PP2Cγ: a human protein phosphatase with a unique acidic domain

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Abstract We have cloned a novel cDNA from human skeletal muscle which encodes a protein phosphatase with a unique acidic domain. It is 34% identical to mammalian PP2Cα and PP2Cβ, and we call it PP2Cγ. It more closely resembles PP2Cs from *Paramecium tetraurelia* and *Schizosaccharomyces pombe* than mammalian PP2Cs. Northern blot analysis shows that PP2Cγ is widely expressed, and is most abundant in testis, skeletal muscle, and heart. Like known PP2Cs, recombinant PP2Cγ requires Mg^{2+} or Mn^{2+} for activity. Unlike any other known phosphatase, PP2Cγ has a highly acidic domain: 75% of the 54 residues are glutamate or aspartate.

Key words: Protein phosphatase 2C;
Protein phosphorylation, acidic domain; cDNA

1. Introduction

Protein phosphorylation and dephosphorylation are key regulatory mechanisms in many cellular processes. Four major classes of serine/threonine protein phosphatases have been described: PP1, PP2A, PP2B, and PP2C. These proteins differ in their substrate specificity, divalent cation requirements, and sensitivity to inhibitors [1–4]. PP1, PP2A, and PP2B have catalytic subunits with significant sequence similarity, and regulatory subunits which are believed to regulate activity or cellular localization. In contrast, PP2C is a monomeric phosphatase with an amino acid sequence distinct from that of other phosphatases.

Two highly conserved isoforms of PP2C (α and β) with approximately 70% sequence identity have been found in mammals [5–7]; each isoform is over 90% identical across species. Non-mammalian PP2Cs show much less amino acid identity to mammalian PP2Cs, yet several regions of sequence are highly conserved [8,9].

Here we report a novel protein phosphatase, PP2Cγ, that is widely expressed in human tissues. PP2Cγ is only 34% identical to PP2Cα and PP2Cβ, and it has a large acidic domain not previously observed in a protein phosphatase.

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Abbreviations: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, basepair(s); kb, kilobase(s)

The sequence reported in this paper has been deposited in the EMBL/GenBank database (accession no. Y13936).

2. Materials and methods

2.1. Materials

We obtained human cDNAs and RNA from Clontech, β-Agarase from New England Biolabs, the pCRII vector from InVitrogen, Pfu polymerase from Stratagene, and Taq polymerase from Boehringer Mannheim. Oligonucleotides were prepared on an automated Applied Biosystems Synthesizer at the University of Iowa DNA Core, Genosys Corp., or GIBCO-BRL. DNAs were sequenced on an Applied Biosystems automated sequencer using fluorescent dye terminators. The catalytic subunit of cAMP-dependent protein kinase was from Promega.

2.2. Cloning

The 3′-end of the PP2Cγ cDNA was found by a rapid amplification of cDNA ends (RACE) technique using human fetal brain and skeletal muscle cDNAs. To obtain the 5′-end of the coding sequence, a series of 5′-RACE reactions were required. Results from the BLAST searches and Northern blots guided us to use human fetal brain and skeletal muscle cDNAs and human testis poly(A+) RNA. RACE and PCR products were purified from agarose gels using β-agarase, cloned into the pCRII vector, and sequenced.

The complete coding sequence was amplified from human skeletal muscle cDNA using Pfu DNA polymerase and primers based on the sequence amplified from human testis. The sense primer was CACGGGTCGCGCCCAGCTTT, which begins 52 bp upstream of the start codon; and the antisense primer was GGAGCCGCCAATAAAAAAGAATGTCCTT, which ends 241 bp downstream of the stop codon. Thermal cycling was done in a Thermolyne Temptronic thermocycler (Barnstead-Thermolyne) using a program of one cycle at 94°C for 2 min; 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min; with a final incubation at 72°C for 15 min. Taq polymerase was added to each reaction and incubated for 10 min at 72°C to incorporate deoxyadenosine at the ends of the PCR products. PCR products were gel-purified, cloned into the pCRII vector, and fully sequenced from both strands. Identical sequences were obtained from two separate PCR reactions using human skeletal muscle cDNA, and from two separate PCR reactions using human lung cDNA as the template.

The relationships of proteins in the phylogenetic tree was derived using the GCG Pileup alignment program (Program Manual for the Wisconsin Package, Genetics Computer Group, Version 8, Madison WI). The diagram was generated using the GCG Distances program with Kimura substitution, followed by the Growtree program with the UPGMA option.

2.3. Northern blot analysis

Northern blots contained 2 μg of poly(A)$^+$ RNA isolated from specific tissues. Probe corresponding to amino acid residues 324–434 was prepared by random prime labeling (Pharmacia). Filters were hybridized overnight at 42°C in 50% formamide, 5×SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 2% SDS, 10×Denhardt's solution, and 100 μg/ml salmon sperm DNA. Filters were washed with 0.1% SSC, 0.1% SDS at 55°C, and exposed to Kodak X-Omat AR film with an intensifying screen for 2 h at −70°C. After additional washes at 68°C, the same pattern of hybridization was observed.

2.4. Bacterial expression and analysis of phosphatase activity

To prepare the DNA for protein expression in bacteria, the full coding sequence was reamplified with Pfu using a sense primer containing an *Nde*I site, and an antisense primer containing an *Xba*I site after the stop codon. PCR products were gel-purified and cloned into

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the pCRII vector. The PP2Cγ DNA was excised from the pCRII vector as an *NdeI*/*XbaI* fragment, and ligated into the compatible sites of the pCW vector [10]. The *Escherichia coli* strain DH5α was transformed with pCW-PP2Cγ. Cells were grown at 37°C until an OD_{600} of 0.5–0.6 was obtained, induced with 1 mM β-D-thiogalactopyranoside, then harvested after 20 h of growth at 28°C. Cells were resuspended in 20 mM Tris-HCl, pH 7.9, with 500 mM NaCl, and lysed by sonication. Lysates from 4 ml of DH5α cells expressing PP2Cγ, or from 4 ml of an identically treated culture of DH5α cells containing an irrelevant plasmid (pTM-CFTR4) were resolved by anion exchange chromatography on a Mono-Q column (Pharmacia). Lysates were diluted in H_2O to reduce the salt concentration to 100 mM, then loaded on the column, which was equilibrated in 20 mM imidazole, pH 6.0. Proteins were eluted with a linear gradient of NaCl in the same buffer, and fractions of 1 ml were collected and assayed for phosphatase activity. Myelin basic protein was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase for 60 min in 50 mM potassium phosphate, pH 6.8, 10 mM $MgCl_2$, 0.1 mM ATP, 100 μCi [γ-^{32}P]ATP, and 0.1 mg/ml bovine serum albumin, then dialyzed to remove the ATP. Approximately 2000 cpm of phosphorylated myelin basic protein was incubated for 30 min at 37°C with 10 μl of column fraction and 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, 10 μg/ml bovine serum albumin, 1 mM $MgCl_2$, and 2 mM $MnCl_2$. Released $^{32}P_i$ was collected by filtration through a Multiscreen filtration unit (Millipore), and Cerenkov radiation was measured. For the phosphatase assays described in Table 1, PP2Cα was amplified from human lung, expressed in *E. coli*, and purified (unpublished results); for PP2Cγ, we used the peak fractions from the anion exchange column described in Fig. 4A. The specific activities toward myelin basic protein were 1 nmol·min·mg for PP2Cα and 20 pmol·min·mg for PP2Cγ.

2.5. Other methods

In vitro translation of DNAs was performed with the TNT system (Promega). Proteins were analyzed using 8% SDS-polyacrylamide gels and autoradiography.

3. Results and discussion

3.1. Cloning and sequence analysis

To identify new mammalian protein phosphatases, we used the BLAST sequence alignment program [11] to search the database of expressed sequence tags (EST) with the amino acid sequence of human PP2Cα [7]. We found several overlapping nucleotide sequences derived from human fetal brain cDNA. We extended the sequence using RACE and reverse transcriptase PCR methods, and identified cDNA containing a 1641 bp open reading frame.

We amplified the complete coding sequence from human skeletal muscle and lung cDNAs; their sequences were identical. The first ATG of the longest open reading frame conformed very well to the Kozak consensus sequence for eukaryotic translation [12]; it was GCCGCCATGG. The predicted protein sequence was only 34% identical to human PP2Cα, yet it contained specific sequences that are highly conserved among PP2Cs from mammals and other organisms [8,9]. Fig. 1 shows that the conserved sequences are distributed throughout the protein. The sequence contained all 11 PP2C motifs identified by Bork and colleagues [9] as well as the 10 invariant residues identified by Das and colleagues [13]. This sequence similarity suggests that the protein is a new member of the PP2C family, and we have named it PP2Cγ.

PP2Cγ mRNA is predicted to encode a 546 amino acid protein with a mass of 59 kDa, significantly larger than the 40–45 kDa mammalian PP2Cα and PP2Cβ. The larger size is due to an additional region of approximately 200 residues not found in other PP2Cs (Fig. 1). A striking feature of this region is its abundance of acidic amino acids, with 72 aspartate or glutamate residues; within this region is a highly acidic 54 amino acid subregion containing 40 acidic residues. The calculated isoelectric point of the entire protein was 4.1; that of the 54 residue acidic region was 2.2. PP2Cγ has potential PEST sequences [14] in the region of amino acids 172–197, 197–209, and 255–320 which may target the protein for rapid degradation. The primary structure shows numerous potential sites for phosphorylation by casein kinase II, particularly within the acidic domain. Casein kinase II is selective for serines and threonines with acidic residues immediately C-terminal to the phosphoacceptor, and the +3 acidic residue is especially important [15–17].

Such an intensely acidic stretch has not previously been observed in any known protein phosphatase. However, similar highly acidic domains have been found in a variety of unrelated proteins such as calsequestrin, bone sialoprotein, nucleolin, *N*-arginine dibasic convertase, centromere protein B, ryanodine receptor, and cyclic nucleotide-gated channel, but their functions are largely undefined. The acidic region of the murine upstream binding factor (UBF) is required for nuclear targeting of the protein and transactivation [18,19]. The acidic region of the serine/threonine kinase p130^{PITSLRE} is important in binding SH2 domains [20]. Interestingly, both transactivation by UBF and SH2-binding by p130^{PITSLRE} appear to require serine/threonine phosphorylation of their acidic regions by casein kinase II [18,20]. Possible regulation of PP2Cγ by casein kinase II phosphorylation remains to be explored. Based on the crystal structure of human PP2Cα [13], we predict the acidic domain would insert near the protein surface, immediately after alpha helix 2, where it could provide an interface for interactions. We speculate that the acidic region of PP2Cγ may be important for intracellular targeting or localization, or for interactions with substrates or regulators.

Hydrophobicity analysis revealed no highly hydrophobic

Table 1
Effects of cations and inhibitors on PP2Cα and PP2Cγ activity

Addition	PP2Cα activity(%)	PP2Cγ activity(%)
None	0.0 ± 1.1	0.0 ± 2.6
Mg^{2+}	100.0 ± 2.6	100.0 ± 0.9
Mn^{2+}	137.8 ± 10.1	254.3 ± 27.6
Ca^{2+}	0.0 ± 0.5	3.9 ± 5.7
Mg^{2+} + Mn^{2+}	123.6 ± 1.9	260.5 ± 3.6
Mg^{2+} +1 μM okadaic acid	82.6 ± 2.9	83.1 ± 5.6
Mg^{2+} +100 μM Na vanadate	78.3 ± 1.1	76.2 ± 7.8
Mg^{2+} +10 mM Na fluoride	25.9 ± 1.7	18.9 ± 1.2

PP2Cα and PP2Cγ were tested for dephosphorylation of myelin basic protein in 20 mM HEPES, pH 7.5, 5 mM dithiothreitol, 0.1 mM EDTA, and 10 μg/ml bovine serum albumin with the indicated additions. Divalent cations were each present at 1 mM as the Cl^- salt. For each phosphatase, activity is expressed as percent of that observed with Mg^{2+}. Results are mean ± SEM of triplicate determinations.

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hPP2Cα   MGAFLDKPKMEKHNAQGQGNG---LRYGLSSMQGWRVEMEDAHTAVIGLPSGLESWSFFAVYDGHAGSQVAKYCCEHLLDHITNNQDFK-----GSAGAP   92
mPP2Cβ   MGAFLDKPKTEKHNAHGAGNG---LRYGLSSMQGWRVEMEDAHTAVVGIPHGLDNWSFFAVYDGHAGSRVANYCSTHLLEHITTNEDFRAADKSGSALEP   97
pPP2C    MGPYLSQPKRDKTTTTGQGKS---VIFAASEMQGWRNTMEDAHIHRHDIIQDV---SVFGVFDGHGGREVAQFVEKHFVDELLKNKNFK-----------   83
hPP2Cγ   MGAYLSQPNTVKCSGDGVGAPRLPLPYGFSAMQGWRVSMEDAHNCIPELDSET---AMFSVYDGHGGEEVALYCAKYLPDTIKDQKAYK-----------   86

hPP2Cα   SVENVKNGIRTGFLEIDEHMRVMSEKKH-------------------------------------------------------------------   120
mPP2Cβ   ----------------------------------------------------------------------------------------------   97
pPP2C    -EQKFEEALKETFLKMDELLLTPEGQKELNQYKATDTDE-----------------------------------------------------------   121
hPP2Cγ   -EGKLQKALEDAFLAIDAKLTTEEVIKELAQIAGRPTEDEDEKEKVADEDDVDNEEAALLHEEATMTIEELLTRYGQNCHKGPPHSKSGGGTGEEPGSQG   185

hPP2Cα   ----------------------------------------------------------------------------------------------   120
mPP2Cβ   ----------------------------------------------------------------------------------------------   97
pPP2C    ----------------------------------------------------------------------------------------------   121
hPP2Cγ   LNGEAGPEDSTRETPSQENGPTAKAYTGFSSNSERGTEAGQVGEPGIPTGEAGPSCSSASDKLPRVAKSKFFEDSEDESDEAEEEEEDSEECSEEEDGYS   285

hPP2Cα   ------------------------------------GADRSGSTAVGVLISPQHTYFINCGDSRGLLCRNRKVHFFTQDHKPSNPLEKERIQNAGG-SV   182
mPP2Cβ   ------------------------------------GMDRSGSTAVGVMVSPTHMYFINCGDSRAVLCRNGQVCFSTQDHKPCNPVEKERIQNAGG-SV   187
pPP2C    ------------------------------------SYAGCTANVALIYKNTLYVANAGDSRSVLCRNNTNHDMSVDHKPDNPEEKSRIERAGG-FV   179
hPP2Cγ   SEEAENEEDEDDTEEAEEDDEEEEEEMMVPGMEGKEEPGSDSGTTAVVALIRGKQLIVANAGDSRCVVSEAGKALDMSYDHKPEDEVERLARIKNAGGKVT   385

hPP2Cα   MIQRVNGSLAVSRALGDFDYKCVHGKGPTEQLVSPEPEVHDIERSEEDDQFIILACDGIWDVMGNEELCDFVRSRLEVTDD------LEKVCNEVVDTCL   276
mPP2Cβ   MIQRVNGSLAVSRALGDYDYKCVDGKGPTEQLVSPEPEVYEIVRAEEDE-FVVLACDGIWDVMSNEELCEFVKSRLEVSDD------LENVCNWVVDTCL   280
pPP2C    SDGRVNGNLNLSRALGDLEYKRDNKLRSNEQLIIALPDVKKTELTPQDK-FILMGCDGVFETLNHQELLKQVNSTIGQAQVTEE--LLKKAAEDLLDQLL   276
hPP2Cγ   MDGRVNGGLNLSRAIGDHFYKRNKNLPPEEQMISALPDIKVLTLTDDHE-FMVIACDGIWNVMSSQEVVDFIQSKISQRDENGELRLLSSIVEELLDQCL   484

hPP2Cα   YKGSR------DNMSVILICFPNAPKVSPEAVKKEAELDKYLECRVEEIIKKQGE-GVPDLVHVMRTLASENIPSLPPGGELASKRNVIEAVYNRLNPYK   369
mPP2Cβ   HKGSR------DNMSVVLVCFSNAPKVSEEAVKRDSELDKHLESRVEEIMQKSGEEGMPDLAHVMRILSAENIPNLPPGGGLAGKRHVIEAVYSRLNPHK   374
pPP2C    APDTS-QGTGCDNMTTILVYLRR                                                                              298
hPP2Cγ   APDTSGDGTGCDNMTCIIICFKPRNTAELQPESGKRKLEEVLSTEGAEENGNSDKKKAKRD                                       546

hPP2Cα   NDDTDSTSTDDMW          382
mPP2Cβ   DNDGVSLHLFPKYLK         390
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Fig. 1. Amino acid sequence of PP2Cγ as deduced from the nucleotide sequence. The primary sequence of PP2Cγ was compared with those of human PP2Cα (GenBank S87759), mouse PP2Cβ2 (D17412), *Paramecium* PP2C (Z36985), using the BESTFIT and PILEUP programs (Genetics Computer Group, Madison, WI). Amino acid sequences were displayed with the DNA Draw program [24]. Gaps introduced to optimize the alignments are indicated by a dash. Shading indicates identity with PP2Cγ. The highly acidic region is underlined. The predicted sites for phosphorylation by casein kinase II are boxed. The predicted myristylation site is double-underlined.

regions (data not shown), suggesting that PP2Cγ is probably cytosolic like other PP2Cs, although membrane association cannot be ruled out. Fig. 1 shows that there is a potential myristylation site which might function to anchor the protein to the membrane. This site is conserved in many PP2Cs, but it has not been shown to be myristylated, and other PP2Cs are considered to be soluble proteins.

3.2. Distribution of PP2Cγ

We examined the tissue distribution of PP2Cγ by Northern blot analyses of human RNAs. Fig. 2 shows that a single transcript of approximately 2.3 kb was detected in all tissues examined, with the highest levels in testis, skeletal muscle, and heart. Reverse transcriptase PCR also revealed PP2Cγ in the human colonic epithelial cell line T84, primary cultures of human airway epithelial cells, as well as canine Madin Darby kidney cells (data not shown). These results indicate that this phosphatase is widely expressed.

3.3. Phylogenetic analysis

We asked whether the PP2Cγ sequence more closely resembled the α or β isoform of mammalian PP2Cs by performing phylogenetic analysis. Surprisingly, PP2Cγ resembled the PP2Cs from the protozoan *Paramecium tetraurelia* [8] and the yeast *Schizosaccharomyces pombe* [21] more closely than it resembled mammalian PP2Cα or PP2Cβ (Fig. 3). The yeast PP2Cs are important for maintaining cell shape and osmotic stability, and the *Paramecium* PP2C was isolated from cilia, which are membrane-covered microtubule structures. It is tempting to speculate that these phosphatases may have related functions, perhaps in regulating microtubule or cytoskeletal function. Identifying the in vivo substrates of PP2Cγ will be helpful for better understanding its function.

3.4. PP2Cγ encodes an active protein phosphatase

To examine the properties of PP2Cγ phosphatase activity, we expressed it in bacteria and partially purified it by anion exchange chromatography. Fig. 4A shows that lysates from bacteria expressing PP2Cγ dephosphorylated myelin basic protein, a common serine/threonine phosphatase substrate, whereas lysates from bacteria containing an irrelevant plasmid did not have phosphatase activity. PP2Cγ binding to the anion exchange column is consistent with a protein with a high density of negative charge. The enzymatic properties of PP2Cγ were similar to those of PP2Cα (Table 1). PP2Cγ phosphatase activity required Mg^{2+} or Mn^{2+}; Ca^{2+} did not support activity. In addition, PP2Cγ activity was insensitive to okadaic acid and vanadate, but it was inhibited by fluoride. These enzymatic similarities are interesting considering that PP2Cγ is only 34% identical to PP2Cα. This suggests that the metal-binding and fluoride-binding residues may be conserved between these phosphatases. Indeed, all four metal-

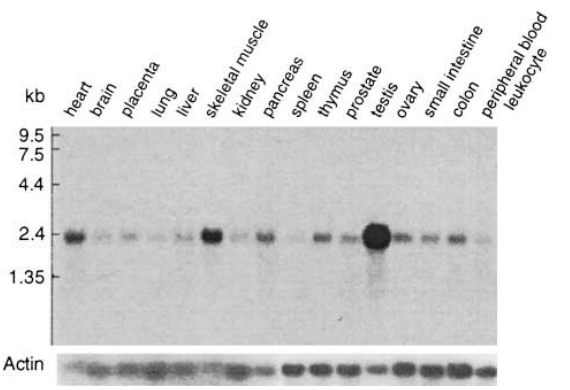

Fig. 2. Tissue distribution of human PP2Cγ. Each lane contains approximately 2 μg of polyA(+) RNA; the amount of RNA in each lane was adjusted to observe similar levels of β-actin expression. The actin results were provided by Clontech. For PP2Cγ, filters were hybridized as described in Section 2.

binding aspartate residues revealed in the PP2Cα crystal structure [13] are completely conserved in PP2Cγ.

Although the predicted size of the PP2Cγ protein is 59 kDa, translation of PP2Cγ yielded a protein of 75 kDa (Fig. 4B). The anomalous mobility may be due to the acidic region; proteins with similar acidic regions can show lower mobility on SDS gels, presumably because of decreased SDS binding [20]. PP2Cγ may be a human homolog of a 76 kDa Mg^{2+}-dependent protein phosphatase purified from bovine brain [22]. A 24 amino acid fragment of that protein (DHEFMVIACDGIWNVMSSQEVIDF) is very similar to the PP2Cγ sequence. The nucleotide sequence of PP2Cγ also strongly resembles a partial cDNA sequence identified in mouse fibroblasts (FIN-13) [23]. Interestingly, this mouse gene is induced by fibroblast growth factor, and is proposed to be involved in cell proliferation.

3.5. Conclusion

PP2Cγ is a novel member of the PP2C family of serine/threonine phosphatases. Although the PP2Cγ sequence is only 34% identical to human PP2Cα and mouse PP2Cβ, it contains the highly conserved signature sequences of PP2C. Moreover, its enzymatic activity is Mg^{2+}-stimulated, as observed with known PP2Cs. The most striking feature of PP2Cγ that distinguishes it from all other known phosphatases is the presence of a large acidic domain. This unique domain may be important for intracellular targeting or localization, or for interactions with substrates or regulators. Its ubiquitous expression suggests that PP2Cγ may play an significant role in determining the phosphorylation state of proteins in a wide variety of tissues.

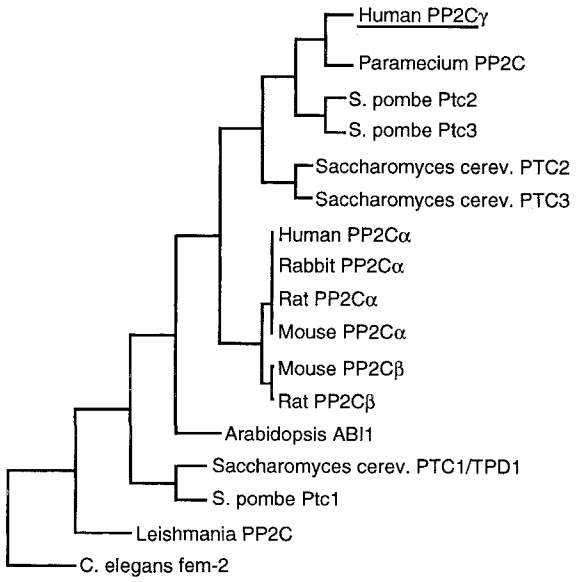

Fig. 3. Phylogenetic tree of PP2C family members. The nucleotide sequence of PP2Cγ (underlined) was compared with those of *Paramecium tetraurelia* PP2C (Z36985); *Schizosaccharomyces pombe* Ptc1 (L26970), Ptc2 (L34881), Ptc3 (L34882); *Saccharomyces cerevisiae* PTC1/TPD1 (L14593), PTC2 (U72498), PTC3 (U72346); human PP2Cα (S87759); rabbit PP2Cα (S87757); rat PP2Cα (J04503), PP2Cβ (S90449); mouse PP2Cα (D28117), PP2Cβ1 (D17411); *Arabidopsis thaliana* PP2C (X77116, X78866); *Leishmania chagasi* PP2C (L15559); and *Caenorhabditis elegans* fem-2 (U29515).

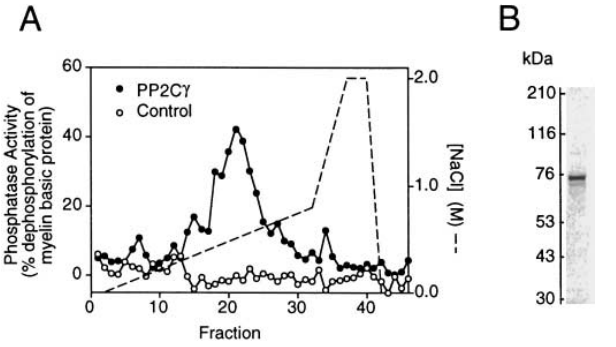

Fig. 4. Analysis of PP2Cγ protein and activity. A: Partial purification of PP2Cγ. Lysates from induced DH5α cells containing pCW-PP2Cγ (●), or from DH5α cells containing no plasmid (○) were separated by Mono Q anion exchange chromatography. Proteins were eluted with a NaCl gradient (dashed line), and fractions were assayed for protein phosphatase activity. B: In vitro translation of PP2Cγ. PP2Cγ was translated in vitro using [^{35}S]methionine as described in Section 2, and analyzed by SDS-PAGE and autoradiography.

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