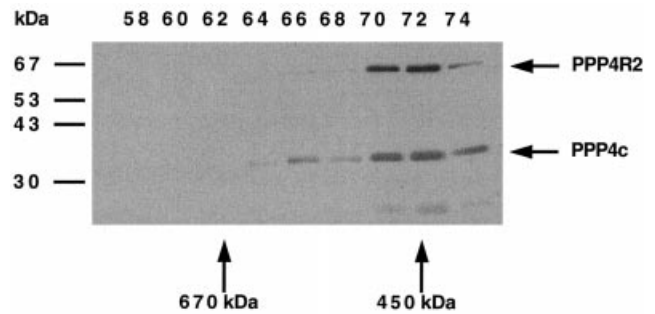
Bacterially expressed PPP4R2 fused to GST was incubated with PPP4c and then with GSH–Sepharose. Figure 4(A) shows



**Figure 2** Analysis of the 600 kDa PPP4<sub>3</sub> species from pig testis following Superose 6 chromatography

(A) Fractions eluting from the Superose 6 column were subjected to SDS/PAGE and immunoblotted using anti-PPP4c and anti-PPP4R2 antibodies. (B) Proteins present in the same fractions detected with Coomassie Blue. The positions of PPP4R2 and PPP4c are indicated by arrows. Superose 6 fraction numbers are indicated above each lane. (C) Affinity purification of the 600 kDa PPP4<sub>3</sub> species from pig testis using microcystin-Sepharose. Fraction 64 of the 600 kDa PPP4<sub>3</sub> preparation eluting from the Superose 6 column was incubated with microcystin-Sepharose. Bound proteins were eluted with SDS buffer, resolved by SDS/PAGE, and stained with Coomassie Blue. Molecular mass markers for SDS/PAGE are phosphorylase *b* (94 kDa), BSA (67 kDa), glutamate dehydrogenase (53 kDa), chicken ovalbumin (43 kDa) and carbonic anhydrase (30 kDa). Molecular mass markers for Superose 6 gel filtration, thyroglobulin (670 kDa) and ferritin (450 kDa), are indicated by arrows.

that PPP4c co-sedimented with GST-PPP4R2, but not with GST or GST-G<sub>M</sub>(1-243), demonstrating that PPP4c is capable of binding to PPP4R2 *in vitro*. The interaction was judged to be specific since the related protein serine/threonine phosphatase, PP1, did not co-sediment with GST-PPP4R2 but did form a complex, as expected, with a GST fusion of the N-terminal amino acids 1-243 of the PP1 glycogen targeting subunit G<sub>M</sub> (Figure 4B).



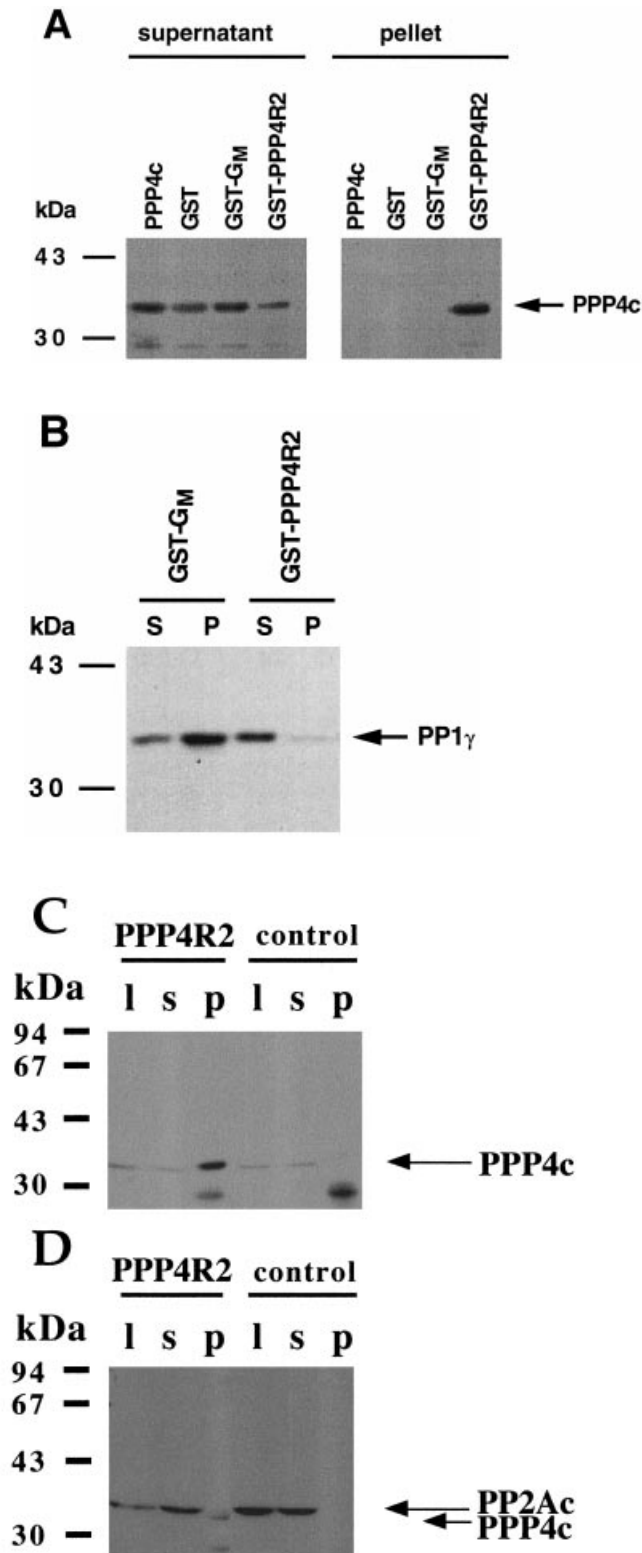
**Figure 3** Analysis of the 450 kDa PPP4<sub>2</sub> species from pig testis following Superose 6 chromatography

Fractions eluting from the Superose 6 column were subjected to SDS/PAGE, transferred to a membrane and analysed using anti-PPP4c and anti-PPP4R2 antibodies. The positions of PPP4R2 and PPP4c are indicated by arrows. Superose 6 fraction numbers are indicated above each lane. Molecular mass markers are described in the legend for Figure 2.

To test the specificity of the interaction of PPP4c and PPP4R2 in a cellular environment, PPP4R2 was engineered with a FLAG-tag at the N-terminus and transfected into 293 cells. Examination of immunoprecipitates obtained with anti-FLAG antibodies by SDS/PAGE and subsequent immunoblotting revealed the presence of PPP4c (Figure 4C). Transfection of cells with vector expressing a FLAG-tagged control protein did not yield any PPP4c in the immunoprecipitate. In addition, PPI (results not shown) and PP2A (Figure 4D) were not observed in the immunoprecipitates from FLAG-PPP4R2 transfected cells. However, since the PP2A antibodies cross-reacted weakly with PPP4c, a band (PPP4c) migrating slightly faster than PP2A is visible in the FLAG-PPP4R2 immune pellet (Figure 4D, lane 3). Overall, the results confirm that PPP4R2 specifically interacts with PPP4c and not with closely related phosphatase catalytic subunits in a cellular environment.

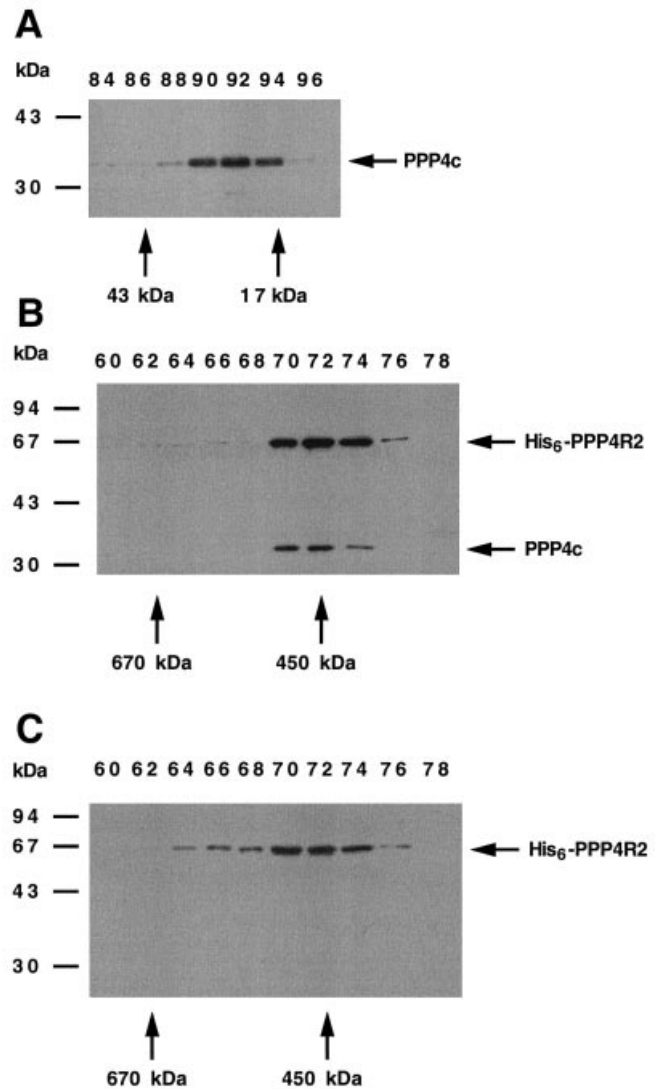
#### Formation of a high-molecular-mass complex of PPP4c and PPP4R2

Since PPP4R2 had a mass of 50.4 kDa and was the only protein in addition to PPP4c identified in the 600 and 450 kDa species of PPP4, it was important to examine the size of the complex formed between PPP4c and PPP4R2. For these studies PPP4R2 was expressed in the baculovirus/insect cell system with 6 histidine residues at the N-terminus. Gel filtration revealed that, although PPP4c eluted with a molecular mass of 35 kDa (Figure 5A), after incubation with either a 2-fold molar excess or an equimolar ratio of His<sub>6</sub>-PPP4R2, PPP4c eluted at the same position as ferritin, which has a molecular mass of 450 kDa (Figure 5B). In contrast, incubation of PP2Ac with His<sub>6</sub>-PPP4R2 did not alter the elution position of PP2Ac from 35 kDa (results not shown). We next examined the elution position of PPP4R2 by itself and found it to be similar to that detected for the complex of PPP4c and His<sub>6</sub>-PPP4R2 (Figure 5C). In order to determine whether PPP4R2 was a polymeric species, a highly asymmetric protein or both, the protein was examined by glycerol-density gradient centrifugation and found to sediment 0.68 of the distance sedimented by BSA (4.5 S, 67 kDa, Stokes radius 3.5 nm, partial specific volume 0.725 ml/g). From the density-gradient estimated sedimentation coefficient of 3.07 S for PPP4R2, a Stokes radius identical with ferritin (7.9 nm) and a partial specific volume of 0.704 ml/g calculated from the amino



**Figure 4** Analysis of the interaction between PPP4c and PPP4R2 *in vitro* and in human 293 cells

(A) PPP4c was incubated by itself, with GST, GST-G<sub>M</sub>(1–243) or GST-PPP4R2 and then with GSH-Sepharose beads. Supernatant (S) and pellet (P) fractions were obtained by centrifugation and proteins in these fractions separated by SDS/PAGE, transferred to a membrane and analysed with anti-PPP4c antibodies. (B) A control experiment was performed in which PP1 $\gamma$  replaced PPP4c. (C and D) Vector expressing FLAG-PPP4R2 (lanes 1–3) and vector expressing a control protein (lanes 4–6) was transfected into 293 cells. Supernatant (s) and



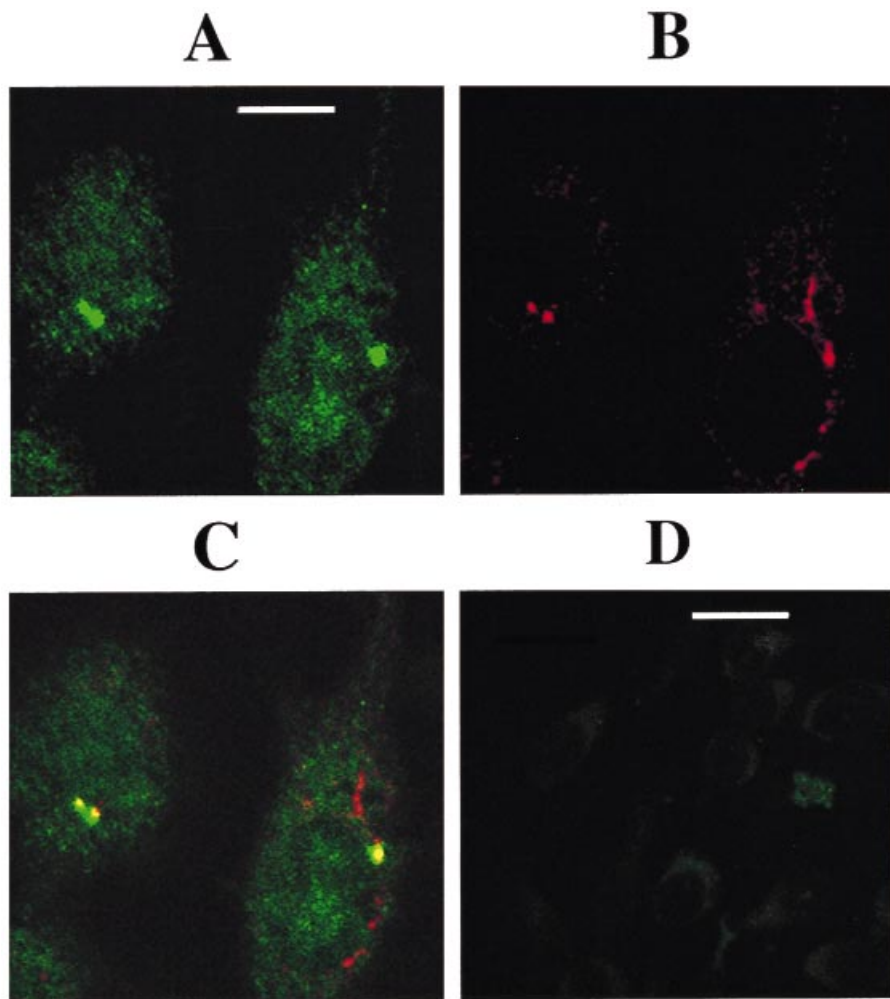
**Figure 5** Assessment of the interaction of PPP4c and PPP4R2 by gel filtration on Superose 6

His<sub>6</sub>-PPP4R2 was incubated with either PPP4c or PP2Ac. Following gel filtration on Superose 6, fractions were analysed by SDS/PAGE and immunoblotting with anti-PPP4R2, anti-PPP4c and anti-PP2A antibodies. (A) PPP4c; (B) His<sub>6</sub>-PPP4R2 incubated with PPP4c; (C) His<sub>6</sub>-PPP4R2. The positions of PPP4R2 and PPP4c are indicated by arrows. Superose 6 fraction numbers are indicated above each lane. Molecular mass markers are ovalbumin (43 kDa) and myoglobin (17 kDa), and as described in the legend for Figure 2.

acid composition, PPP4R2 was calculated to have a molecular mass of 95.9 kDa [38]. Since the monomeric molecular mass from the amino acid sequence is 50.4 kDa, PPP4R2 is likely to be a dimer, that is also highly asymmetric since it elutes from Superose 6 with a Stokes radius similar to ferritin, a globular protein of 450 kDa. Overall the experiments demonstrate that PPP4R2 interacts with PPP4c and is therefore likely to be a subunit of the 450 and 600 kDa PPP4 native complexes, and that

pellet (p) fractions were obtained by centrifugation following immunoprecipitation from cell lysates (l) with anti-FLAG antibodies. The proteins in each fraction were analysed by SDS/PAGE and subsequent immunoblotting with anti-PPP4c (C) and anti-PP2A antibodies (D). Anti-PP2A antibodies show a slight cross-reactivity towards PPP4c.





**Figure 6** Immunofluorescent localization of PPP4R2 and  $\gamma$ -tubulin in human A431 cells fixed with methanol

PPP4R2 (green) was detected with sheep anti-PPP4R2 peptide antibodies and donkey anti-sheep FITC-conjugated antibodies.  $\gamma$ -Tubulin (red) was detected with mouse  $\gamma$ -tubulin antibodies and donkey anti-mouse Texas Red-conjugated antibodies. Fluorescent micrographs were taken with a confocal microscope. **(A)** Staining of the centrosomes and weak staining of the nucleus with anti-PPP4R2 peptide antibodies in two A431 cells. In the cell on the left the centrosome has duplicated. **(B)** The same cell stained with anti- $\gamma$ -tubulin antibodies. **(C)** Merged image of **(A)** and **(B)** showing co-localization (yellow stain) of PPP4R2 (green) and  $\gamma$ -tubulin (red) at centrosomes. **(D)** Cells stained with anti-PPP4R2 peptide antibodies that had been preincubated with 20-fold molar excess of peptide antigen for 1 h. The scale bar represents 40  $\mu\text{m}$  in **(A–C)** and 120  $\mu\text{m}$  in **(D)**.

PPP4R2 exhibits the apparent molecular size of a 450 kDa globular protein by gel filtration because of dimerization and asymmetry.

#### Cytological localization of PPP4R2

Previous immunocytological studies have shown that PPP4c displays a prominent, although not exclusive, localization at centrosomes in mammalian cells [17], we therefore examined the cytological location of PPP4R2. Intense immunofluorescent staining of PPP4R2 was observed with anti-PPP4R2 peptide antibodies at centrosomes in epidermal carcinoma (A431) cells (Figure 6A) and HeLa cells (results not shown). A weaker staining of the nucleus was also observed. Staining of the same cells with anti- $\gamma$ -tubulin antibodies confirmed the positions of the centrosomes (Figure 6B). Similar anti-PPP4R2 immunofluorescent staining at centrosomes was observed irrespective of whether methanol or paraformaldehyde was employed as the fixative. The staining was judged to be specific since it was

abolished by preincubation of anti-PPP4R2 antibodies with excess PPP4R2 peptide antigen (Figure 6D). Anti-PPP4R2 protein antibodies produced a similar immunofluorescent stain at centrosomes (results not shown).

#### Identification of proteins related to mammalian PPP4R2

A tBLASTn search of the Institute for Genomic Research database with human PPP4R2 sequence identified a related *D. melanogaster* cDNA sequence. Two cDNA clones LD28993 and LD29930 (IMAGE consortium) were both found to encode a 44 kDa protein of 393 amino acids with 28% identity (39% similarity) to PPP4R2 (Figure 7). A search of the *Caenorhabditis elegans* database (Sanger Centre, Cambridge, U.K.) identified a cosmid D2092 that encoded a protein with 26% identity (34% similarity) to PPP4R2. A protein from *S. cerevisiae* (ORF-YBL046w/SPTREMBL. P38193) with 22% identity (32% similarity) to PPP4R2 was identified in the NCBI database. Partial sequences



Human	1	MCQAPCWRAGGSLGRCSLCRSCSLARFPRLPSFPPPGRLRAGVCAREGE
Drosophila		.....
Human	51	GVGGVGGVVPVKRPAEGGGCEGLREAMDVERLQEA LKDFEKRKKKEVC
Drosophila	1	.....MATMENSDEIMQI LQRFTDLKQKEIP
Human	101	PVLDQRLCHVAKTGETMIQWSQKGYFIFKLEKVMDDFRTSAPFPRGPPN
Drosophila	27	KELEFYLYVAKTGDITLFWSSLLKYLFRKLLSVLKHFNEDRRGLEEIPN
Human	151	-PNVEYI PFDEMKERILKIYTGPNGI PFTIQRLCELLTDPKRN YTGTDKF
Drosophila	77	YPNVDFPHYETMKSSLLERLDL FNAA PFTVQRMCELLTDPKRN YSRI DKF
Human	200	LRGV EKNVMVVS CVYPSSE RNN SNLNRMGVMFPGNAPS YTERSN I---
Drosophila	127	MRALEKNILVVSTIDEGPKRQTQSENGDLLDSVV---NGDLFL E-VNIDIE
Human	247	--NGFGTFRPRNRPKVLSLAPMTTNGWPESTD-----SKRANLQQNEEKT
Drosophila	173	MQNNNGNADEGSSPGAGSAGCAQKASCPRSDDNDQPKAKRANLEIDGEEER
Human	290	HSDSSTSESEVSS-VSPLRNKHPDEDAVEAEGHEVKKLRF-DKEGEVRET
Drosophila	223	SEASDETDT EVA TRVKNKDEKNDNDETDSP-HEAAEIEEFDEEVDEADQ
Human	338	ASQTSS-EISSVMVGETEAS--SSQDKDKDSRCRTRQCTEED---EEE
Drosophila	272	ETKTKQPAYGSQKEGEQESFPSSADDEAEDPMVSKSIEAEKELVAQEK
Human	382	DEEEESFMTSREMI PERK NQEKESD DALTVNEETSEENNQMEESDVSQ
Drosophila	322	KREEDKKAIEPKEEI-VKKEVVEESDKPDGKVAQLGDKAVVKKSTFPAD
Human	432	AE-KDLLHSEGSENE GPESK WFF
Drosophila	371	GENQEPV KVKGRKRKGG EK TCTN

Human	PFTIQRLCELLTDPKRN Y--TGTDKFLRGVEKNVMVVS
Zebrafish	PFTIQRLCELLTDPKRN Y--TGTEKFLRGVEKNVMVVS
Drosophila	PFTVQRMCELLTDPKRN Y--SRIDKFLRALEKNILVVS
Nematode	PFTWQRLCELLTDPKRN Y--NTIDKFLRAVDEKVINVT
Barley	PFTLQRLCELLTDPKRN Y--TKVSKLALALEKNLLVTS
Yeast	PFTILRLICELCYDEPKY YKINELEKFNALAEKCCMVT S

**Figure 7** Comparison of human PPP4R2 with related proteins in other species

Amino acid sequence of human PPP4R2 aligned with the related sequence in *D. melanogaster* (upper panel). Alignment of amino acids 176–211 of human PPP4R2 with related sequences in *Danio rerio* (zebrafish) A1657846, *D. melanogaster* LD28993 and LD299930, *C. elegans* (nematode) AAB42229.1/P91198, *Hordeum vulgare* (barley) AJ2344445, and *S. cerevisiae* (yeast) YBL046w/P38193 (lower panel). Identical amino acids are highlighted in black and similarities are shaded grey.

from zebrafish and barley also exhibited significant sequence similarity to PPP4R2 over amino acids 176–211 (Figure 7).

## DISCUSSION

### PPP4R2 is a 50 kDa regulatory subunit of PPP4c and a major determinant for the assembly of PPP4c into complexes with apparent molecular masses of 450 and 600 kDa

We have identified at least three pools of immunologically detectable PPP4c, two of which exhibited high apparent molecular masses of 450 kDa (PPP4<sub>2</sub>) and 600 kDa (PPP4<sub>3</sub>). Virtually identical results were obtained from three tissues despite the level of PPP4c varying from 0.4  $\mu$ M in testis [22] to barely detectable in skeletal muscle (results not shown and [39]). The 450 kDa PPP4<sub>2</sub> complex purified to near homogeneity from skeletal muscle and testis was found to comprise PPP4c and a protein of 50.4 kDa (PPP4R2). Specific interaction of PPP4R2 with PPP4c was confirmed by co-sedimentation of native PPP4c with GST-PPP4R2 *in vitro* and co-immunoprecipitation of PPP4c, but not the related phosphatases PP2Ac and PP1c, with FLAG-epitope tagged PPP4R2 from 293 cells.

The complex formed by incubation of PPP4c with His<sub>6</sub>-PPP4R2 eluted at the position of a globular protein of 450 kDa on gel filtration. Surprisingly, His<sub>6</sub>-PPP4R2 in the absence of PPP4c also eluted with an apparent size of approximately 450 kDa, indicating a large Stokes radius of 7.9 nm and suggesting that PPP4R2 may be an asymmetric and/or polymeric protein. Analysis of the mass of His<sub>6</sub>-PPP4R2 by density gradient sedimentation revealed a sedimentation coefficient of 3.1 S and a calculated molecular mass of 95.9 kDa. Since PPP4R2 cDNA encodes a protein of 50.4 kDa, His<sub>6</sub>-PPP4R2 is likely to be a dimeric molecule that is also highly asymmetric.

The molar ratio of PPP4R2/PPP4c is inferred to be 1:1 from their equal Coomassie Blue staining in the 600 kDa PPP4<sub>3</sub> preparations (Figures 2B and 2C) and the 450 kDa PPP4<sub>2</sub> preparations (results not shown), indicating that the PPP4R2-PPP4c complex is probably a tetramer comprising two molecules of PPP4R2 and PPP4c. The highly asymmetric nature of the PPP4R2 may explain the negligible shift in gel-filtration elution position of PPP4R2/PPP4c complex compared with PPP4R2 alone.

PPP4R2 appears to be the only subunit present in stoichiometric proportions to PPP4c in both the 450 kDa PPP4<sub>2</sub> and

the 600 kDa PPP4<sub>3</sub> complexes, as judged by microcystin–Sepharose analysis. This suggests that the 600 kDa PPP4<sub>3</sub> species may simply be a higher polymeric species of the 450 kDa PPP4<sub>2</sub>. However, conversion of the 600 kDa PPP4<sub>3</sub> species into the 450 kDa PPP4<sub>2</sub> species during some purifications could also occur because PPP4<sub>3</sub> contains an additional subunit(s) to PPP4<sub>2</sub> that is/are easily lost in successive purification steps. Recently, a 105 kDa protein [26], with some sequence similarities to the repeats in the A (PR65) subunit of PP2A, has been identified in a PPP4 species eluting from anion-exchange columns in fractions of lower NaCl concentrations (250–300 mM) than either PPP4<sub>2</sub> or PPP4<sub>3</sub>. These fractions were used to purify free PPPc [22], but were discarded from the PPP4<sub>2</sub> and PPP4<sub>3</sub> preparations, and antibodies raised to peptides in the 105 kDa protein did not cross-react with either PPP4<sub>2</sub> or PPP4<sub>3</sub>. Antibodies to cRel and  $\alpha 4$  recently reported to interact with PPP4c in two-hybrid analyses [24,25] also did not recognize any component present in our PPP4<sub>2</sub> and PPP4<sub>3</sub> preparations.

Native PPP4<sub>2</sub> and PPP4<sub>3</sub> complexes are inactive, but were found to be activated > 10-fold by basic proteins such as protamine. Transfection of FLAG–PPP4R2 into 293 cells and assay of the anti-FLAG immunoprecipitates containing FLAG–PPP4R2–PPP4c complexes, revealed a very low phosphorylase phosphatase activity which was activated 5–6-fold by addition of protamine (results not shown). In contrast, the 450 kDa complex formed *in vitro* by incubation of His<sub>6</sub>-PPP4R2 and PPP4c was active even in the absence of basic proteins. This suggests the possibility that a small inhibitor protein could be present in the 450 kDa complex. However, no proteins in the 10–40 kDa range were visible in the 450 kDa preparation, and antibodies to the PP2A inhibitor proteins I<sub>1</sub><sup>PP2A</sup> and I<sub>2</sub><sup>PP2A</sup> (kindly provided by Dr Z. Damuni, Pennsylvania State University College of Medicine, U.S.A.) did not cross-react with PPP4<sub>2</sub> or PPP4<sub>3</sub> complexes. The inability of His<sub>6</sub>-PPP4R2 to block the activity of PPP4c may arise because the expressed His<sub>6</sub>-PPP4R2 is not folded into the precise conformation required to inhibit PPP4c activity. Nevertheless, the results demonstrate that PPP4R2 interacts with PPP4c to form complexes of high apparent molecular mass. The 450 kDa complex appears to comprise only PPP4R2 and PPP4c. The 600 kDa complex contains PPP4R2 and PPP4c and although other subunit(s) may be associated to this species, it is equally possible that the 600 kDa complex is a higher polymeric form of the 450 kDa complex. As might be expected for a regulatory subunit of the ubiquitously expressed PPP4c, PPP4R2 has a wide tissue distribution, since we not only purified it from skeletal muscle and testis, but also identified cDNAs encoding PPP4R2 in heart, liver, spleen and retina expressed-sequence-tag libraries.

#### **PPP4R2 localizes and may target PPP4c to centrosomes and PPP4R2 is likely to regulate the phosphatase activity of PPP4c in these microtubule organising centres**

As might be expected for a regulatory subunit of PPP4, PPP4R2 exhibited a similar subcellular location to PPP4c [17], with a predominant localization at centrosomes, in addition to its presence in the nucleus and the cytoplasm. Two different antibodies gave intense immunofluorescent staining of PPP4R2 at centrosomes in more than one type of cell, irrespective of the method of fixation of the cells. The location of PPP4R2 at centrosomes suggests that the PPP4 complex that is targeted to centrosomes is likely to be the 450 kDa PPP4<sub>2</sub>, the 600 kDa PPP4<sub>3</sub> species or both. Many PP1 complexes and some PP2A species are located in distinct regions of the cell and are targeted to these subcellular locations by their regulatory subunits. For example, PP1c is targeted to glycogen particles by glycogen-

binding subunits, while the same catalytic subunit is targeted to myofibrils by myosin-binding subunits [40]. A specific trimeric species of PP2A has been reported to be targeted to microtubules [41]. It is therefore likely that PPP4c is targeted to centrosomes by PPP4R2, although at present we cannot completely exclude the possibility that an additional associating protein in the 600 kDa complex might be involved. The protein(s) with which PPP4R2 might interact at these microtubule-organizing centres is/are unknown. Since ring complexes which nucleate microtubule growth [4,5] contain  $\alpha$ -,  $\beta$ - and  $\gamma$ -tubulin, it may be relevant that some highly purified preparations of the 450 kDa PPP4<sub>2</sub> and the 600 kDa PPP4<sub>3</sub> species contained a protein migrating at a similar position to  $\alpha$ -tubulin and recognized by  $\alpha$ -tubulin antibodies (results not shown) although  $\gamma$ - and  $\beta$ -tubulin could not be detected.

The finding that the 450 kDa PPP4<sub>2</sub> and the 600 kDa PPP4<sub>3</sub> species are inactive, even though PPP4c is active against a variety of substrates, indicates that PPP4R2 may inhibit PPP4c activity against these substrates. Many regulatory substrates of PP1 and some regulatory subunits of PP2A are known to inhibit activity against certain substrates, while enhancing activity against others [42]. It is therefore possible that 450 kDa PPP4<sub>2</sub> and the 600 kDa PPP4<sub>3</sub> species are inactive against casein and phosphorylase, which are unlikely to be physiological substrates of PPP4, but are active against specific centrosomal substrate(s). Alternatively the PPP4 complexes may be inactive against all substrates until they receive a signal for activation. Many binding subunits of protein phosphatases allow the activity of the catalytic subunit to change in response to extracellular signals. The fact that protamine can activate 450 kDa PPP4<sub>2</sub> and the 600 kDa PPP4<sub>3</sub> species suggests that the PPP4R2/PPP4c complex may be regulated by (as yet unidentified) small molecules or by phosphorylation, which would allow the activity of PPP4c to respond to signals that influence cell cycle events. Potential phosphorylation sites for several protein kinases exist within the PPP4R2 sequence. *Drosophila* mutants deficient in PPP4c have been found to be blocked in mitosis due to aberrant growth, organization or stability of microtubules at centrosomes [18], indicating that the activity of a centrosomally located PPP4 complex is essential for one or more of these processes. The results presented here suggest that this activity may be regulated through PPP4R2.

A search of several databases with the sequence of PPP4R2 identified proteins with low sequence identity with human PPP4R2 in lower eukaryotes. However, the PPP catalytic subunits are among the most highly conserved proteins known [43]. *Drosophila* PPP4c and PP2Ac show 91% and 94% identity respectively with their human homologues [44,45]. PPP regulatory subunits show more variation, but many are fairly highly conserved: *Drosophila* A(PR65) and B(PR55) subunits of PP2A exhibit 71–78% identity with their mammalian counterparts [46,47]. Consequently, the *Drosophila* protein possessing only 26% identity with PPP4R2 is not necessarily the homologue of PPP4R2 but may be an isoform of the true homologue. In *S. cerevisiae* Pph3p is the most closely related phosphatase to PPP4c, but is reported to have overlapping functions with PP2A [48], whereas PPP4c function cannot be performed by PP2Ac in *Drosophila* [18]. Spindle poles bodies in yeast have a number of distinct properties from centrosomes in mammals and  $\gamma$ -tubulin, which is essential for microtubule nucleation [3], is highly divergent in *S. cerevisiae*, showing an identity of only 36% with the  $\gamma$ -tubulin of higher eukaryotes [49]. It will therefore be relevant to test whether the yeast PPP4R2 related protein interacts with Pph3p, despite its low identity with PPP4R2.

Comparison of the *D. melanogaster*, *S. cerevisiae* and *C. elegans* PPP4R2 related proteins with human PPP4R2 identified

a conserved region of 36 amino acids (Figure 7) in the N-terminal half of the protein which is likely to have an  $\alpha$ -helical structure, although the predicted  $\alpha$ -helical content for the whole PPP4R2 protein is low. Experiments are in progress to see if this domain may be involved in binding PPP4c or another protein, such as a tubulin isoform. The sequence of charged residues (amino acids 376–388) near the C-terminus may also be important for function, since it is found in mammals, *Drosophila* and yeast, although it appears to be absent from the *C. elegans* protein, which terminates prior to this region. The presence of PPP4R2-related proteins in mammals, insects, worms, yeasts and plants suggests this family of proteins serve important functional role(s) in all eukaryotes.

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