Mutational analysis of the domain structure of mouse protein phosphatase 2C β

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The structures of five distinct isoforms of mammalian protein phosphatase 2C β (PP2C β -1, -2, -3, -4 and -5) have previously been found to differ only at their C-terminal regions. In the present study, we performed mutational analysis of recombinant mouse PP2C β -1 to determine the functional domains of the molecule and elucidate the biochemical significance of the structural differences in the isoforms. Differences in affinity for [³²P]phosphohistone but not for [³²P]phosphocasein were observed among the five PP2C β isoforms. Deletion of 12 amino acids from the C-terminal end, which form a unique sequence for PP2C β -1, caused a 35 % loss of activity against [³²P]phosphohistone but no loss of activity against [³²P]phosphocasein. Deletion of up to 78 amino acids from this end did not cause any further alteration in activity, whereas deletion of 100 amino acids totally eliminated the activity against both [³²P]phosphohistone

and [³²P]phosphocasein. On the other hand, deletion of 11 amino acids from the N-terminal end caused a 97 % loss of enzyme activity, and further deletions caused a total loss of activity. Substitution of any of the six specific amino acids among 16 tested in this study, which were located among the 250 N-terminal residues, caused 98–100 % loss of enzyme activity. Among these amino acids, three (Glu-38, -60 and -243) have recently been reported to be essential for the binding of metal ions in the catalytic site of the PP2C molecule [Das, Helps, Cohen and Barford (1996) EMBO J. 15, 6798–6809]. These observations indicate that PP2C β is composed of at least two distinct functional domains, an N-terminal catalytic domain of about 310 amino acids and the remaining C-terminal domain, which is involved in determination of substrate specificity.

INTRODUCTION

Reversible phosphorylation of cellular proteins plays a pivotal role in the regulation of cellular functions. Protein phosphatases, in combination with protein kinases, are key factors in the regulation of cellular protein phosphorylation. The protein phosphatases of eukaryotic cells are divided into three groups (protein serine/threonine, tyrosine and serine/threonine/tyrosine phosphatases) based on their specificities for phospho-amino acids. Protein serine/threonine phosphatases (PPs) are further classified into four major groups (PP1, PP2A, PP2B and PP2C) depending on their substrate specificities, bivalent cation dependencies and sensitivities to various inhibitor molecules [1]. Various isoforms of each major group have been found in mammalian cells [2], and cloning of cDNAs encoding novel PPs such as PPX [3] and PP5 [4] has also been reported.

PP2C is distinct from the other three major PPs because of its absolute requirement for Mg²⁺ or Mn²⁺ and because its amino acid sequence shows little similarity to those of other PPs [5]. Recent studies on the function of mammalian PP2C have suggested that it participates in the regulation of AMP-activated protein kinase [6] and Ca²⁺/calmodulin-dependent protein kinase II [7] activities and regulation of the DNA-repair system [8]. Molecular genetic analysis of the function of PP2C in plants has revealed that *Arabidopsis* ABI1 protein has an EF-hand Ca²⁺-binding site and a PP2C-related sequence in its N-terminal and C-terminal domains respectively, and is involved in intracellular

signalling induced by the plant hormone abscisic acid [9,10]. In *Saccharomyces cerevisiae* the PTC1 (TPD1) gene product, which shares 35 % similarity of its amino acid sequence with mammalian PP2C, participates in the regulation of the osmosensing mitogen-activated protein kinase cascade, splicing of tRNAs and regulation of sporulation and mitotic growth [11–13]. The participation of a PP2C-like gene product in osmoregulation and the heat-shock response has also been reported in *Schizosaccharomyces pombe* [14].

The presence of two isotypes, PP2C α and PP2C β , which are separate gene products, has been reported in mammalian cells [5,15,16]. Subsequently, cDNA clones encoding five distinct PP2C β isoforms (PP2C β -1, -2, -3, -4 and -5) have been isolated, and these isoforms have been identified as the products of alternative splicing of a single pre-mRNA [15–18]. The amino acid sequences of these five PP2C β isoforms differ only in their C-terminal regions (11–24 amino acids) [17]; however, they also differ in the tissue specificity of their expression [17]. A detailed analysis of the expression of PP2C β in mouse testis revealed that PP2C β -3, -4 and -5 were expressed specifically in pachytene spermatocytes and in more highly differentiated germ cells in the seminiferous tubules [19]. In addition, the substrate specificity of PP2C β -4 was found to be different from those of PP2C β -3 and -5 [19]. These lines of evidence indicate that the differences in the structures of the PP2C β isoforms reflect unique physiological functions of each isoform.

Das et al. [20] have recently reported the molecular architecture

Abbreviations used: PP, protein serine/threonine phosphatase; GST, glutathione S-transferase; ORF, open reading frame; HA, haemagglutinin; NLS, nuclear localization signal.

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of PP2C α based on crystallographic analysis. PP2C α consists of two domains: an N-terminal catalytic domain with six α -helices and eleven β -strands, and a 90-residue C-terminal domain of three α -helices. They suggested that the overall structures of PP2C α and PP2C β are similar. They also identified five acidic amino acids that co-ordinated two Mn²⁺ ions at the catalytic site and proposed that the metal ions participate in the catalysis. However, biochemical evidence was required for their proposal to be verified.

Therefore, we were interested in determining biochemically the structure–function relationship of the PP2C β molecule. To this end, we prepared various deletion and point mutants of recombinant PP2C β -1 using a bacterial expression system and tested the effects of these mutations on the enzymic properties of the molecule. We provide evidence supporting the proposal that PP2C has two functional domains and that metal ions participate in catalysis.

EXPERIMENTAL

Materials

Restriction endonucleases, T4 DNA ligase and polynucleotide kinase were purchased from Toyobo (Osaka, Japan). Taq polymerase and the site-directed mutagenesis kit Mutan-Express Km were obtained from Takara Shuzo (Kyoto, Japan). [γ -³²P]ATP was from DuPont–New England Nuclear (Boston, MA, U.S.A.), and a DNA sequencing kit was from Perkin Elmer (Foster, CA, U.S.A.). The PROTRAN nitrocellulose membrane was purchased from Schleicher & Schuell (Dassel, Germany). Anti-rabbit IgG (Fc)-alkaline phosphatase conjugate was obtained from Promega (Madison, WI, U.S.A.). Glutathione–agarose beads, glutathione, thrombin, whole histone and α -casein were purchased from Sigma (St. Louis, MO, U.S.A.). The pGEX2T glutathione S-transferase (GST) gene fusion vector was obtained from Pharmacia (Uppsala, Sweden), and the pEUK-C-1 vector from Clontech (Palo Alto, CA, U.S.A.). Monoclonal antibody 12CA5 was purchased from Boehringer (Mannheim, Germany). All other reagents were purchased from Wako Pure Chemical (Osaka, Japan). The *Escherichia coli* strain used for expression of the cDNAs was DH-5 α .

Construction of GST–PP2C β -1 fusion protein

To construct pGEX2T–PP2C β -1, which expresses GST–PP2C β -1 fusion protein, the *Nco*I–*Eco*RI fragment was excised from the expression plasmid pKMC–MPP β -1 [16], which harbours PP2C β -1. The end of the *Nco*I site of this fragment was blunted using a Klenow fragment. The resulting fragment was ligated into pGEX-2T, which had been double digested by *Sma*I and *Eco*RI. The resultant plasmid was named pGEX2T–PP2C β -1.

Construction of deletion mutants of GST–PP2C β -1

PCR was performed to introduce N-terminal deletions into PP2C β -1. The upstream primers used were 5'-A-A-T-C-G-G-A-T-C-C-C-T-A-A-A-G-C-A-C-A-A-T-G-C-T-C-A-C-G-G-3' for Δ N(1–11), 5'-A-A-T-C-G-G-A-T-C-C-C-T-A-A-A-T-G-G-T-C-T-G-C-G-T-T-A-T-G-G-C-3' for Δ N(1–19) and 5'-A-A-T-C-G-G-A-T-C-C-C-T-A-A-A-G-C-A-G-T-A-T-G-C-A-A-G-G-A-T-G-G-3' for Δ (1–26), which contain the sequences of the 5' region of the PP2C β -1 open reading frame (ORF). The sequence in italics shows the *Bam*HI site. The downstream primer was 5'-G-A-T-T-T-G-T-C-A-G-C-T-G-C-C-C-T-G-3', which corresponds to the complementary sequence downstream of the *Bam*HI site in the ORF of PP2C β -1. PCR was carried out using these oligonucleotides as primers and pGEX2T–PP2C β -1 as the template.

The resultant PCR products were digested by *Bam*HI and ligated into pGEX2T–PP2C β -1, which had previously been digested by *Bam*HI. The resultant plasmids were named pGEX-2T–PP2C β -1- Δ N(1–11), pGEX-2T–PP2C β -1- Δ N(1–19) and pGEX-2T–PP2C β -1- Δ N(1–26). Introduction of C-terminal deletions into PP2C β -1 was also performed using PCR. The upstream primer used was 5'-A-G-A-G-C-A-G-C-T-T-G-T-T-T-C-T-C-C-3', which corresponds to the sequence upstream of the *Sac*I site in the PP2C β -1 ORF. The downstream primers were 5'-T-A-T-C-G-A-A-T-T-C-C-T-A-A-A-C-A-C-T-C-A-T-G-T-T-A-T-C-T-C-G-3' for Δ C(291–390), 5'-C-A-T-C-G-A-A-T-T-C-C-T-A-C-A-A-C-T-C-T-G-A-A-T-C-T-C-T-T-C-3' for Δ C(313–390) and 5'-C-A-T-T-G-A-A-T-T-C-T-T-A-C-C-C-A-T-C-A-T-T-G-T-C-T-T-T-G-T-G-3' for Δ C(379–390), which contain the complementary sequences of the 3' end region of the PP2C β -1 ORF. The italic sequence shows the *Eco*RI site. PCR was carried out using these oligonucleotides as primers and pGEX2T–PP2C β -1 as the template. The PCR products were digested by *Sac*I and *Eco*RI and ligated into pGEX2T–PP2C β -1, which had previously been double digested by *Sac*I and *Eco*RI. All deletion mutants were verified by nucleotide sequence analysis using the dideoxy method. The resultant plasmids were named pGEX-2T–PP2C β -1- Δ C(291–390), pGEX-2T–PP2C β -1- Δ C(313–390) and pGEX-2T–PP2C β -1- Δ C(379–390).

Construction of site-directed mutants of GST–PP2C β -1

Site-directed mutagenesis was performed by the oligonucleotide-directed dual amber method [21] using the Mutan-Express Km kit. The 0.65 kb *Nco*I–*Sac*I fragment was excised from pGEX2T–PP2C β -1 and ligated into a pKF18 k plasmid as follows. The end of the *Nco*I site of this fragment was blunted using a Klenow fragment. The resulting fragment was ligated into the *Sma*I/*Sac*I site of pKF18 k, and the resulting plasmid was named pKF18 k–PP2C β N. Various PP2C β -1 mutants were then prepared using the pKF18 k–PP2C β N-generated single-stranded DNA as a template. All mutants were verified by nucleotide sequence analysis. The mutated PP2C β -1 cDNA fragments were then ligated into pGEX2T–PP2C β -1, which had previously been double digested by *Nco*I and *Sac*I. The mutagenic oligonucleotides used for each specific mutation are listed in Table 1.

Expression of GST–PP2C β -1 fusion proteins in *E. coli* and preparation of PP2C β -1 proteins

Expression and purification of GST–PP2C β -1 and its mutants were achieved using the protocols described by Smith and Johnson [22]. Cultures of *E. coli* strain DH5 α , transformed by pGEX2T–PP2C β -1 and its mutants, were grown in Luria–Bertani medium containing ampicillin (50 μ g/ml) at 30 °C. When the A_{600} of the culture medium reached 0.6, isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and the cells were cultured for another 5 h. The cells were then harvested and resuspended in ice-cold PBS. After incubation for 15 min on ice, the cells were sonicated with a Model UR-200 sonicator (Tomy Seiko, Tokyo, Japan) and centrifuged at 10 000 g for 5 min. The resultant supernatants were collected. The GST-fusion proteins in the cell extracts were adsorbed to glutathione–agarose beads and treated with thrombin [0.001 % (w/v) thrombin in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl and 2.5 mM CaCl₂, for 1 h at 25 °C] to release recombinant PP2C β -1 and its mutants from the fusion proteins. Protein determination was performed by Bradford's method using BSA as the standard [23].

Table 1 Oligonucleotides used to introduce site-directed mutations into mouse PP2C β -1

The bases underlined were site-directed mutagenized to introduce new amino acids to replace the original ones.

Protein	Amino acid changed	Codon changed	Oligonucleotide sequences
R33G	Arg ³³ → Gly	AGA → GGA	5'-AAGGATGGGGAGTAGAAATG-3'
E37A	Glu ³⁷ → Ala	GAA → GCA	5'-AGAAATGGCAGATGCACAC-3'
D38A	Asp ³⁸ → Ala	GAT → GCT	5'-AAATGGAAGCTGCACACAC-3'
A39D	Ala ³⁹ → Asp	GCA → GAT	5'-GAAATGGAAGATGATCACACAGCTGTTG-3'
H40L	His ⁴⁰ → Leu	CAC → CTC	5'-GAAGATGCACTCAGCTGTTG-3'
D60A	Asp ⁶⁰ → Ala	GAC → GCC	5'-CAGTTTATGCCGGTCATGC-3'
H62L	His ⁶² → Leu	CAT → CTT	5'-ATGACGGTCTTGTGGATC-3'
D168A	Asp ¹⁶⁸ → Ala	GAT → GCT	5'-CTACCCAGGCTCACAAC-3'
H169L	His ¹⁶⁹ → Leu	CAC → CTC	5'-ACCCAGGATCTCAACCTTGT-3'
R179G	Arg ¹⁷⁹ → Gly	CGC → GGC	5'-CAGTATGCGGGTCATGC-3'
R191G	Arg ¹⁹¹ → Gly	CGT → GGT	5'-ATGATCCAGGGTGTGAACG-3'
R200G	Arg ²⁰⁰ → Gly	CGG → GGG	5'-GCAGTGTCTGGGGCTCTGG-3'
D204A	Asp ²⁰⁴ → Ala	GAC → GCC	5'-TCTGGGGGCTATGACTAC-3'
D243A	Asp ²⁴³ → Ala	GAT → GCT	5'-TGGCCTGTCTGGGATCTG-3'
W246G	Trp ²⁴⁶ → Gly	TGG → GGG	5'-GATGGGATCGGGGATGTG-3'
D247A	Asp ²⁴⁷ → Ala	GAT → GCT	5'-GATCTGGGCTGTGATGAG-3'

Assay of PP activities

Phosphorylation of whole histone by protein kinase A and of α -casein by casein kinase II using [γ -³²P]ATP was performed essentially as described previously [24]. PP activities were measured in the presence or absence of 10 mM MgCl₂ and 1 μ M okadaic acid using [³²P]phosphohistone or [³²P]phosphocasein as the substrate as described previously [16]. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of 1 nmol of phosphate in 1 min at 30 °C.

Immunoblotting

A polyclonal antibody, AB106, directed against a synthetic mouse PP2C β oligopeptide (Thr-Thr-Asn-Glu-Asp-Phe-Arg-Ala-Ala-Asp-Lys-Ser-Gly-Ser-Ala-Leu-Glu) was raised in rabbits. Purified recombinant PP2C β -1 and its mutants were analysed by SDS/PAGE. Proteins separated by the SDS/PAGE were electrotitrated on to a PROTRAN nitrocellulose membrane. Following the transfer, the membrane was preincubated for 1 h at 25 °C in blocking solution containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl and 1 % (w/v) non-fat milk. After 1 h incubation with the AB106 antibody at 25 °C, the membrane was washed with blocking solution then incubated for 30 min at 25 °C with anti-rabbit IgG(Fc)-alkaline phosphatase conjugate. After washing with the blocking solution, colour was developed with 0.1 M Tris/HCl, pH 9.0, containing 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium.

Construction of expression plasmids for epitope-tagged PP2C β -1 and its deletion mutant

An expression plasmid for epitope-tagged PP2C β -1 was made as follows. A DNA fragment encoding the nine-amino-acid epitope (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) of influenza virus haemagglutinin (HA), which is recognized by the monoclonal antibody 12CA5, was inserted into the 5' end of the ORF of PP2C β -1 cDNA by oligonucleotide-directed mutagenesis using the PCR. The first PCR product was generated using the upstream primer 5'-A-T-G-T-A-T-C-C-A-T-A-T-G-A-T-G-T-

T-C-C-A-G-A-T-T-A-T-G-C-T-G-G-T-G-C-A-T-T-T-T-G-G-A-T-A-A-A-3' and the downstream primer 5'-A-A-T-T-T-G-C-C-A-C-T-C-G-G-G-A-T-C-C-3', with PP2C β cDNA as the template. A second PCR was performed using the upstream primer 5'-G-C-T-C-T-A-G-A-G-G-C-A-C-G-A-T-G-T-A-T-C-C-A-T-A-T-G-A-T-G-T-T-3' and the downstream primer 5'-A-A-T-T-T-G-C-C-A-C-T-C-G-G-G-A-T-C-C-3', with the first PCR product as the template. The second PCR product was digested by *Xba*I and *Bam*HI and inserted into the expression vector pEUK-C-1, which had previously been digested by *Xba*I and *Bam*HI. The remaining 3' portion of mouse PP2C β -1 (the *Bam*HI fragment excised from pKMC-MPP β -1) was introduced into the *Bam*HI site of the expression vector containing the *Xba*I/*Bam*HI fragment of the second PCR product. The final product was named pEUK-C-PP2C β -1. To construct an expression plasmid for the deletion mutant of HA-PP2C β -1, the *Sac*I-*Eco*RI fragment was excised from pGEX-2T-PP2C β -1- Δ C(313–390), and the 3' end of the DNA fragment was blunted using a Klenow fragment. This DNA was ligated into pEUK-C-PP2C β -1 previously double digested by *Sac*I and *Sma*I. The resultant plasmid was named pEUK-C-PP2C β -1- Δ C(313–390).

Cell culture and transfection

COS7 cells were grown in Dulbecco's minimal essential medium with 10 % (v/v) fetal calf serum, 100 units/ml penicillin G and 100 μ g/ml streptomycin. The epitope-tagged PP2C cDNA and the cDNA of its deletion mutant were transiently expressed in COS7 cells using the DEAE-dextran transfection procedure. After culturing for 24 h, the cells were harvested, lysed in a buffer containing 10 mM Tris/HCl, pH 7.5, 1 % (v/v) Triton X-100, 150 mM NaCl and 1 mM EDTA. The supernatants obtained by centrifugation (12 000 *g* for 10 min) were incubated with anti-HA antibody (12CA5, 1 μ g/ml) for 1 h at 4 °C. The immune complexes were adsorbed to Protein A-agarose beads, and the beads were washed twice with the buffer containing 10 mM Tris/HCl, pH 7.5, 1 % (v/v) Triton X-100, 150 mM NaCl and 1 mM EDTA, and once with TE buffer containing 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA. The suspension of the washed beads in TE buffer was used for the enzyme assay. Alternatively, the cells were fixed and immunostained with the anti-HA antibody.

RESULTS

Comparison of the biochemical properties of wild-type PP2C β isoforms

The five distinct isoforms of PP2C β (-1, -2, -3, -4 and -5) were expressed in *E. coli* cells as GST-fusion proteins. Recombinant GST-PP2C β s were purified and digested by thrombin to release the PP2C portions from the fusion proteins. On SDS/PAGE analysis, each recombinant PP2C β isoform was stained as a single band of the expected molecular size (Figure 1A).

The enzyme activities of the five distinct recombinant PP2C β isoforms were determined using [³²P]phosphocasein and [³²P]phosphohistone as the substrates. The activity of the recombinant PP2C β -1 against [³²P]phosphohistone was comparable with that of the PP2C purified from rat liver [25]. As shown in Figure 1B, little difference in activity was observed between the five isoforms when [³²P]phosphocasein was used as the substrate. In contrast, the activities of PP2C β -2 and -4 for [³²P]phosphohistone were substantially lower than those of the other PP2C β isoforms. As a result the activity ratios (histone phosphatase activity/casein phosphatase activity) of PP2C β -1, -3 and -5 (4.1–4.5) were substantially higher than those of PP2C β -2 and -4 (1.6–2.6).

Figure 1 PP activities of recombinant mouse PP2C β isoforms

(A) PP2C β isoforms released from GST–PP2C β s by thrombin treatment were subjected to SDS/PAGE analysis. After electrophoresis the gel was stained with Coomassie Brilliant Blue. Size markers are shown on the left. (B) PP activities of the recombinant PP2C β isoforms were assayed using [32 P]phosphohistone and [32 P]phosphocasein as substrates. The results represent the means of three experiments $\pm$ S.E.M.

Table 2 Enzyme kinetic parameters of PP2C β isoforms

The results represent the means of three experiments $\pm$ S.E.M.

	PP2C β -1	PP2C β -2	PP2C β -3	PP2C β -4	PP2C β -5
Casein					
$V_{\max}$ (units/mg)	24 $\pm$ 3	23 $\pm$ 2	23 $\pm$ 3	25 $\pm$ 3	23 $\pm$ 1
K_m (μ M)	11 $\pm$ 1	11 $\pm$ 2	9 $\pm$ 1	11 $\pm$ 1	11 $\pm$ 2
K_a for MgCl ₂ (mM)	2.0 $\pm$ 0.2	2.1 $\pm$ 0.1	1.9 $\pm$ 0.2	2.1 $\pm$ 0.1	1.9 $\pm$ 0.3
Histone					
$V_{\max}$ (units/mg)	125 $\pm$ 11	125 $\pm$ 13	100 $\pm$ 9	140 $\pm$ 15	100 $\pm$ 10
K_m (μ M)	25 $\pm$ 3	50 $\pm$ 4	17 $\pm$ 2	42 $\pm$ 3	11 $\pm$ 2
K_a for MgCl ₂ (mM)	3.8 $\pm$ 0.2	2.5 $\pm$ 0.1	4.2 $\pm$ 0.3	2.5 $\pm$ 0.1	7 $\pm$ 0.4

Figure 2 Schematic illustration, immunoblot analysis and PP activities of deletion mutants of PP2C β -1

(A) Structures of the deletion mutants of PP2C β -1. The amino acid residues on the left indicate the remnant originating from the GST portion. The black boxes represent the region unique to PP2C β -1. Size markers are shown on the right. (B) Purified recombinant PP2C β -1 and its deletion mutants were immunoblotted using anti-PP2C β antibody (AB106) as the first antibody. Size markers are shown on the left. (C) PP activities of wild-type PP2C β -1 and its deletion mutants were assayed using [32 P]phosphohistone and [32 P]phosphocasein as substrates. The results represent the means of three experiments $\pm$ S.E.M.

These lines of evidence strongly suggest that the differences in the amino acid sequences of the C-terminal regions of PP2C β isoforms are related to the substrate specificity of these enzyme molecules. Therefore, we performed enzyme kinetic analysis to determine whether the kinetic parameters of the PP2C β isoforms reflected the differences in their substrate specificities (Table 2). As expected, no differences in K_m (9–11 μ M) or $V_{\max}$ (23–25 units/

Figure 3 Alignment of amino acid sequences of PP2Cs from various organisms and PP activities of point mutants of PP2C β -1

(A) The partial amino acid sequences of mouse PP2C β -1, *S. cerevisiae* PTC1, *Leishmania* PP2C and *Arabidopsis* KAPP are shown. The conserved amino acids are boxed. The numbers on the right indicate the amino acid positions in the protein. The amino acids of mouse PP2C β -1 substituted by other amino acids during mutagenesis are denoted by asterisks. (B) The histone phosphatase activities of the point mutants of PP2C β -1 were assayed in the presence of both 10 mM MgCl₂ and 1 μ M okadaic acid using [³²P]phosphohistone as the substrate as described previously [16]. The results represent one of two reproducible experiments.

mg) were observed for any of the five PP2C β isoforms when [³²P]phosphocasein was used as the substrate. In contrast, the K_m values for isoforms PP2C β -1, -2, -3, -4 and -5 were 25, 50, 17, 42 and 11 μ M, respectively, when [³²P]phosphohistone was employed as the substrate. Since there was no essential difference in V_{max} (100–140 units/mg) among the five isoforms, it was concluded that the differences in the C-terminal region structures of the different isoforms caused the variation in their affinity for [³²P]phosphohistone.

We then determined whether there was any difference in Mg²⁺ sensitivity among the five isoforms (Table 2). The K_a for MgCl₂ was determined using both [³²P]phosphohistone and [³²P]phosphocasein as substrates. The K_a for MgCl₂ was about 2.0 mM for all five isoforms when [³²P]phosphocasein was used as the substrate. However, the K_a differed when [³²P]phosphohistone was employed as the substrate. The K_a values for MgCl₂ for PP2C β -2 and -4 were 2.5 mM and those for PP2C β -1 and -3 were about 4 mM, whereas that for PP2C β -5 was 7 mM.

Figure 4 Immunoblot analysis, PP activities and subcellular localization of HA-tagged PP2C β -1 and its deletion mutant expressed in COS7 cells

(A) COS7 cell extracts harbouring the expression plasmids [vector, pEUK-C-PP2C β -1 and pEUK-C-PP2C β -1- Δ C(313–390)] were subjected to immunoblot analysis using anti-HA antibody (12CA5) as the first antibody as already described. Size markers are shown on the left. (B) Cells harbouring the expression plasmids were directly fixed and immunostained with anti-HA antibody. Colour development was performed with the peroxidase reaction using diaminobenzidine tetrahydrochloride as the substrate. Counterstaining was performed with haematoxylin.

Analysis of deletion mutants of PP2C β -1

In order to obtain more detailed information on the functional domains of the PP2C β molecule, we prepared various deletion mutants of PP2C β -1 and studied their structure–activity relationships. The deletion mutants of PP2C β -1 shown in Figure 2A were expressed in *E. coli* cells as GST-fusion proteins. The PP2C β -1 mutant proteins obtained after digestion with thrombin were subjected to immunoblot analysis. As shown in Figure 2B, single bands of the expected sizes were immunostained by anti-PP2C β antibody for all these recombinant proteins except Δ C(313–390) and Δ C(291–390). Two immunoreactive protein bands were observed for Δ C(313–390) and Δ C(291–390); the lower-molecular-mass bands might have been caused by anomalous thrombin digestion of the mutant PP2C β -1s.

Determination of the enzyme activity of each mutant protein demonstrated that deletion of 11 amino acids from the N-terminal end (Δ N(1–11)) caused 97 % loss of enzyme activity and that further deletions (Δ N(1–19) and Δ N(1–26)) from this end caused total loss of activity (Figure 2C).

On the other hand, deletion of the 12 amino acids from the C-terminal end [Δ C(379–390)], which are unique to PP2C β -1, generated a molecular species whose enzyme activity level was about 65 % of that of the wild-type enzyme when [32 P]-phosphohistone was used as the substrate (Figure 2C). The enzyme kinetic analysis revealed that the K_m for [32 P]-phosphohistone was substantially increased by the deletion of the C-terminal 12 amino acids (results not shown). However, the

enzyme activity was not affected by this deletion when [32 P]-phosphocasein was employed as the substrate. Although deletion of 78 amino acids from the C-terminal end [Δ C(313–390)] did not cause any further change in activity, deletion of 100 amino acids [Δ C(291–390)] caused total loss of catalytic activity. The K_a values of wild-type PP2C β -1 and the deletion mutant Δ C(379–390) for MgCl $_2$ were 4.0 and 2.5 mM, respectively, when [32 P]phosphohistone was used as the substrate (results not shown).

Amino acid substitution analysis of PP2C β -1

Since the results obtained from the analysis of PP2C β -1 deletion mutants strongly suggested that a region containing about 310 amino acids at the N-terminal end comprised the catalytic domain and that the C-terminal domain of the molecule was not responsible for basal enzyme activity, we prepared recombinant PP2C β -1 molecules with various point mutations in the putative catalytic domain to determine the amino acids essential for enzyme activity. Alignment of the amino acid sequences of PP2Cs from various organisms showed that more than 30 amino acids were conserved in the putative catalytic domain of these organisms, whereas no amino acid was conserved in the region downstream of this domain (Figure 3A) [26]. Of the conserved amino acids, 16 (seven acidic, seven basic, one hydrophobic and one aromatic) were selected, and each was substituted by another amino acid with properties different from the original one. The

choices of mutations were basically made so that the charged or aromatic amino acids were replaced by hydrophobic or neutral ones and the hydrophobic amino acid was replaced by a charged one. Each point-mutated PP2C β -1 protein was expressed in *E. coli* cells as a GST-fusion protein, and the mutant GST-PP2C β -1 protein was purified. As judged by SDS/PAGE, all the mutant proteins as well as wild-type PP2C β -1 were close to homogeneity, with major bands at about 46 kDa (results not shown).

Most of the mutants retained substantial levels of enzyme activity, although the levels were generally decreased in comparison with that of the wild-type PP2C β -1 (Figure 3B). However, the mutants Asp-38-Ala, His-62-Leu, Arg-179-Gly and Arg-200-Gly showed only 0.1–2.2 % of the wild-type activity, and no activity was detected in mutants Asp-60-Ala and Asp-243-Ala. The activities of all the mutants except Asp-60-Ala and Asp-243-Ala were dependent on Mg²⁺ and insensitive to okadaic acid. Essentially the same results were obtained when [³²P]phosphocasein was used as the substrate (results not shown).

Das et al. [20] have recently reported the results of an X-ray crystallographic analysis of human PP2C α protein. They identified five amino acids (Glu-37, Asp-38, Asp-60, Asp-239 and Asp-282) that hold metal ions within the PP2C α molecule. All these amino acids were also conserved in the mouse PP2C β -1 molecule, although Asp-239 and Asp-282 in PP2C α corresponded to Asp-243 and Asp-286 in PP2C β respectively. Four of these five amino acids (Glu-37, Asp-38, Asp-60 and Asp-243) were included in our list of point mutants, and the mutants Asp-38-Ala, Asp-60-Ala and Asp-243-Ala showed little or no enzyme activity (Figure 3B). Since the mutant Glu-37-Ala showed a substantial level of activity, albeit much lower than that of wild-type PP2C β -1, we tested whether this point mutation affected the enzyme-kinetic parameters when [³²P]phosphohistone was used as the substrate. The $V_{\max}$ value of mutant Glu-37-Ala was 22.0 % of that of wild-type PP2C β -1 (results not shown). Of interest was that the K_m of this mutant for [³²P]phosphohistone was about half that of wild-type PP2C β -1 and that its K_a for MgCl₂ was at least twice as high as that of wild-type PP2C β -1 (results not shown).

Subcellular distribution of epitope-tagged PP2C β -1 and its deletion mutant expressed in COS7 cells

It has recently been reported that epitope-tagged PP2C β -1 and wild-type PP2C β -1 are expressed in both the cytosol and nucleus of COS7 and BHK cells [27]. It has also been suggested that the basic-amino-acid-rich domain conserved in the C-terminal regions of the six distinct isoforms of PP2C might act as a nuclear localization signal (NLS) (see the Discussion section). Therefore, we tested whether deletion mutants lacking the putative NLS were excluded from the nucleus when expressed in mammalian cells.

We constructed expression vectors of epitope (HA)-tagged PP2C β -1 and its C-terminal deletion mutant Δ C(313–390) for COS7 cells. Immunoblot analysis of COS7 cell extracts harbouring the expression plasmid for HA-tagged wild-type PP2C β -1 and that of its deletion mutant using anti-HA antibody showed that recombinant proteins of the expected sizes were expressed in the cells (Figure 4A). The immunoprecipitates from both cell extracts transfected with the expression plasmid for HA-tagged wild-type PP2C β -1 and that of its deletion mutant Δ C(313–390) showed Mg²⁺-dependent and insensitive-to-okadaic-acid activities (results not shown). Immunostaining of the transfected COS7 cells with anti-HA antibody demonstrated that there was little difference in subcellular distribution between HA-tagged

wild-type PP2C β -1 and Δ C(313–390) and that they were localized mainly in cell nucleus (Figure 4B).

DISCUSSION

In a previous study we demonstrated using artificial substrates that the substrate specificities of PP2C β -3 and -5 were different from that of PP2C β -4 [19]. A more detailed analysis was performed in the present study using all five distinct PP2C β isoforms, and the activity ratios (histone phosphatase activity/casein phosphatase activity) of PP2C β -1, -3 and -5 (4.1–4.5) were found to be substantially higher than those of PP2C β -2 and -4 (1.6–2.6) (Figure 1). This difference resulted from the higher affinities of PP2C β -1, -3 and -5 for [³²P]phosphohistone (Table 2). Of interest is the observation that the affinity of PP2C β isoforms for [³²P]phosphohistone is reciprocal to the efficacy of MgCl₂ in stimulating the various PP2C β isoforms. Thus the group with lower affinity for [³²P]phosphohistone (PP2C β -2 and -4) shows lower K_a values for MgCl₂ than the higher-affinity group (PP2C β -1, -3 and -5). In addition, the group with higher affinity for [³²P]phosphohistone contain a cluster of four hydrophobic amino acids at their C-terminal ends, whereas the lower-affinity group does not [17]. These findings suggest that a hydrophobic C-terminal region confers higher affinity for [³²P]phosphohistone and that the formation of this enzyme–substrate complex causes a lower affinity of the enzyme for MgCl₂.

It is known that the cellular Mg²⁺ level is not stable but changes dynamically, depending on alterations in physiological conditions. It has been reported that in primary cultures of rat brain cells the cellular Mg²⁺ concentration varied from 0.63 to 11 mM following stimulation of the cells with glutamic acid [28]. Alterations in cellular Mg²⁺ levels depending on the culture conditions have also been reported in chicken cardiac cells [29]. Therefore, it may be reasonable to speculate that a physiological alteration in cellular Mg²⁺ levels is also a factor that defines the substrate specificity of PP2C β isoforms.

The deletion of 12 amino acids from the C-terminal end of PP2C β -1 caused a lower activity against [³²P]phosphohistone, whereas that against [³²P]phosphocasein was not changed by this deletion (Figure 2C). In addition, the K_a value for MgCl₂ was lowered by this deletion only when [³²P]phosphohistone was used as the substrate for the enzyme assay. As a result, the 12-amino-acid-deleted molecule showed enzyme properties similar to those of PP2C β -2 and -4. These lines of evidence further support the speculation that the hydrophobic amino acid clusters observed in PP2C β -1, -3 and -5 are related to the higher activity for [³²P]phosphohistone and the higher K_a for MgCl₂.

The mutant molecule, Δ C(313–390), showed the same enzymic properties as those of the Δ C(379–390) mutant (Figure 2C). Therefore, the region Asp-313–Gly-378 of the C-terminal portion of PP2C β -1 may have function(s) other than the regulation of substrate specificity. One such possibility is regulation of the subcellular localization of PP2C β -1. It has been suggested that PP2C α is a nuclear protein and that the basic-amino-acid-rich region of PP2C α immediately upstream of the putative phosphorylation sites by casein kinase II might be the NLS of the molecule [30]. Subsequently, phosphorylation of the C-terminal region (Ser-375 and Ser-377) of PP2C α by casein kinase II has been demonstrated [31]. The basic-amino-acid-rich region in the C-terminal region is also conserved in all PP2C β isoforms (Lys-359–Lys-374), although the phosphorylation sites by casein kinase II are not conserved in these isoforms [16,17]. In addition, it has recently been reported by Wenk and Mieskes [27] that wild-type PP2C β -1 and Myc-tagged PP2C β -1 are expressed in

both the cytoplasm and nucleus of COS7 and BHK cells. They further demonstrated, using a stepwise permeabilization technique, that PP2C β -1 is not simply attached to the outer membrane of the nucleus but is located inside it. In the present study we have demonstrated that the HA-tagged PP2C β -1 mutant Δ C(313–390), which lacks the putative NLS, is localized mainly in cell nucleus when expressed in COS7 cells, as is observed with the undeleted HA-tagged PP2C β -1 (Figure 4B). Therefore, it is unlikely that the region Asp-313–Gly-378 is related to the nuclear localization of the molecule.

Study of the deletion mutants suggests that the region from the N-terminal end to Leu-312 comprises the catalytic domain of the PP2C β -1 molecule. This region contains all the amino acids conserved in the PP2C proteins of various organisms so far reported [26]. Analysis of point mutants for the 16 conserved amino acids demonstrated that the amino acids required for full enzyme activity are distributed over a wide range from Arg-33 to Asp-247 (Figure 3B). These observations are in accordance with the speculation that the 4/5 region from the N-terminus of PP2C β -1 comprises the catalytic domain.

Das et al. [20] have recently reported the molecular architecture of PP2C α based on crystallographic analysis. They revealed that two Mn²⁺ ions are co-ordinated by four aspartate residues (Asp-38, -60, -239 and -282) and a glutamate residue (Glu-37) at the catalytic site. These amino acids are also conserved in PP2C β . It has been proposed that dephosphorylation is catalysed by metal-activated water molecules that act as nucleophiles to attack the phosphoester bond. We substituted Glu-37, Asp-38, Asp-60 and Asp-243 (corresponding to Asp-239 of PP2C α) with alanine in the amino acid substitution analysis and found that enzyme activity was completely abolished in mutants Asp-60-Ala and Asp-243-Ala, and was decreased by 67 and 97% compared with the wild type in mutants Glu-37-Ala and Asp-38-Ala respectively (Figure 3B). The K_m for MgCl₂ of Glu-37-Ala was at least twice that of wild-type PP2C β -1. These results indicate that metal ions participate in catalysis.

The substitution of His-62, His-169, Arg-179 or Arg-200 with another amino acid also resulted in near complete loss (92.0–99.9%) of enzyme activity. The molecular architecture of PP2C α has revealed that a catalytic site channel is created with β -strands 1, 2, 3, 11 and 10 on one side and β -strands 4–9 on the other side [20]. In addition, one side of the channel is dominated by a 60-residue segment that connects strands β 8 and β 9, fold α 3 and an extensive loop region possessing no secondary structure. Interestingly, His-164, Arg-174 and Arg-195 (corresponding to His-169, Arg-179 and Arg-200 of PP2C β respectively) are in this 60-residue segment. Therefore, the results obtained by the analysis of the point mutants suggest that the proper structure of the 60-residue segment is also essential for PP2C to operate its catalytic activity.

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