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Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle

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Abstract The cDNA encoding a phosphorylation-dependent inhibitory protein of protein phosphatase-1 (PP1) was isolated from a porcine aorta library. The coding region represented the complete amino acid sequence of this protein comprised of a novel 147-residue polypeptide, which we termed CPI17, a 17-kDa PKC-potentiated inhibitory protein of PP1. As well as the native CPI17 from porcine aorta, the recombinant protein completely suppressed the PP1 activity (IC_{50} = 0.18 nM) by the stoichiometric thiophosphorylation. The CPI17 mRNA is expressed in smooth muscle tissues such as aorta and bladder, whereas little expression was observed in heart, skeletal muscle, and non-muscle tissues. These results suggest a specific regulatory mechanism of the PP1 activity through CPI17 in smooth muscle.

Key words: Protein phosphatase; Inhibitory protein; Protein kinase C; Smooth muscle

1. Introduction

The Ser/Thr phosphatase of type-1 (PP1) controls many cellular functions including muscle contraction, cell division, transcription and metabolisms by dephosphorylating various target proteins [1]. PP1 molecules consist of a 38-kDa catalytic subunit (PP1c) and various kinds of the non-catalytic regulatory subunits, in vivo. These regulatory subunits modulate the substrate specificity of PP1c and localize PP1 molecules to some targets in cells [2].

PP1 activity is also regulated by modulator proteins, and some of them have been cloned from mammalian tissues; these include inhibitor-1, inhibitor-2, DARPP32, NIPP-1, and the homolog of yeast sds22 [3–7]. Inhibitor-1 and DARPP32 are well characterized as phosphorylation-dependent inhibitory proteins. On the basis of an immunological study, inhibitor-1 was shown to be distributed in various tissues [8] and regulates metabolisms responding to hormonal signals [1]. On the other hand, DARPP32 was specifically seen in brain, suggesting to be involved in the dopamine-mediated signal transduction in nerve systems [9,10]. Both inhibitors were potentiated by the PKA[1] phosphorylation [1]. However, the inhibitory activity was blocked by the regulatory subunits bound to PP1c [11–13].

In our earlier work, we have isolated the inhibitory protein for PP1 from porcine aorta smooth muscle [14]. This inhibitory protein was potentiated by the PKC phosphorylation. It is noteworthy that the phosphorylated inhibitory protein could suppress the activity of the PP1 holoenzyme comprised of a non-catalytic 69-kDa subunit as well as that of PP1c. With regard to the potentiating kinase and the inhibitory activity to the holoenzyme, the inhibitory protein from aortic smooth muscle was clearly distinct from the well-known inhibitors[2]. Now we have cloned and characterized the cDNA for this inhibitory protein from porcine aorta smooth muscle. The isolated cDNA encodes a novel 16.7-kDa protein, compared with any known modulators of PP1. Therefore, we have termed it CPI17, a PKC-potentiated PP1 inhibitory protein of 17 kDa. The expression of CPI17 mRNA was specific to smooth muscle tissues. These findings indicate a characteristic regulatory system for PP1 activity in smooth muscle cell.

2. Materials and methods

2.1. Preparation of RNA and cDNA library of porcine aorta smooth muscle

Total cellular RNA of porcine aorta smooth muscle was prepared using the guanidinium thiocyanate-acid phenol procedure [15]. Polyadenylated (poly(A)$^+$) RNA was fractionated by oligo(dT) cellulose (NEB) chromatography. An oligo(dT)-primed cDNA library in λEx-Cell vector was prepared from poly(A)$^-$ RNA using the TimeSaver cDNA synthesis kit with Directional cloning toolbox (Pharmacia), and packaged using Gigapack II packaging extracts (Stratagene), as described by the manufacturers.

2.2. Isolation of a porcine aorta CPI17 cDNA clone

The specific cDNA fragment of CPI17 of porcine aorta was sequentially amplified by PCR method using Ex-taq polymerase (Takara). Nucleotide sequences of PCR products were determined by the dideoxy procedure using a Hitachi SQ5500 DNA-sequencer after subcloning into pCRII vector using the Original TA cloning kit (Invitrogene). The degenerate primers A, B, and C were designed from the available partial amino acid sequence of CPI17 [14], whose nucleotide sequences were 5′-TGGAT(T/C/A)GA(T/C)GGI(C/A)GI(T/C)TIGA(A/G)GA-3′, 5′-GA(A/G)GCIGA(T/C)ATGCCIGA(T/C)GA-3′, and 5′-(T/C)TTIA(G/A)IA(G/A)ICC(T/C)TG(A/G/T)AT(T/C)TT-3′, respectively. At the first step, PCR was performed with primer A and C. The oligo(dT)-primed single-strand cDNA was used as a template, which was synthesized from total RNA of porcine aorta using the cDNA cycle kit (Invitrogene). The second PCR was carried out with the primer B and C using the first PCR product as a template. The nucleotide sequence of the 93-bp product from the second PCR was identical to those expected from the amino acid sequences. To determine the 5′-terminal nucleotide sequence of CPI17, the rapid amplification of cDNA 5′-end method was performed using Marathon cDNA amplification kit (Clontech), with the CPI17-specific primer indicated by a dotted line in Fig. 1. The oligo(dT)-primed double-

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Abbreviations: PP1, type-1 Ser/Thr phosphatase; PP1c, catalytic subunit of PP1; PP2A, type-2A Ser/Thr phosphatase; PP2Ac, catalytic subunit of PP2A; CaN, Ca^{2-}/calmodulin-dependent Ser/Thr phosphatase; PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}/phospholipid-dependent protein kinase; CPI17, PKC-potentiated inhibitory protein of PP1, M_r = 17 kDa; PCR, polymerase chain reaction

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strand cDNA was used as a template. A 0.3-kbp product was identified to contain the 5′-terminal region of the cDNA encoding CPI17. In order to obtain the full-length cDNA, the porcine aorta cDNA library constructed in the λExCell vector was screened using this 0.3-kbp fragment as a probe after ^{32}P-labeling. Ten positive clones in 280000 plaque forming units (pfu) of the library were identified, and each of them was isolated through the secondary and tertiary screenings. The phagemid (pExCell) containing the insert encoding CPI17 was excised in vivo from the phage DNA according to the instruction provided by the manufacturer.

2.3. Bacterial expression and purification of hexahistidine-tagged CPI17 (HtCPI17)

The DNA fragment corresponding to the coding region of CPI17 gene was amplified by PCR using the mutagenic primers, 5′-AGGACCGccatggCAGCTCAGCGGCTGGGCAAGC-3′, and 5′-CGGGgtcgacGTCAGGGCGGCGCGGTCCGGGCGC-3′, in which the initiation and termination codons were indicated by underlines, and nucleotide sequence giving restriction sites for *Nco*I and *Sal*I were shown by small letters. The isolated pExCell vector containing the full-length CPI17 cDNA was used as a template. After confirming the nucleotide sequence of the PCR product subcloned into pCRII, the 0.45-kbp *Nco*I–*Sal*I fragment was cloned into *Nco*I/*Sal*I site of pET30 plasmid (Novagene). The constructed plasmid, named pHtCPI17, encodes the 44-residues of tag sequence, containing a $(His)_6$-segment at N-terminus, followed by the complete amino acid sequence (147 residues) of CPI17. *E. coli* strain BL21(DE3) was transformed with pHtCPI17 and grown overnight at 37°C in 5 ml of LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl/l, pH 7.0) containing 30 μg/ml kanamycin. The culture was inoculated to 1 l of the same medium. Transformed cells were grown to the absorbance at 600 nm of 0.6 at 37°C, and the expression of HtCPI17 was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside into the culture for 3 h at 37°C. The bacteria were collected by centrifugation at 4500×*g* for 10 min, and the cell pellet was washed 3 times with 100 ml of 0.9% NaCl, 25 mM Tris-HCl, pH 7.5. The pellet was suspended on ice with 50 ml of buffer A (0.5 M NaCl, 20 mM potassium phosphate, pH 7.0, 1 mM benzamidine) containing 10 mM imidazole-HCl, pH 7.0, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by twice passage through a French press. The lysate was heated at 90°C for 5 min. After cooling on ice, the heat-treated lysate was mixed with 1:1000 volume of 0.1 M diisopropyl fluorophosphate, and then clarified by centrifugation at 22000×*g* for 20 min. HtCPI17 in the lysate was adsorbed to Ni^{2+}-bound Chelating-Sepharose fast flow (Pharmacia) column (φ 1.5×5.7 cm), at 4°C. The column was successively washed with buffer A containing 10 mM and 0.1 M imidazole-HCl, pH 7.0. The bound proteins were eluted by buffer A with 0.5 M imidazole-HCl, pH 7.0. HtCPI17 was further purified by the gel filtration using a Sephacryl S-200 HR column (φ 2.7×60 cm) equilibrated with buffer B (0.5 M NaCl, 0.5 mM EDTA, 0.1% 2-mercaptoethanol (2-ME), 10 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS)-NaOH, pH 7.0). After detection of proteins in each fraction by SDS-PAGE, the fractions containing the homogeneous HtCPI17 were pooled, and dialyzed against 0.4% ammonium bicarbonate, 0.1% 2-ME. The dialysate of purified HtCPI17 was lyophilized and stored at −20°C. More than 30 mg of HtCPI17 was routinely purified to homogeneity from 1 l of the cell culture (data not shown).

HtCPI17 was thiophosphorylated with 0.08 U/ml PKC in 50 mM MOPS–NaOH, pH 7.0, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM $CaCl_2$, 20 μg/ml phosphatidylserine, 2 μg/ml 1,2-diolein, 1 mM ATPγS at 30°C for 60 min. One unit of PKC was defined as 1 nmol of phosphorylation per minute. The reaction was terminated by heating at 70°C for 20 min. After centrifugation, the tp-HtCPI17 was purified by MonoS 5/5 column chromatography [14]. The stoichiometric thiophosphorylation was confirmed by polyacrylamide gel electrophoresis in the presence of 8 M urea.

2.4. Northern hybridization

The formamide-denatured total cellular RNA samples (10 μg each) were separated by electrophoresis on 1.2% formaldehyde–agarose gel and transferred to a nylon membrane (Gene Screen; NEN) in 10×SSC (1×SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0). Filters were prehybridized for 2 h at 42°C in 30% formamide, 5×SSC, 5×Denhardt's solution [15], 1% SDS, 0.1 mg/ml salmon sperm DNA, 0.05% sodium pyrophosphate, before hybridizing at 42°C overnight with ^{32}P-labeled probe (4.1×10^8 cpm/μg) in the same solution. The ^{32}P-labeled probe was prepared using the random primer DNA labeling kit (Takara) in the presence of [α-^{32}P]CTP with the 0.45-kbp *Eco*RI fragment of pCR-CPI17. After washing at 20°C for 5 min with 2×SSC, 0.1% SDS, 0.05% sodium pyrophosphate for 4 times, the blot was washed 4 times each for 30 min: twice at 42°C and then twice at 53°C, in 0.2×SSC, 0.1% SDS, 0.05% sodium pyrophosphate. The blot was exposed to an imaging plate (Type BAS-III; Fujix), and the hybridized bands were visualized by BAS-2000 (Fujix).

2.5. Others

CPI17 of porcine aorta smooth muscle was isolated as described previously [14], and thiophosphorylated as described above. The catalytic subunits of PP1 (PP1c) and PP2A (PP2Ac) were purified from rabbit skeletal muscle as described by Cohen et al. [16], or Martin et al. [17]. Calcineurin (CaN; PP2B) was extracted from porcine brain and purified by DEAE-Cellulofine A500m column (Seikagaku Co.) and calmodulin (CaM)-conjugated Sepharose column chromatography [18]. 20-kDa subunit of smooth muscle myosin (LC20) was prepared from frozen chicken gizzard and phosphorylated in the presence of 0.1 mM [γ-^{32}P]ATP as described by Yoshida and Yagi [19]. Porcine brain PKC and chicken gizzard CaM were purified according to the method described by Kikkawa et al. [20] and Yoshida et al. [21], respectively. Bovine serum albumin (BSA) was purchased from Sigma. The protein phosphatase activity was measured by determining the released ^{32}Pi from ^{32}P-labeled p-LC20 (2 μM). Assays for PP1c and PP2Ac were performed at 25°C in 50 mM MOPS–NaOH, pH 7.0, containing 0.1 mM EGTA 1 mg/ml BSA, 1 mM dithiothreitol, 1 mM benzamidine, 0.02% Brij-35. For CaN activity, 0.2 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 μM CaM was added to the condition described above. The reaction was initiated by the addition of phosphatases. Concentrations of phosphatases were given in the figure legend. One unit of phosphatase (U) was defined as 1.0 μmol of dephosphorylation per minute. Protein concentration was determined by the improved method of Bradford [22,23] using BSA as standard. The values of molecular mass for CPI17 and HtCPI17 were 16.7 and 21.5 kDa, respectively. Polyacrylamide gel electrophoresis in the presence of urea was performed using the gel containing 8 M urea, 9% acrylamide, 0.3% *N*,*N*′-methylene bisacrylamide, 80 mM 2,6-lutidine, and 80 mM *N*-((trishydroxylmethyl)methyl)-2-aminoethanesulfonic acid (TES). Electrophoresis was from anode to cathode in the running buffer (40 mM 2,6-lutidine, 40 mM TES). Unphosphorylated and (thio)phosphorylated (Ht)CPI17s were completely separated by this system (data not shown).

3. Results

3.1. cDNA clones of CPI17 from porcine aorta smooth muscle

Ten positive clones were isolated from the cDNA library of porcine aorta smooth muscle using the 278-bp nucleotide fragment produced by the 5′-RACE PCR as a probe (Section 2.2.). One of the clones termed pai8 had the longest insert consisting of 637 bp. The 586-bp nucleotide sequence of pai8 excluding a 51-bp polyadenylated sequence at a 3′-end is shown in Fig. 1. Sequences from other clones were identical to those of pai8 at corresponding regions (data not shown). The homology search for this sequence in GenBank and EMBL databases did not detect significant similarities to any characterized genes. However, the search found out near identical segments (>85% identical) in the expressed sequence tags (EST) database [24] from human fetal heart, lung, brain and uterus, and senescent fibroblast, and from mouse placenta and embryo.

The sequence around the first ATG from 5′-end is conformable to the initiation signal for translation in vertebrates as described by Kozak [25]. As shown in Fig. 1, the amino acid sequence of 147 residues was deduced from the first ATG (23–25) to TGA (464–466), with a calculated molecular mass of 16672 Da. All of amino acid sequences determined from pep-

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      CCGGGGCCGGGCCAGGACCGCGATGGCAGCTCAGCGGCTGGGCAAGCGGGTGCTGAGCAA   60
1                          m  a  a  q  r  l  g  k  R  V  L  S  K

      GCTGCAGTCTCCGTCGCGGGCCCGGGGGCCGGGGGGCAGCCCCGGGGGGCTGCAGAAGCG  120
14     L  Q  S  P  S  R  A  R  G  P  G  G  S  P  G  G  L  Q  K  R

      ACACGCGCGTGTCACCGTCAAGTACGATCGGCGGGAGCTGCAGCGGCGGCTGGACGTGGA  180
34     H  A  R  V  T  V  K  Y  D  R  R  E  L  Q  R  R  L  D  V  E
                  ==

      GAAGTGGATCGACGGGCGCTTGGAGGAGCTGTACCGTGGCAGGGAGGCAGACATGCCCGA  240
54     K  W  I  D  G  R  L  E  E  L  Y  R  G  R  E  A  D  M  P  D

      TGAAGTCAACATCGATGAACTGCTGGAATTAGAGAGTGAAGAGGAGAGAAGCCGGAAAAT  300
74     E  V  N  I  D  E  L  L  E  L  E  S  E  E  E  R  S  R  K  I

      TCAGGGACTCCTAAAGTCATGCACGAACCCCACAGAGAACTTTGTCCAGGAGCTACTGGT  360
94     Q  G  L  L  K  S  C  T  N  P  T  E  N  F  V  Q  E  L  L  V

      GAAGCTGCGAGGCCTCCACAAGCAGCCGGGCCTCCGCCAGCCCAGCCCCTCCGGCGACGG  420
114    K  L  R  G  L  H  K  Q  P  G  L  R  Q  P  S  P  S  G  D  G

      CAGCCTGAGTCCCCGCCAGGACCGCGCCCGGACCGCGCCGCCCTGACGCATGCCCCGGCC  480
134    S  L  S  P  R  Q  D  R  A  R  T  A  P  P

      TCTGTGACCCCCCTCCGCCGCCCCTGCTTCCCCCCGGCCCTCAACACGGCCCGGCTTGTT  540
      TATACGTTGTATTTAATGGTTCTGTAACAATAACTGCGTGCCTGTT                586
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Fig. 1. Complementary DNA and predicted protein sequence of the PKC-potentiated PP1 inhibitory protein of porcine aorta smooth muscle. The left and right numbers indicate the position of amino acid and nucleotide, respectively. The dotted line indicates the position of an antisense primer used for RACE PCR. The amino acid sequence determined from protein sequencing are indicated by capital letters. The *underline* marks the TGA stop codon. The threonine at position 38 is the phosphorylation site, and indicated by a double underline [14].

tide fragments of CPI17 protein were identical to the sequence deduced from cDNA, and indicated by capital letters. The residue at position 38 is a phosphorylatable threonine necessary to the potentiation of CPI17 [14]. CPI17 is basic (theoretical $pI = 10.34$), so the molecular mass of CPI17 has been overestimated by SDS-PAGE as 20 kDa [14]. The homology search using FASTA program against non-redundant protein sequence database libraries found out a polypeptide which has been termed phospholipase C neighboring gene (PNG) product [26]. The PNG product was 44.9% identical to CPI17 at positions 7–120, when were permitted four insertions (data not shown). The amino acid sequence of CPI17 was distinct from those of other proteins in databases including any known modulator proteins and subunits of PP1.

3.2. Characterization of gene product from CPI17 cDNA

In order to determine whether the isolated gene product acts as an inhibitor of PP1, we expressed the recombinant CPI17 as a fusion protein (HtCPI17) as described in Section 2.3. HtCPI17 was phosphorylated or thiophosphorylated by PKC stoichiometrically, similarly to the native CPI17 from porcine aorta [14].

The effect of HtCPI17 on phosphatase activities was shown in Fig. 2. The PP1c activity was inhibited by the thiophosphorylated HtCPI17 as indicated by closed circles with an IC_{50} of 0.18 nM. The inhibition reached to the maximum at 10 nM tp-HtCPI17, where the PP1c activity was completely suppressed. The inhibitory activity of tp-HtCPI17 was indistinguishable from that of tp-CPI17 as indicated by crosses. With high concentration of CPI17, which could not be analyzed in earlier work [14], it appeared that unphosphorylated HtCPI17 (u-HtCPI17) inhibited PP1c activity with IC_{50} of 1.3 μM. The tp-HtCPI17 suppressed PP2Ac activity to 60% of the maximum at 1.8 μM, but was not effective to suppress CaN activity. Any decreases in activities of PP2Ac and CaN were not observed in the presence of 4.6 μM u-HtCPI17 (data not shown).

3.3. Expression of CPI17 mRNA

As shown in Fig. 3, the northern hybridization analysis revealed a 0.7-kb CPI17 mRNA in 10 μg of total cellular RNA of porcine aorta and bladder smooth muscle. The size was consistent to that of the cDNA clone pai8 (Fig. 1). A weak signal of 0.7 kb was also detected in the total RNA fractions of brain. In skeletal and cardiac muscle, and liver, any specific signals were scarcely found (Fig. 3). A 0.7-kb mRNA of CPI17 was also present in the extracts from rabbit

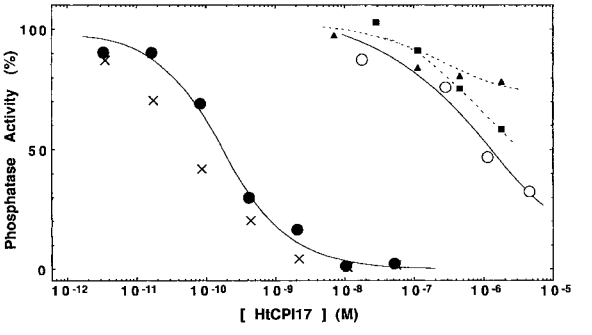

Fig. 2. Inhibition of protein phosphatases by the recombinant CPI17. PP1c (●,○,×), PP2Ac (■), and CaN (▲) were added in the assay mixtures to 86, 74 and 126 μU/ml, respectively. The results from thiophosphorylated and unphosphorylated HtCPI17 were indicated by closed and open symbols, respectively. The cross indicated the result of thiophosphorylated native CPI17. Other conditions were described in Section 2.

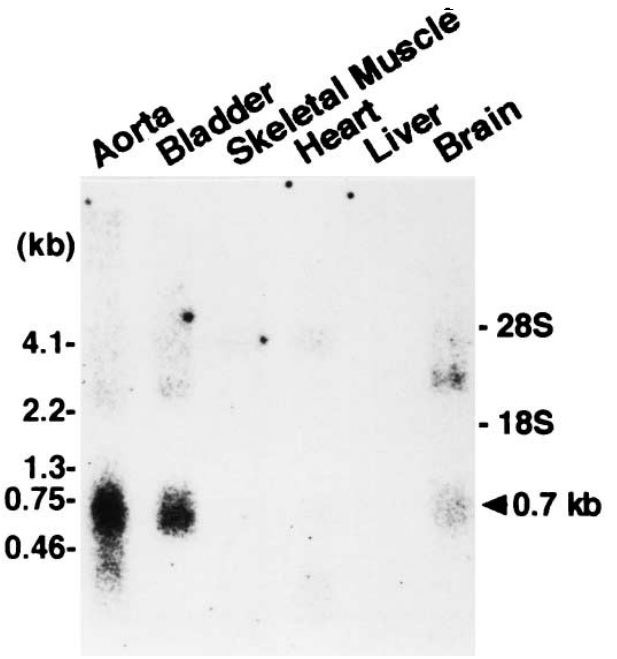

Fig. 3. Northern hybridization analysis of various tissues from pig. Total cellular RNA (10 μg) was loaded on 1.2% formaldehyde–agarose gels. The size indicated at the left side was determined using an RNA size marker (NEB). The blot was hybridized with ^{32}P-labeled cDNA corresponding to CPI17 coding region.

aorta and brain, but any positive bands could not be detected from rabbit skeletal and cardiac muscle, and kidney (data not shown).

4. Discussion

We have previously purified a phosphorylation-dependent inhibitory protein of PP1 (CPI17) with molecular mass of 20 kDa from porcine aorta smooth muscle [14]. On the basis of the available partial amino acid sequence of the protein, we isolated here a cDNA clone encoding a 16.7-kDa polypeptide from a porcine aorta cDNA library. The amino acid sequence deduced from this clone was consistent with those of peptide fragments derived from CPI17 protein, indicating that the gene product of this clone is CPI17.

The thiophosphorylated recombinant CPI17 fused with the tag sequence suppressed the PP1c activity completely. PP2Ac and CaN activities were not significantly inhibited by the recombinant protein. Therefore, CPI17 is defined as an inhibitory protein specific for PP1. The complete amino acid sequence of CPI17 is novel compared with those of any known modulator proteins and subunits of PP1. As shown in our earlier work, phosphorylated CPI17 could inhibit the holoenzyme containing non-catalytic subunit as well as PP1c, completely and rapidly [14]. It had been shown that the well-known inhibitory proteins of PP1, inhibitor-1 and inhibitor-2 had less effect on PP1 holoenzymes [11–13]. It is expected that the primary structure of CPI17 responsible for binding to PP1 molecule is distinguishable from those of other inhibitory proteins.

With use of the recombinant CPI17 protein, the unphosphorylated CPI17 also inhibited the PP1c activity with IC_{50} of 1.3 μM, a value over 7000-fold higher than that of the thiophosphorylated form. The result suggests CPI17 is a potent inhibitor for PP1 even in the unphosphorylated form. The phosphorylation of the threonine at position 38 of CPI17 increases the affinity for PP1. It has been previously reported that DARPP32, which is the well-known phosphorylation-dependent inhibitor, also could suppress the PP1c activity, in the unphosphorylated form [27]. The phosphorylation of DARPP32 induced about 2000-fold decrease in the IC_{50} for PP1c inhibition. On the basis of analysis using analog peptides, residues 9-14 of DARPP32 were defined as the inhibitory domain [28]. Fig. 4 shows the N-terminal region of DARPP32 and inhibitor-1, containing two sites critical for the inhibition. The residues 8–14 (indicated by the box line) and residues around phosphorylation site (Thr-34, indicated by the black box) of DARPP32 are highly conserved in the corresponding region of inhibitor-1. The sequence of CPI17 is distinct from these inhibitors even in the inhibitory domain (Fig. 4). However, in the alignment of the two sequences at Thr-38 of CPI17 and Thr-34 of DARPP32, three residues, KIQ, in the inhibitory domain of DARPP32 are homologous to corresponding three residues, KLQ, of CPI17. It is possible that the KLQ sequence shares a part of the inhibitory domain of CPI17. Although the C-terminal residues adjacent to KIQ of DARPP32 are hydrophobic, the corresponding residues of CPI17 are not. Such variant residues in CPI17 may give its conspicuous character as the potent inhibitor against PP1 holoenzyme.

Molecular cloning of CPI17 also confirmed that the expression of CPI17 mRNA is specific to smooth muscle tissues such as aorta and bladder. The specific PP1 holoenzyme (smPP1M) exists to control the extent of phosphorylation of myosin in smooth muscle [13,29]. While the 110-kDa subunit of smPP1M is widely expressed [30], smPP1M is a candidate for the target of CPI17 in smooth muscle. The present data clearly indicated the novel mechanism for PP1 regulation through CPI17 in smooth muscle cells. This finding will give a clue to understand the signal transduction involving the specific PP1 for smooth muscle functions.

In contrast to the northern analysis, some homologous cDNAs to CPI17 were found out in EST database of non-muscle tissues. These genes possibly encode non-muscle homologs of CPI17. Another homolog detected in protein databases, which has been characterized as a phospholipase C neighboring gene (PNG) product. The PNG was shown to be widely distributed and the function of this gene product

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CPI17          MAAQRLGKRVLSKLQSPSRARGPGGSPGGL-QKRHARVTVKYDRREL
                        * *       *     * *  *
DARPP32          MDPKDRKKIQFSVPAPPSQLDPRQVEMIRRRRPTPAMLFRLS
Inhibitor-1    MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTS
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Fig. 4. Comparison of the amino acid sequence of CPI17 to the N-terminal regions of DARPP32 and inhibitor-1. Identities are marked by *asterisks*, and an insertion was indicated by a *dash*. The amino acid sequences of bovine brain DARPP32 and rat skeletal muscle inhibitor-1 are from [5] and [3], respectively. The inhibitory domain [28] for DARPP32 and the corresponding region of inhibitor-1 is indicated by a *box line*. The phosphorylation residue required for the potentiation is indicated by a *black* box.

remains unknown [26]. It is interesting that the conserved region of PNG to CPI17 involves the threonine corresponding to Thr-38 of CPI17. Thus, we expect that the PKC-potentiated inhibitory proteins like CPI17 may control the PP1-mediated cellular events in various tissues.

References

[1] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[2] Hubbard, M.J. and Cohen, P. (1993) Trends Biochem. Sci. 18, 172–177.
[3] Elbrecht, A., DiRenzo, J., Smith, R.G. and Shenolikar, S. (1990) J. Biol. Chem. 265, 13415–13418.
[4] Park, I.-K., Roach, P., Bondor, J., Fox, S.P. and DePaoli-Roach, A.A. (1994) J. Biol. Chem. 269, 944–954.
[5] Kurihara, T., Lewis, R.M., Eisler, J. and Greengard, P. (1988) J. Neurosci. 8, 508–517.
[6] Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W. and Bollen, M. (1995) J. Biol. Chem. 270, 28068–28074.
[7] Renouf, S., Beullens, M., Wera, S., Van Eynde, A., Sikela, J., Stalmans, W. and Bollen, M. (1995) FEBS Lett. 375, 75–78.
[8] MacDougall, L.K., Campbell, D.G., Hubbard, M.J. and Cohen, P. (1989) Biochim. Biophys. Acta 1010, 218–226.
[9] Hemmings Jr., H.C., Girault, J.-A., Nairn, A.C., Bertuzzi, G. and Greengard, P. (1992) J. Neurochem. 59, 1053–1061.
[10] Hemmings Jr., H.C. and Nairn, A.C. (1991) Adv. Prot. Phosphatases 6, 525–537.
[11] Stralfors, P., Hiraga, A. and Cohen, P. (1985) Eur. J. Biochem. 149, 295–303.
[12] Dent, P., Macdougall, L.K., Mackintosh, C., Campbell, D.G. and Cohen, P. (1992) Eur. J. Biochem. 210, 1037–1044.
[13] Alessi, D., Macdougall, L.K., Sola, M.M., Ikebe, M. and Cohen, P. (1992) Eur. J. Biochem. 210, 1023–1035.
[14] Eto, M., Ohmori, T., Suzuki, M., Furuya, K. and Morita, F. (1995) J. Biochem. 118, 1104–1107.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd EDN., Cold Spring Harbor Labratory, Cold Spring Harbor, NY.
[16] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, 390–408.
[17] Martin, B.L., Shriner, C.L. and Brautigan, D.L. (1994) Prot. Express. Purif. 5, 211–217.
[18] Moriya, M., Fujinaga, K., Yazawa, M. and Katagiri, C. (1995) Cell Tiss. Res. 281, 273–281.
[19] Yoshida, M. and Yagi, K. (1988) J. Biochem. 103, 380–385.
[20] Kikkawa, U., Go, M., Koumoto, J. and Nishizuka, Y. (1986) Biochem. Biophys. Res. Commun. 135, 636–643.
[21] Yoshida, M., Minowa, O. and Yagi, K. (1983) J. Biochem. 94, 1925–1933.
[22] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[23] Friedenauer, S. and Berlet, H.H. (1989) Anal. Biochem. 178, 263–268.
[24] Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., Moreno, R.F., Kerlavage, A.R., McCombie, W.R. and Venter, J.C. (1991) Science 252, 1651–1656.
[25] Kozak, M. (1987) Nucl. Acids Res. 15, 8125–8148.
[26] Lagercrantz, J., Carson, E., Larsson, C., Nordenskjold, M. and Weber, G. (1996) Genomics 31, 380–384.
[27] Desdouits, F., Cheetham, J.J., Huang, H.-B., Kwon, Y.-G., da Cruz e Silva, E.F., Denefle, P., Ehrlich, M.E., Nairn, A.C., Greengard, P. and Girault, J.-A. (1995) Biochem. Biophys. Res. Commun. 206, 652–658.
[28] Hemmings Jr., H.C., Nairn, A.C., Elliott, J.I. and Greengard, P. (1990) J. Biol. Chem. 265, 20369–20375.
[29] Shirazi, A., Iizuka, K., Fadden, P., Mosse, C., Somlyo, A.P., Somlyo, A.V. and Haystead, T.A.J. (1994) J. Biol. Chem. 269, 31598–31606.
[30] Okubo, S., Ito, M., Takashiba, Y., Ichikawa, K., Miyahara, M., Shimizu, H., Konishi, T., Shima, H., Nagao, M., Hartshorne, D.J. and Nakao, T. (1994) Biochem. Biophys. Res. Commun. 200, 429–434.