Purification and Identification of a Novel Subunit of Protein Serine/Threonine Phosphatase 4*

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The catalytic subunit of protein serine/threonine phosphatase 4 (PP4C) has greater than 65% amino acid identity to the catalytic subunit of protein phosphatase 2A (PP2A). Despite this high homology, PP4 does not appear to associate with known PP2A regulatory subunits. As a first step toward characterization of PP4 holoenzymes and identification of putative PP4 regulatory subunits, PP4 was purified from bovine testis soluble extracts. PP4 existed in two complexes of approximately 270–300 and 400–450 kDa as determined by gel filtration chromatography. The smaller PP4 complex was purified by sequential phenyl-Sepharose, Source 15Q, DEAE2, and Superdex 200 gel filtration chromatographies. The final product contained two major proteins: the PP4 catalytic subunit plus a protein that migrated as a doublet of 120–125 kDa on SDS-polyacrylamide gel electrophoresis. The associated protein, termed PP4R1, and PP4C also bound to microcystin-Sepharose. Mass spectrometry analysis of the purified complex revealed two major peaks, at 35 (PP4C) and 105 kDa (PP4R1). Amino acid sequence information of several peptides derived from the 105 kDa protein was utilized to isolate a human cDNA clone. Analysis of the predicted amino acid sequence revealed 13 nonidentical repeats similar to repeats found in the A subunit of PP2A (PP2A). The PP4R1 cDNA clone engineered with an N-terminal Myc tag was expressed in COS M6 cells and PP4C co-immunoprecipitated with Myc-tagged PP4R1. These data indicate that one form of PP4 is similar to the core complex of PP2A in that it consists of a catalytic subunit and a “PP2A-like” structural subunit.

The phosphorylation of proteins on serine or threonine residues by protein kinases is responsible for the communication of many intracellular signals (1). The dephosphorylation of proteins by serine/threonine phosphatases is equally important for the occurrence of this signaling phenomenon. Four major cellular protein serine/threonine phosphatase activities (PP1, PP2A, PP2B, and PP2C) have been reported and classified according to substrate selectivity, inhibitor sensitivity, and requirement for divalent cations (2–6). Various molecular biology techniques have led to the identification of several additional phosphatases including protein phosphatase 4, formerly known as protein phosphatase X (7, 8).

PP2A is one of the best studied protein serine/threonine phosphatases. Several reports have demonstrated an important regulatory role for PP2A in a variety of cellular processes. Because PP2A is involved in many cellular events, its activity must be tightly controlled in vivo. Two major mechanisms of regulation of PP2A have been described in the literature. The first involves post-translational modifications of the catalytic subunit of PP2A (PP2A). Phosphorylation of a tyrosine residue near the C terminus (9) or phosphorylation of an unidentified threonine residue (10) decreases catalytic activity in vitro. Conversely, carboxymethylation of the C-terminal leucine causes an increase in phosphatase activity (11). The second major mechanism of regulation of PP2A is through its association with other proteins. PP2A exists as a heterotrimERIC complex consisting of a catalytic subunit (C) associated with a structural subunit (A) and a variable subunit (B). The A subunit is thought to act as a scaffold for the B and C subunits (5, 12, 13). Three families of B subunits have been identified and include B, B’, and B” (2–6, 12). The B subunit can modulate substrate selectivity in vitro (14, 15) and is hypothesized to target PP2A to discrete compartments within the cell (2–6, 13). In addition to the regulation of PP2A by associated subunits and post-translational modification, the PP2A holoenzyme forms complexes with other cellular proteins, such as Tau (16) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase IV (17).

The catalytic subunit of protein phosphatase 4 (PP4C) is 65% identical to PP2A at the amino acid level (18) and has been placed in the type 2A family of phosphatases. PP4C has been highly conserved between species sharing 91% amino acid identity between human and Drosophila (19). PP4C is predominantly localized in the nucleus in rat brain and liver but is most highly expressed in testis (20). Additionally, PP4C was demonstrated to be an essential enzyme in the development of Drosophila embryos (21). The expression of PP4C was reduced to 25% of the normal protein level in a mutant strain of Drosophila termed centrosomes minus microtubules (cmm) (21). An interesting characteristic of the cmm phenotype is the presence of regions of cells that are unable to complete mitosis because no microtubules exist to connect chromatin and centrosomes. This phenotype implicates PP4C in the regulation of the nucleation and/or stabilization of microtubules. These data taken together indicate that PP4 has a crucial cellular function, al-

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The abbreviations used are: PP1, -2A, -2B, -2C, and -4, protein serine/threonine phosphatase 1, 2A, 2B, 2C, and 4, respectively; PP4C, PP4 catalytic subunit; PP2A, PP2A catalytic subunit; PP4A, PP4-associated protein; PP2A, A or PP65 subunit of PP2A; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CAPS, 3-(cyclohexylamino)propanesulfonic acid; FPLC, fast protein liquid chromatography.
though a physiological substrate for PP4 has not yet been identified.

Appropriate regulation of PP4 is likely critical to its normal physiological function. The close homology of PP2Aγ and PP4C sequence suggests that they may share certain regulatory properties. Indeed, it was recently shown that PP4C, like PP2Aγ, is carboxymethylated (20). Furthermore, by analogy with both PP1 and PP2A, it is unlikely that PP4 exists as free catalytic subunits in the cell (5). However, PP4C does not associate with the A subunit of PP2A in vitro (18). In order to better understand and characterize the physiologically relevant forms of PP4, we now report the purification of a heterodimeric form of PP4, and identification of the first PP4C-associated protein, termed PP4R1.

MATERIALS AND METHODS

Materials—Goat anti-rabbit IgG-alkaline phosphatase conjugate, Bio-Scale DEAE2 column, and preadsorbed SDS-polyacrylamide gel electrophoresis (PAGE) molecular mass standards were obtained from Bio-Rad.

Phenyl-Sepharose, Source 15Q, Superdex 200, and GammaBind Plus Sepharose resin and gel filtration standards were from Amersham Pharmacia Biotech. Microcystin-Sepharose and microcystin were obtained from Upstate Biotechnology, Inc.

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Cloning and Sequencing of PP4R1—The cDNA for PP4R1 was identified by using the peptide sequences (derived from the purified proteins) to search the expressed sequence tag database with the BLAST protocol. The cDNA clones were obtained from The Institute for Genomic Research/American Type Culture Collection Human cDNA Special Collection (Rockville, MD) and sequenced in their entirety by fluorescent dideoxy terminator-based DNA sequencing (performed on an ABI310 sequencer in the Center for Molecular Neuroscience DNA Sequencing Facility, Vanderbilt University).

Purification of Phosphatase 4—A cDNA fragment encoding the NH2-terminal Myc-tagged (MEQKLISEEDL) PP4R1 was produced by successive polymerase chain reactions (PCRs) and restriction enzyme digestion. The first PCR product was made using sense oligonucleotide 1 (5'-CTC ATC TCA GAA GAG GAT CTG GGC GAC CTC TCG CTG CT-3'), antisense oligonucleotide 2 (5'-AAA AAG CAT ATG GTA TTG-3'), and human PP4R1 cDNA as template. Successive PCR using sense oligonucleotide 3 (5'-GGC CGA AGT CAG GAT GGA ACA AAA ACT CTC AGA AGA GGA TCT GCC-3'), antisense oligonucleotide 2, and the first PCR product as template generated a PCR product that was digested with the restriction enzymes EcoRI and Ndel. The resulting cDNA fragment and the cDNA fragment of PP4R1 from the Ndel site to the XhoI site (5'-end of PP4R1) were ligated into the EcoRI/XhoI cloning site of the mammalian expression vector pcDNA3.1 to produce the NH2-terminal epitope-tagged PP4R1 cDNA under the control of the cytomegalovirus promoter. The construct was verified by restriction enzyme analysis and DNA sequencing.

Cell Transfection—COS M6 cells maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum were transiently transfected (10-cm tissue culture dishes) using the DEAE-dextran procedure as described previously (28, 29). Transfected COS cells (one 10-cm dish) were dislodged by scraping in 1 ml of lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM diithiothreitol, 0.5% Triton X-100, 6.3 µg/ml aprotinin, 4 µg/ml leupeptin, 10 µg benzamidine, 4 µg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. Cells were sonicated with two 10-s bursts followed by centrifugation for 15 min. The protein concentration of clarified supernatants was estimated by the BioRad protein assay using bovine serum albumin as the standard.

Immunoprecipitation of Myc-PP4R1 and PP4C—Approximately 1 mg of protein from cell lysates (1 ml of lysis buffer) was incubated with 40 µl of anti-Myc ascites for 6 h while rotating at 4 °C. Immune complexes were isolated by adding 40 µl of a 50% slurry of GammaBind Plus Sepharose. After washing the beads six times with lysis buffer, bound proteins were eluted with 20 µl of Laemmli sample buffer. The proteins were analyzed by immunoblot analysis with affinity-purified antibodies directed against the C terminus of PP4C.
RESULTS

Observation of Multiple PP4 Complexes in Bovine Testis Soluble Extracts—During the initial stages of purification of PP4 holoenzymes from various tissues, a broad elution profile of PP4 immunoreactivity was observed on several chromatography columns including anion exchange and gel filtration (data not shown). One possible explanation of this behavior is that PP4, like PP2A, exists in multiple oligomeric complexes. To address this possibility, the elution of distinct partially purified PP4 species from gel filtration chromatography were compared. Because PP4C protein is expressed in greatest quantity in the testis (20), this tissue was utilized for subsequent biochemical manipulations and holoenzyme purification. Bovine testis soluble proteins recovered from 25–50% ammonium sulfate precipitation were separated by sequential hydrophobic interaction and anion exchange chromatography. Fractions containing PP4 were identified by immunoblot analysis using a PP4C antibody previously described (20). PP4 eluted over several fractions with two noticeable peaks from Source 15Q (fractions 20 and 25; Fig. 1A). Pools of each PP4 peak were individually applied to a gel filtration column (Fig. 1B). Immunoblot analysis of select gel filtration fractions indicated that PP4 in pool 1 exhibited an apparent molecular mass of approximately 270–300 kDa (fractions 31–34), whereas PP4 in pool 2 was slightly larger, approximately 400–450 kDa (fractions 29–32). These data suggest that PP4C exists in at least two distinct complexes in bovine testis. Immunoblot analysis of the gel filtration fractions using affinity-purified antibodies to the A and Ba subunits of PP2A revealed that the PP2A subunits did not co-fractionate with PP4C (data not shown).

Purification of One PP4 Holoenzyme—As a first step toward identifying PP4-associated subunits, the purification of a PP4 holoenzyme from bovine testis was undertaken. PP4 (270–300 kDa) was purified by sequential chromatography on hydrophobic, strong anion exchange, weak anion exchange, and gel filtration columns. Fig. 2 shows fractions from the gel filtration column as analyzed by immunoblot and silver stain. Silver stain analysis revealed a protein doublet (120–125 kDa) co-fractionating with the catalytic subunit of PP4. The molecular mass of the co-purifying protein doublet was carefully determined by comparing its migration with the migration of SDS-PAGE standards (data not shown). Taking into account the differences in molecular masses of these two proteins (see mass spectrometry analysis below), one might predict a 3:1 protein staining ratio if the complex existed in a 1:1 molar stoichiometry. The protein silver staining ratio of the 120–125-kDa co-purifying proteins to PP4C ranged from 1.4 to 2.7 as quantitated by NIH Image, suggesting a near stoichiometric complex. Extracts from 200 g of bovine testis (1200 mg of total protein) contained approximately 400 ng of PP4C, as determined by quantitative immunoblot analysis (see “Materials and Methods”). The final PP4 holoenzyme contained 7 ng of PP4C (based on the intensity of the silver-stained protein; see “Materials and Methods”), indicating an overall yield of approximately 2%. PP2AC was not detected in the silver-stained gels.
of the purified PP4 preparation, but very low levels of PP2A
immunoreactivity were observed. The purified PP4 holoenzyme was further characterized by FPLC on Mono Q and phenyl-
Superose columns. Silver stain analysis of these column fractions confirmed that the 120–125-kDa protein doublet also co-purified with PP4C on these analytical resins (data not shown). Moreover, when partially purified PP4 holoenzyme was incubated with microcystin-Sepharose (an affinity resin frequently used to identify PP1- and PP2A-associated proteins Refs. 30–32), both PP4C, and the 120–125-kDa protein doublet bound to microcystin (data not shown; see below). Together, these data provide compelling evidence that a PP4C-associated protein, termed PP4R1, had been isolated.

To obtain a more accurate determination of the size of the individual components of the PP4 complex, the sample was analyzed by mass spectrometry. This technique has recently been introduced as an excellent method to dissect the size of multiprotein complexes (33). The mass spectrum of cross-linked purified PP4 holoenzyme revealed two major peaks and several minor peaks (Fig. 3). The two major peaks are proteins of 35.1 and 104.5 kDa, presumably corresponding to PP4C and PP4R1. The molecular mass of PP4R1 measured by mass spectrometry (104,499 Da) was somewhat different from that observed by SDS-PAGE (120–125 kDa). In contrast to the sharp PP4C peak, the peak corresponding to PP4R1 is quite broad. This broad PP4R1 peak may be due to the presence of multiple species of similar molecular weight as detected by SDS-PAGE. Since the size of the 140-kDa peak is virtually identical to the sum of the molecular weights of PP4C and PP4R1, it probably represents a heterodimeric complex containing stoichiometric amounts of the two proteins cross-linked with glutaraldehyde.

Phosphatase Activity of PP4—Purified PP4 was assayed for phosphatase activity using casein, protein kinase A-phosphorylated histone H1, protein kinase C-phosphorylated histone H1, and phosphorylase a as substrates. The specific activity of purified PP4 for these phosphoproteins ranged from 0.624 to 13.1 pmol/min/μg of PP4C, contained in the purified PP4 holoenzyme, whereas the specific activity of purified PP2A toward identical substrates ranged from 67 to 1197 pmol/min/μg. An immobilized phosphatase assay similar to the one described by Ding et al. (34) also was developed to test PP4 phosphatase activity toward proteins phosphorylated in vitro (bovine testis soluble extracts) or in vitro (HEK cells). Okadaic acid-sensitive protein dephosphorylation could be detected in this assay with purified PP2A but not with purified PP4 (data not shown).

Identification of the PP4R1 cDNA—Multiple peptides from both proteins of the putative bovine PP4R1 were subjected to amino acid sequence analysis (Fig. 4). A total of nine peptides were sequenced; peptides 1 and 4, 3 and 9, and 5 and 6 had nearly identical sequences. The peptide sequences were used to search the expressed sequence tag data base to identify potential cDNA clones. Several human cDNA clones with translated sequences highly homologous to the sequenced peptides (Fig. 4) were identified and obtained from The Institute for Genomic Research/American Type Culture Collection Human cDNA Special Collection. One clone was of particular interest because it contained a Kozak consensus start sequence (35), an open reading frame of 933 amino acids with a predicted molecular weight of 105,189, and a poly(A) tail. Both the cDNA and predicted amino acid sequences of this clone are shown in Fig. 5A. The predicted molecular weight of the encoded protein is similar to the molecular weight determined by mass spectrometry of the purified PP4R1 protein (104,500). Thus, these data indicate that the isolated cDNA clone contains most, and perhaps all, of the PP4R1 coding sequence. Furthermore, all of the bovine peptides could be identified in one human cDNA sequence, and two peptides were shared by both proteins of the doublet, suggesting that the proteins of the doublet are very similar. Interestingly, PP4R1 has 13 repeats similar to the nonidentical or "heat" repeats found in the A subunit of PP2A (PP2Aa) (36, 37). The repeats identified in PP4R1 are indicated by white type on black in Fig. 5A and are also listed in Fig. 5B. Amino acid residues in boldface type in Fig. 5B are residues identified in the consensus repeat sequence in PP2Aa (38). Seven of the PP4R1 repeats (indicated by an asterisk) were matched to the PP2A repeats by employing the ProfileScan server.4 The remaining repeats were found by manual examination of the amino acid sequence. PP4R1 is distinct from PP2Aa in that these motifs are not contiguous; instead, the repeats are separated by a novel region of approximately 322 amino acids between the sixth and seventh repeats. To date, this divergent region does not have homology to any known protein.

Characterization of an Antibody Generated toward PP4R1 (apoPP4R1-1)—Polyclonal anti-peptide antibodies (designated apoPP4R1-1) directed against a bovine sequence homologous to residues 912–924 of the expected human protein were produced.

4 The ProfileScan server can be found on the World Wide Web at http://www.isrec.isb-sib.ch/software/FFSCAN_form.html.
in rabbits. The antiserum recognized a doublet of identical molecular weight in both bovine testis soluble extracts and in the purified PP4 holoenzyme (Fig. 6). Antibody binding could be blocked in the presence of excess peptide. The anti-peptide antibodies were affinity-purified from the rabbit antiserum and used to monitor the purification of the PP4 holoenzyme. Both PP4R1 and PP4C were significantly enriched throughout the purification protocol as shown in Fig. 6B.

**Fig. 5.** Sequence of PP4R1 (A) and identification of ‘heat’ repeats (B). A. The peptides obtained from purified bovine PP4R1 are underlined. Regions homologous to the repeats found in the A subunit of PP2A are printed in white type on black. B. Amino acid sequence alignment of the PP4R1 repeats. Amino acid residues in boldface type are loosely conserved amino acids previously defined in the repeats of PP2A\(_\alpha\). The repeats with asterisks were identified by ProfileScan as having homology to the PP2A\(_\alpha\) ‘heat’ repeats.

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A

| 1   | 70  | VLERI SRLADDSEPT - VRALMEQVPHIALFCQENRSIP |
| 2*  | 115 | LLPPIVRLADVQQRQ VRKTSQAALLALEQELIRIEDVE |
| 3*  | 156 | VCPV LITEALAPDNQ DSKVRTEAATMCKMAPVQGKDTERL |
| 4*  | 196 | ILPPR FCMCC DCDRFHVRKCAANFGDCESVQGQATEEM |
| 5*  | 236 | LLPPRFQSLCDNVRW - VRKACBPMVSCATCQBIYRTK |
| 6*  | 275 | LSALF INLISLSPSRW - VRQAQFSLQPFSTPANPSSQG |
| 7*  | 647 | LREYETLADSMQW- VRVRTLAQHELAVILQDQTAAD |
| 8   | 686 | LVPI FNGEL DDLDE -VRIGV LKLHDFKLLHIDKREY |
| 9   | 724 | LYQLQFELVTDNSR - VRFRAELAQLI LILEYSPRVY |
| 10  | 765 | LRPI AMLNCADKQSS - VRWISYKLVSEMVRKHLAQTPEF |
| 11  | 808 | INELVNGFCR KWS - GRQAFVFCQTVIEDCCLMDQFRA |
| 12* | 849 | LMPHLLTANDVF - VRVLAKTILQTELKDYFLASAS |
| 13  | 893 | VEQYIALQMDRSD - VRKAFHS T KisEDAMTDSS |

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B

"Co-isolation of PP4\(_{R1}\) and PP4\(_{C}\) on Microcystin-Sepharose—The availability of PP4\(_{R1}\)-specific antibodies (aPP4\(_{R1}\)) al-"
owed examination of PP4 binding to microcystin-Sepharose. This affinity resin previously has been utilized to isolate PP1 and PP2A complexes from crude tissue extracts (30–32). Soluble proteins from bovine testis were incubated with the microcystin-Sepharose in the absence or presence of excess uncomplexed microcystin, and bound proteins were eluted by Laemmli sample buffer. Immunoblot analysis of the applied, flow-through, and bound samples revealed that PP4C and PP4R1 co-purified on microcystin-Sepharose (Fig. 7). The interaction of both proteins with the resin was blocked by preincubation of bovine testis soluble extracts with 5 µM uncomplexed microcystin.

Expression and Immunoprecipitation of PP4R1—To aid in the detection and isolation of the recombinant protein, the PP4R1 cDNA was engineered with a Myc tag on the amino terminus. In Fig. 8A, cell lysates prepared from vector and Myc-PP4R1-transfected COS M6 cells were subjected to immunoblot analysis with the anti-Myc antibody. A protein migrating slightly faster than the purified bovine PP4R1 was observed only in the lysates from cells transfected with Myc-PP4R1 cDNA. To determine if recombinant Myc-PP4R1 protein could associate with endogenous PP4C, Myc-PP4R1 was immunoprecipitated from cell lysates with the anti-Myc antibody, and the immune complexes were analyzed for PP4C and PP4R1 by immunoblotting. PP4C co-immunoprecipitated with Myc-PP4R1 in lysates prepared from Myc-PP4R1-transfected cells, but not in lysates from vector-transfected cells (Fig. 8B). These data confirm that the PP4R1 cDNA clone encodes a protein that can associate with PP4C. Moreover, PP4R1 appears to be a specific PP4C-associated protein because PP2A was not detected in the Myc-PP4C immune complex (data not shown).

DISCUSSION

The high homology of PP2A and PP4 led to the hypothesis that PP4, like PP2A, may associate with regulatory subunits to form multimeric complexes. To identify any proteins associated with the catalytic subunit of PP4, one oligomeric form of this phosphatase was purified. The purified complex consisted of PP4C and a PP4C-associated protein (PP4R1). Sequence information obtained from PP4R1 peptides was utilized to identify a cDNA clone. The amino acid sequence encoded by this cDNA has a predicted molecular weight of 105,189, which is very similar to the size of PP4R1 measured by mass spectroscopy (104,500). Furthermore, the cDNA contains the Kozak consensus start sequence (35), indicating that the most 5′ methionine is an ideal place for the initiation of translation. These data suggest that the cDNA clone may indeed represent a full-length PP4R1 cDNA. Although the Myc-tagged PP4R1 migrated slightly faster on SDS-PAGE than the purified protein, we would like to point out that the expressed protein was of human origin, whereas the purified protein was obtained from bovine tissue. Thus, the differences in sizes could be species variations of PP4R1. Alternatively, the difference in migration on SDS-PAGE of the purified and expressed proteins could be attributed to proteolysis of the expressed PP4R1 protein. The PP4R1 cDNA does, however, encode a functional PP4C binding protein, because when expressed in mammalian cells it can be immunoprecipitated with the catalytic subunit of PP4.

The purified PP4 preparation exhibited very little phosphatase activity toward substrates readily dephosphorylated by PP2A. The activity that was measured could be accounted for with as little as 1% PP2A contamination, and it is important to point out that a hint of PP2A immunoreactivity was observed in the purified PP4 holoenzyme. Two reasonable explanations for the low apparent PP4 activity include inhibition of PP4C catalysis by PP4R1 or a narrow PP4 substrate profile (i.e. the ideal or physiological substrate has not yet been tested). Additionally, we cannot rule out the possibility that PP4 was inactivated during the purification procedure. However, this explanation seems unlikely, because PP2A catalytic activity was not inactivated following similar purification conditions (data not shown), and partially purified PP4 bound to microcystin-Sepharose, suggesting that the catalytic site is intact.

PP4R1 migrated as a doublet of 120–125 kDa on SDS-PAGE and exhibited a fairly broad peak (~105 kDa) as determined by mass spectrometry. The apparent size discrepancy may be due to aberrant migration of PP4R1 on SDS-PAGE. Several possibilities could account for the protein doublet. First, the smaller protein may represent a proteolytic fragment of the larger protein giving rise to the appearance of two proteins on SDS-PAGE and a fairly broad peak on mass spectrometry. We are
aware of some proteolysis, because another minor co-purifying protein (29 kDa) was identified as a fragment of PP4C by amino acid sequence analysis (data not shown). A second explanation is that the two proteins are identical except that one is post-transcriptionally or post-translationally modified. The mass spectrometry data is consistent with this possibility; however, at present no direct evidence exists to support this interpretation. Finally, the two proteins may represent highly homologous isoforms of PP4R1 that differ slightly in molecular weight.

PP2Aα and PP4R1 both contain nonidentical or “heat” repeats. Each of the 15 PP2Aα repeats is predicted to consist of two tightly packed amphipathic helices (36). A linear organization of these repeats would form a rodlike protein with an axial ratio of 10.5:1 (39, 40). PP2AC has been shown to bind to two tightly packed amphipathic helices (36). A linear organization to PP2A, it is quite likely that PP4C binds to the C terminus of PP4R1, since this is where PP2Aα and PP4R1 share the greatest homology. PP4R1 is distinct from PP2Aα in that the nonidentical repeats are not contiguous in PP4R1. It is difficult to interpret the implications of this unique PP4R1 feature both structurally and functionally. Since we did not identify any PP2A “B-like” subunits in our purified preparation, it is conceivable that the divergent region separating the repeats may function similarly to the B subunit in dictating substrate specificity and subcellular localization. However, we cannot rule out the possibility that a B-like subunit of PP4 was lost during the purification. Finally, the larger PP4 complex (440 kDa), which was not extensively characterized, may contain distinct PP4 subunits because the PP4α to PP4C ratio of immunoreactivity in this complex was substantially lower (data not shown) than the ratio in the purified 270–300-kDa complex.

The data presented in this report suggest that the purified PP4 complex is composed of one PP4 catalytic subunit and one associated protein; however, it is possible that two dimers may combine to form a tetramer. A complex corresponding to the predicted size of a tetramer was not detected by mass spectrometry but would be consistent with the gel filtration data. Since the unusual elution of PP2Aα on gel filtration may be explained by the predicted rodlike structure (39, 40), the size discrepancy of purified PP4 estimated by gel filtration and mass spectrometry could be reconciled by similar factors.

Several studies had indicated that PP4 existed in a high molecular weight complex with proteins distinct from the reg-
