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Characterization of a novel human protein phosphatase 2C family member, PP2Cκ

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Abstract. A novel member of human protein phosphatase 2C gene named *PP2Cκ* was isolated from a human fetal brain cDNA library. The 2.0 kb cDNA encodes a 372 amino acid polypeptide with an intact protein phosphatase 2C (PP2C) catalytic domain. Reverse transcription-PCR (RT-PCR) revealed that the *PP2Cκ* was widely expressed in normal human tissues. Transient transfection suggested that PP2Cκ was localized in the nucleus in AD293 cells. Recombinant Trx-His-PP2Cκ showed phosphatase activity toward *p*-nitrophenyl phosphate (*p*NPP), as well as oligopeptides containing phospho-threonine residues. Furthermore, the overexpression of PP2Cκ distinctly activated the heat shock transcription factor pathway in eukaryotic cells.

Introduction

Protein phosphorylation/dephosphorylation is recognized as a major regulatory mechanism of cellular functions such as cell signal transduction and cell cycle controlling. Protein phosphatase 2C (PP2C) is one of four major protein serine/threonine phosphatases (PP1, PP2A, PP2B and PP2C) in eukaryotes (1,2). It is distinguished from other groups of phosphatases by its structural distinction, absolute requirement for divalent cation (Mg^{2+} or Mn^{2+}) and insensitivity to the okadaic acid (1,2). To date, at least 9 different PP2C genes have been identified in mammalian cells, such as *PP2Cα, 2Cβ, 2Cγ, 2Cδ(Wip1), 2Cε, 2Cζ, 2Cη, ILKAP-PP2C*, Ca^{2+}/calmodulin-dependent protein kinase phosphatase (3-12) and so on. There are even more PP2C isoforms in plants and other organisms reported in the past few years (12), and they compose a large gene family.

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Each protein phosphatase 2C isoform has an approximate 250 amino acid PP2C catalytic domain, which includes six typical motifs with highly conserved amino acid residues for metal ion (Mg^{2+} or Mn^{2+}) binding (1). In addition to catalytic domain, PP2C isoforms usually have unique C-terminal or N-terminal regions which may be involved in determination of substrate specificity.

Recent research data demonstrated that some major PP2Cs are involved in the regulation of mitogen-activated protein kinase (MAPK) cascades which deliver signals in response to extracellular stimulus (13-17). MAPK pathways, especially stress-activated protein kinase (SAPK) pathways, are negatively regulated by multiple PP2C isoforms at different levels (18). Evidence also exists indicating that PP2C isoforms play important roles in regulation of cell cycle (19-21), apoptosis (22-24), and the nerve system-related signal pathway (25-27).

In recent work, we have isolated a novel human PP2C isoform gene through the large-scale sequencing analysis of human fetal brain cDNA library. Its predicted protein has a typical PP2C catalytic domain and shows Mn^{2+}/Mg^{2+}-dependent phosphatase activity. We termed it human protein phosphatase 2Cκ (PP2Cκ) according to the guidelines of Human Genome Organization (HUGO) Nomenclature Committee. Herein we report the initial characterization of this novel gene and its potential role in eukaryotic cell signal transduction.

Materials and methods

Cloning of PP2Cκ cDNA. A human cDNA, which encodes a novel human protein phosphatase 2C, was cloned from the human fetal library during large-scale cDNA sequencing. The cDNA library was constructed in a modified pBluescript II SK (+) vector with human fetal brain mRNA purchased from Clontech. A 0.5 kb DNA fragment containing 2 *Sfi*I A (5'-GGCCATTATGGCC-3') and *Sfi*I B (5'-GGCCGCCTCGG CC-3') recognition sites were introduced into the *Eco*RI and *Not*I sites of pBluescript II SK (+) (Stratagene); double-stranded cDNAs were synthesized using SMART™ cDNA Library Construction kit (Clontech) following the manufacturer's instructions. The cDNA inserts were sequenced on an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer) using the BigDye Terminator Cycle Sequencing Kit and BigDye Primer Cycle Sequencing Kit (Perkin-Elmer) with -21M13

primer, M13Rev primer and synthetic internal walking-primers designed according to the obtained cDNA sequence fragments. Subsequent editing and assembly of all the sequences from one clone was performed using Acembly (Sanger's Center).

Bioinformatic analysis. DNA and protein sequence comparisons were carried out using BLAST2.0 at NCBI (http://www.ncbi.nlm.nih.gov/blast). ProfileScan was done by ANTHEPROT V5.0 (http://pbil.ibcp.fr/ANTHEPROT) (author: G. Deleage). Sequence alignment was performed by AlignX [http://www.informaxinc.com/vectorNTI (v6.0)] and GENEDOC (http://www.cris.com/~Ketchup/genedoc.shtml).

Expression pattern of PP2Cκ. Two human adult multiple tissue cDNA (MTC) panels and one human fetal MTC panel (Clontech) were used as PCR templates according to the manufacturer's protocol. Thirty-two PCR cycles for human *PP2Cκ* and 30 cycles for *GAPDH* (as control) were performed using Taqplus DNA polymerase (Sangon) according to the following program: 30 sec at 94˚C, 30 sec at 65˚C and 1.0 min at 72˚C. The PCR products of *PP2Cκ* and *GAPDH* were then electrophoresed on a 2% agarose gel. PCR primers are indicated from 5' to 3' as follows: human *PP2Cκ* sense, ATGTCAACAGCTGCCTTAATTACTT; human *PP2Cκ* antisense, TCAGGCCCATCGTCCACTGGAG; *GAPDH* sense, TGAAGGTCGGAGTCAACGGATTTGGT; *GAPDH* antisense, CATGTGGGCCATGAGGTCCACCAC. The sense primer and the antisense primer of *PP2Cκ* span 1118 bp in the cDNA from 214 to 1332 bp.

Construction of expression plasmids. Expression plasmids were constructed by standard procedures. For bacterial expression of proteins, the open reading frame of *PP2Cκ* was subcloned into the pET32a vector (Novagen) to generate Trx-His-tag fusion protein. Plasmids that express PP2Cκ in mammalian cells (pcDNA-Myc-PP2Cκ and pEGFP-PP2Cκ) were constructed using PP2Cκ cDNA together with the pcDNA4/Myc-His-C (Invitrogen) and pEGFP-C1 (Clontech) vectors, respectively.

Expression in E. coli and purification of PP2Cκ. Transformants of *E. coli* BL21 with the pET32a-PP2Cκ plasmid were grown at 37˚C in 500 ml LB medium with 100 μg/ml ampicillin. When the culture had grown to an OD_{600} of 0.6, isopropyl b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After inducing the expression of the Trx-His-PP2Cκ protein for 4 h at 28˚C, cells were harvested, washed and lysed in 50 ml BugBuster protein extraction reagent (Novagen). Protein was purified from the soluble fraction with Ni-NTA agrose column (Qiagen) according to the manufacturer's protocol. The protein concentration was determined by the method of Bradford using BSA as a standard. The integrity of the fusion protein was checked by SDS-PAGE.

Phosphatase assay. The standard reaction which contained 1 μg of protein, 50 mM Tris-HCl (pH 6.0), 10 mM *p*-nitrophenyl phosphate (*p*NPP) and 5 mM divalent cation (Mg^{2+} or Mn^{2+}) in a total of 1 ml was performed at 37˚C for 30 min and then stopped by 0.1 M NaOH. The increase in the amount of *p*-nitrophenol was monitored by measuring the absorbance at 420 nm on an Ultraspec 4000 (Pharmacia) spectrophotometer. The serine/threonine phosphatase assay system (Promega) was used to detect the serine/threonine phophatase activity of PP2Cκ. The reactions contained 20 μl 5X reaction buffer (provided by the assay system), 10 μl phosphopeptide (provided by the assay system) and different concentrations of PP2Cκ in a total of 100 μl. The reaction was stopped by adding 100 μl of molybdate dye/additive mixture and the standard curve was made according to the manufacturer's protocol. The released phosphate was monitored by measuring the absorbance at 600 nm on Model 450 Microplate Reader (Bio-Rad). The Thr phosphopeptide sequence was RRA (pT)VA.

Cell culture, transfection, and pathway profiling assay. We used Mercury Pathway Profiling System (Clontech) to investigate the potential roles of PP2Cκ in the signal pathways, and firefly luciferase as the reporter gene. AD293 cells and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cells were seeded on a 96-well plate at $1x10^4$/well. After 24 h, PP2Cκ expression vector, pcDNA4-PP2Cκ, and each cis-acting luciferase reporter vector of the Mercury Pathway Profiling System (Clontech) were co-transfected into cells by the Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After 48 h, the transfected cells were lysed and the activity of luciferase reporter gene was measured by dual-luciferase reporter assay system (Promega). We also designed the transfection assay in a plasmid dose-dependent manner after we got our primary results.

Subcellular localization of PP2Cκ. AD293 cells were transiently transfected with pEGFP-PP2Cκ and pEGFP-C1 (as control) using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde for 20 min at room temperature (RT), washed with PBS (pH 7.4), and then nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole). Fluorescence was viewed with a Leica fluorescence microscope. EGFP served as the control.

Results

Identification and cloning of human PP2Cκ cDNA. The GenBank non-redundant and EST databases were searched with individual sequences from our collection of full-length assembled cDNAs using the NCBI BLAST server in order to identify sequences of interest. A 2034 bp cDNA was isolated which contains an open reading-frame from nucleotide 214 to 1332, encoding a 372 amino acid protein with a calculated molecular mass of ~46 kDa. (GenBank accession number for this cDNA is AY157615). The deduced protein has an intact protein phosphatase 2C catalytic domain from amino acid residue 95 to 344 which shows high similarity with the human PP2Cε, hPP2Cα1, and hPP2Cß1 catalytic domains (Figs. 1 and 2). All the typical motifs (I-VI) and conserved amnio acid residues for metal ions binding were also found in the predicted PP2C catalytic domain (Figs. 1 and 2). We termed this gene human *PP2Cκ* (protein phosphatase 2Cκ). The protein phosphatase 2Cκ gene is mapped to human chromosome 4q22 by BLAST search

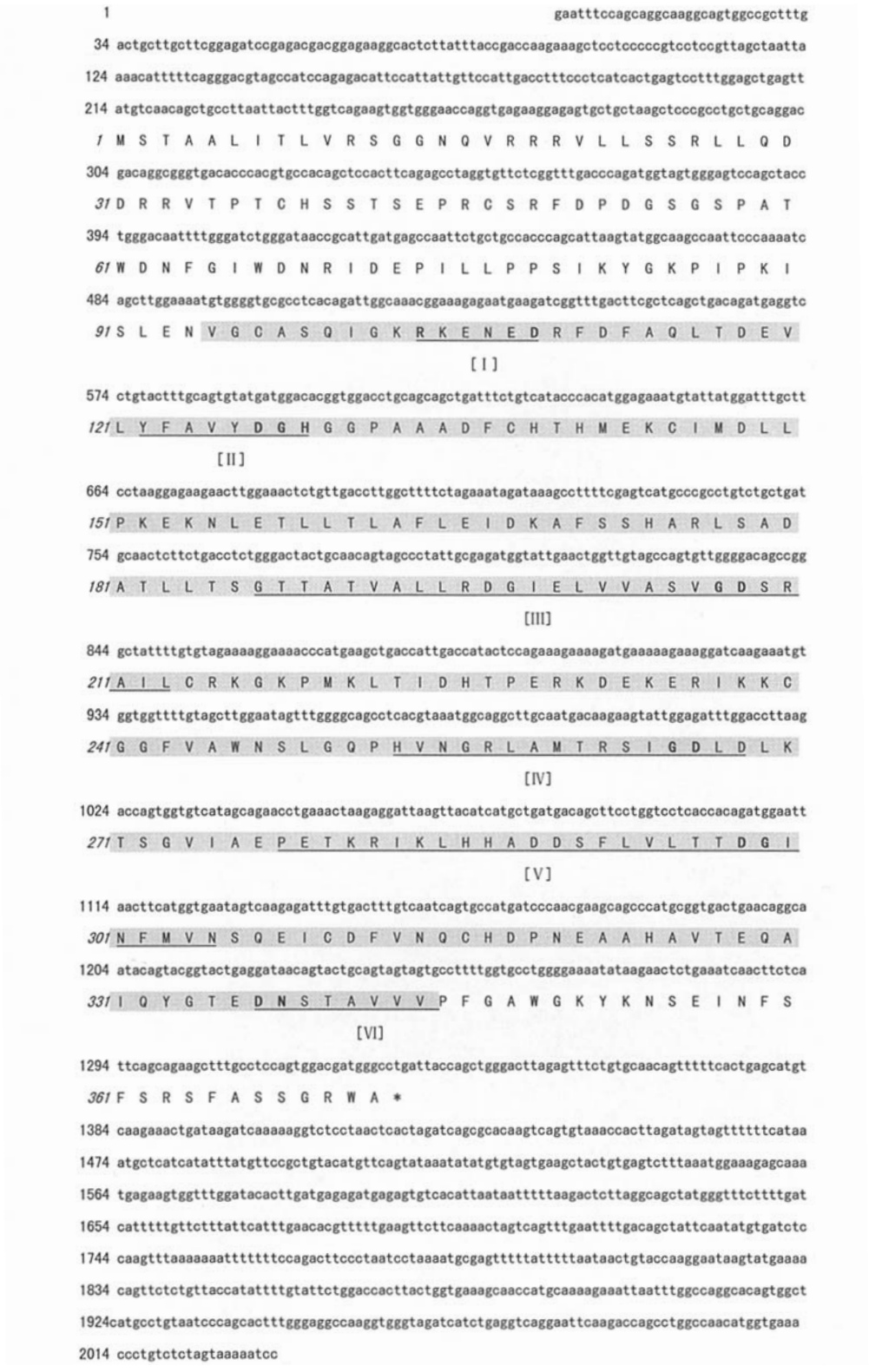


Figure 1. The cDNA and deduced amino acid sequences of human *PP2Cκ* gene. The nucleotides and amino acids are numbered on the left (italics indicates amino acids). The protein phosphatase 2C catalyzed domain (from residue 95 to 344) is shaded. The six PP2C motifs are underlined and the conserved amino acid residues are indicated in bold text.

against human genome database. The cDNA spans about 23 kb on the chromosome and consists of 7 exons.

Expression pattern of the PP2Cκ gene. The tissue distribution of *PP2Cκ* mRNA was determined by RT-PCR using multiple tissue cDNA (MTC) panels (Clontech) as PCR templates. *PP2Cκ* mRNA was detected in all the human tissues we used, which indicates that *PP2Cκ* is widely expressed and relatively abundant. The expression levels in adult heart, brain, kidney, pancreas and ovary are relatively high. In eight fetal tissues, *PP2Cκ* showed high expression in heart and thymus (Fig. 3).

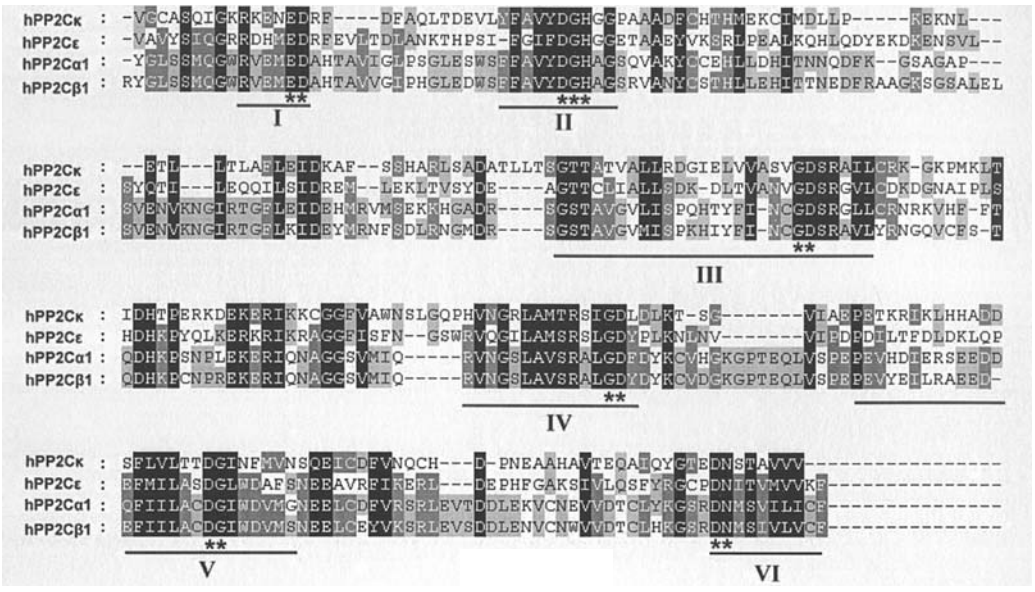


Figure 2. Sequence comparison of PP2Cκ phosphatase catalytic domain with those of hPP2Cε (AAR00269), hPP2Cα1 (P35813), and hPP2Cß1 (CAA06704). Identical amino acids and homologous amino acids are indicated by solid boxes and shaded boxes, respectively. Typical motifs and conserved residues are indicated.

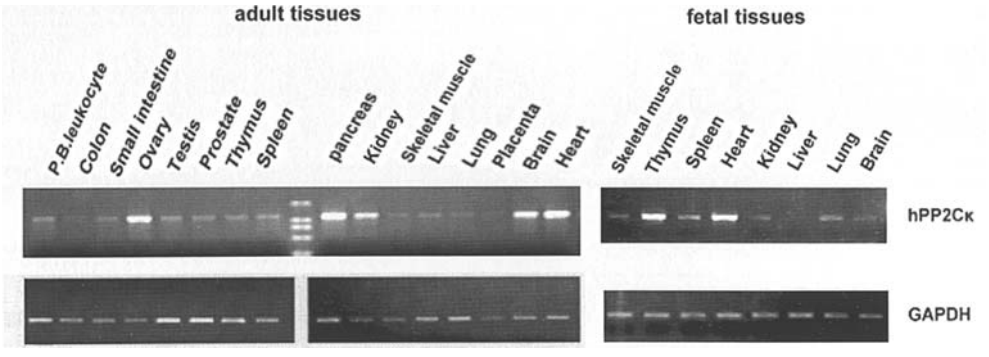


Figure 3. Tissue distribution of *PP2Cκ* mRNA. Reverse transcription-PCR analysis of human cDNA for *PP2Cκ* and *GAPDH* (as a control). Prenormalized cDNAs from sixteen human adult tissues and eight human fetal tissues were employed as templates in PCR reactions.

Expression in E. coli and purification of PP2Cκ. Following induction with IPTG, the Trx-His recombinant PP2Cκ yielded a major band of apparent molecular mass of 66 kDa on an SDS-PAGE gel corresponding to about 15-20% of the total applied protein (Fig. 4A). Purification was performed by Ni-NTA agrose column (Qiagen). The purified protein was deionized and eluted.

In vitro enzymatic properties of PP2Cκ. The phosphatase activity of Trx-His-PP2Cκ on pNPP was first examined. The Trx-His-PP2Cκ had divalent cation (Mg^{2+} or Mn^{2+}) dependent phosphatase activity which was relatively high in the presence of Mn^{2+} (Fig. 4B). The phophatase activity was not affected by 1 μM okadaic acid (OA) (data not shown). We investigated the substrate specificity of Trx-His-PP2Cκ using the serine/threonine phosphatase assay system (Promega). In this assay, the enzyme activity was estimated by measuring the amount of the phosphate released. Trx-His-PP2Cκ showed phosphatase activity toward pThr (Fig. 4C). These data showed that PP2Cκ is a protein phosphatase with serine/threonine phosphatase activity and it is a novel isoform of the PP2C family.

Pathway profiling. To find the potential roles of PP2Cκ in the cell signal transduction pathway, we overexpressed PP2Cκ in eukaryotic cells and investigated its roles by Mercury Pathway Profiling System. We used 6 cis-acting luciferase reporter vectors [cAMP response element (CRE), serum response element (SRE), nuclear factor of κB cells (NF-κB), heat shock response element (HSE), activator protein 1 (AP1), and glucocorticoid response element (GRE)] in AD293 cells and 4 cis-acting luciferase reporter vectors [E-box DNA binding element (Myc), p53 response element (P53), E2F DNA binding element (E2F), and Rb response element (Rb)] in NTH3T3 cells. Considering that the AD293 cell line is a transformed cell line, we assayed the above 4 types of cell-cycle related pathways in NIH 3T3 cells. In all the tests, the TAL promoter weak reporter was used as a control. The results indicate that PP2Cκ distinctly activated the heat shock response element (HSE) pathway (also named heat shock factor pathway) about 7-fold, but had no effect on the other 9 pathways we used (Fig. 5A and B). The dose-depended assays in both AD293 and NIH3T3 cells verified the activation of PP2Cκ to the heat shock factor pathway (Fig. 5C and D).

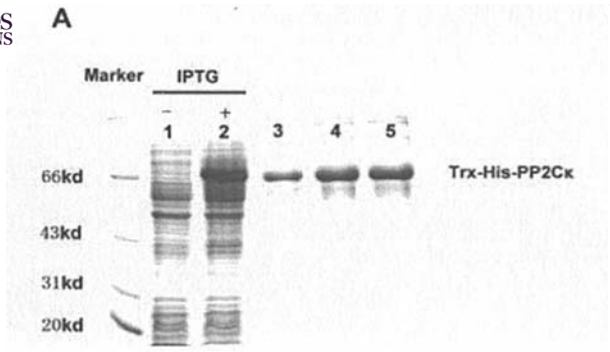
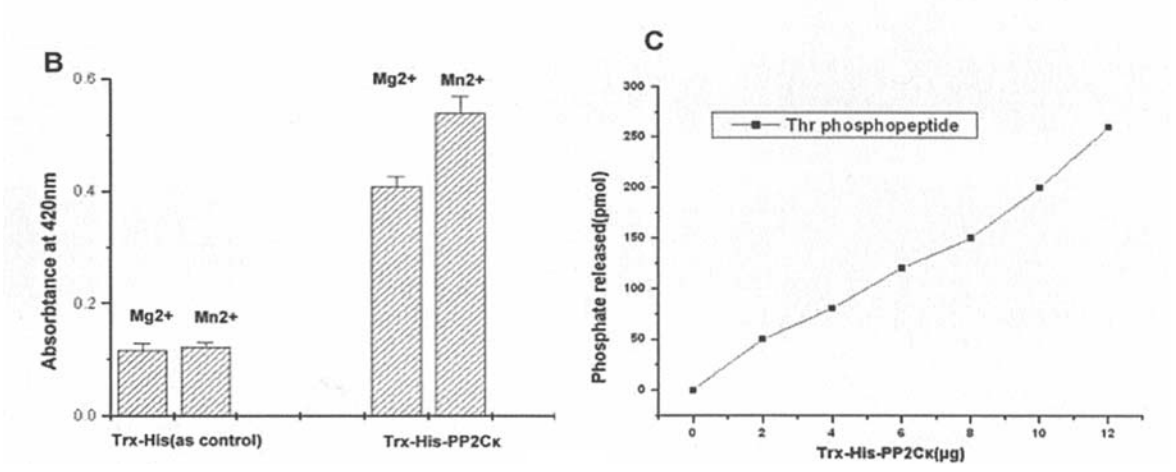


Figure 4. (A) SDS-PAGE gel analysis of protein samples. Lane 1, total cell protein sample before induction by IPTG; lane 2, total cell protein sample after induced by 1 mM IPTG for 4 h; lanes 3-5, recombinant protein sample after purification by Ni-NTA agrose column. (B) Basic enzymatic properties of PP2Cκ: the phosphatase activity of Trx-His-PP2Cκ and Trx-His (as control) using 10 mM pNPP as substrate; (C) Ser/Thr phosphatase activity of PP2Cκ. 0.1 mM Thr phosphopeptide [RRA (pT)VA] was used as substrate. Indicated amounts of PP2Cκ were assayed.



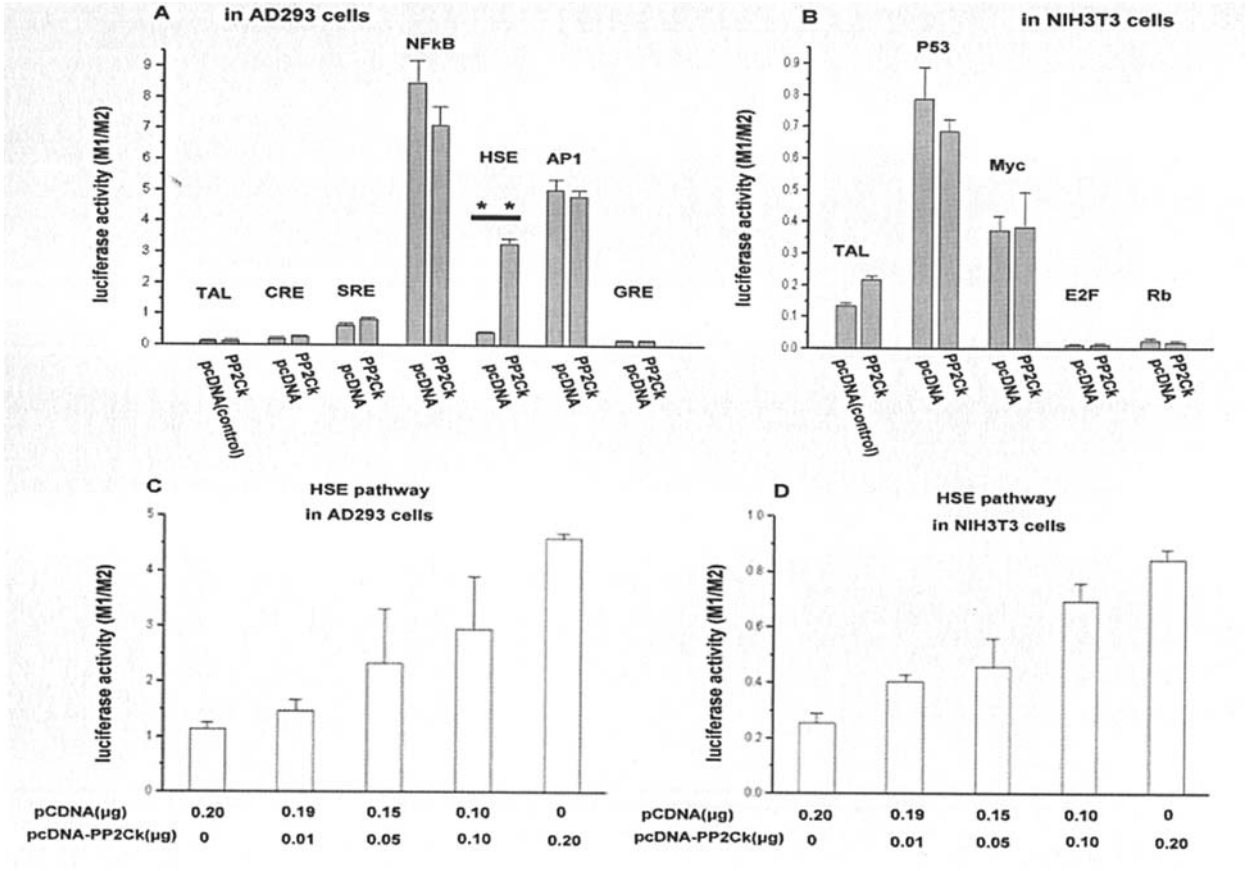


Figure 5. (A) The role of PP2Cκ in 6 signal pathways (CRE, SRE, NF-κB, HSE, AP1 and GRE pathway) in AD293 cells. The distinct difference between PP2Cκ and pcDNA control are indicated by asterisks. (B) The role of PP2Cκ in 4 cell-cycle related signal pathways (P53, Myc, E2F and Rb pathway) in NIH3T3 cells. (C) Activation of HSE pathway by PP2Cκ in a dose-dependent manner in AD293 cells. (D) Activation of HSE pathway by PP2Cκ in a dose-dependent manner in NIH3T3 cells.

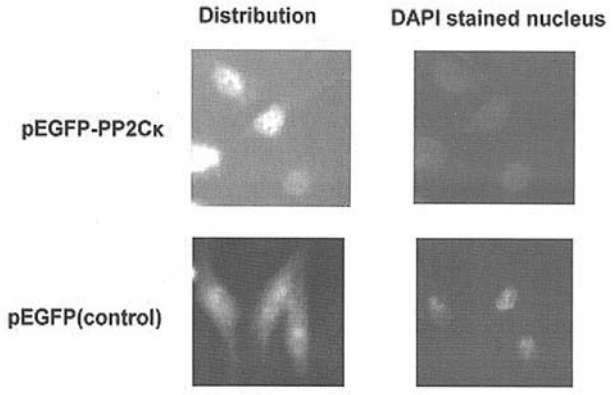


Figure 6. Subcellular localization of PP2Cκ. AD293 cells transfected with pEGFP-PP2Cκ were fixed and stained with 4',6'-diamidino-2-phenylindole dihydrochloride (*DAPI*). The pEGFP-C1 vector was used as a control.

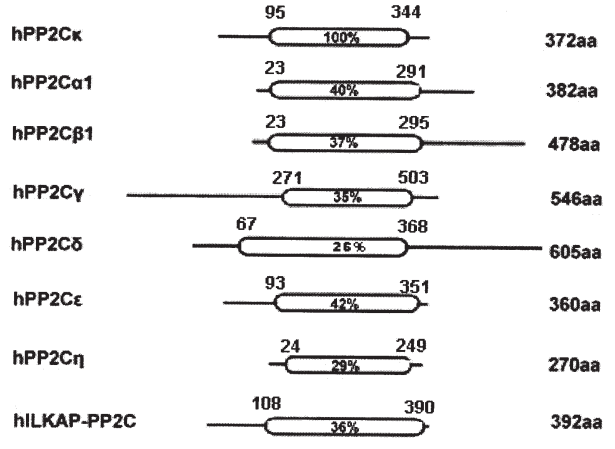


Figure 7. Domain structure of the members of the human PP2C family. The catalytic domains of human PP2Cκ, PP2Cα1 (P35813), PP2Cß1 (CAA06704), PP2Cγ (CAA74245), PP2Cδ (O15297), PP2Cε (AAR00269), PP2Cη (Q96MI6), and ILKAP-PP2C (NP_110395) are depicted with boxes. The residue positions of each catalytic domain are indicated on both ends of the domains. The homology between PP2Cκ with other PP2Cs is shown.

Subcellular localization of PP2Cκ. Subcellular localization of PP2Cκ was analyzed by transient transfection of pEGFP-PP2Cκ recombinant plasmid to AD293 cells. Green fluorescence revealed that PP2Cκ was expressed in the cell nucleus [nucleus was stained with DAPI (4',6'-diamidino-2-phenylindole dihydrochloride)] (Fig. 6).

Discussion

The distinct protein structure features and increasing gene family background of protein phosphatase 2C help us to identify new members of PP2C isoforms in mammals. We report on a new isoform of human PP2C family named *PP2Cκ*. The 2.0 kb cDNA encodes a 372 amino acid protein containing an intact PP2C catalytic domain from residue 95 to 344 and a unique N-terminal region. Six typical motifs and several key amino acid residues (shown in Figs. 1 and 2) are conserved in all known members of PP2C family. The crystal structure reveals that these motifs compose a central ß-sandwich binding two manganese ions surrounded by α-helices. Mn^{2+}-bound water molecules at the binuclear metal center coordinate the phosphate group of substrates and provide a nucleophile and general acid in the dephosphorylation reaction (1,28). Glu-37(G), Asp-38(D), Asp-60(D), Asp-239(D) and Asp-282(D) are essential for the binding of metal ions and H_2O molecules in the catalytic site of PP2C isoforms (residue number is from PP2Cα) (1,28,29). All the key motifs and conserved residues were found in the PP2C domain of PP2Cκ, which suggested that it was a new functional PP2C isoform (Figs. 1 and 2). The primary phosphatase assay verified its basic serine/threonine phophatase activity (Fig. 4).

Apart from the 250-residue PP2C catalytic domains, most mammalian PP2C isoforms have their unique C-terminal regions or N-terminal regions (Fig. 7). Deletion analysis indicated that these polypeptide regions did not affect the catalytic activity and they may play roles in determination of substrate specificity. The low sequence similarity among PP2C members suggests that PP2C gene family is formed by a convergent evolution (Fig. 7).

PP2Cκ is ubiquitously expressed in all the normal tissues we studied and its expression levels display notable differences in various tissues. Previous studies indicate that PP2C isoforms have different expression patterns in mammals. For example, *PP2Cα, ß*, and *δ* are ubiquitously expressed, but *PP2Cε, ζ* show tissues specific expression pattern (3,4,6,8,9). *PP2Cα* and *PP2Cß* even have 2 and 5 isoforms, respectively (17). The different distributions of PP2Cs may determine their distinct substrate specificities and individual functions *in vivo*.

With the help of the Mercury Pathway Profiling System, we found that PP2Cκ could stimulate the activities of the heat shock factor pathway. It has been reported that phosphorylation may provide sophisticated regulation of transcription factors, including heat shock factor 1 (HSF1) (30). At least five phosphorylation sites, Ser230, Ser303, Ser307, Ser206, Ser363, have been reported in heat shock factor 1, and all these sites are phosphorylated constitutively and are inducible. The phosphorylation can positively or negatively regulate the transcriptional activity of HSF1 (30-35). The heat shock factor is inhibited by at least five kinases such as ERK, JNK and GSK3ß (30), but up to now, no phosphatases have been isolated in the regulation of the heat shock factor pathway. We presume that PP2Cκ may regulate the activity of the heat shock factor in two ways: 1) the PP2Cκ directly interacts with the heat shock factor and dephosphoralyted the phospho-serine residues, 2) PP2Cκ influences the heat shock factor pathway indirectly by inactivating the kinases, such as JNK, ERK and GSK3ß. Some other members of PP2C family can influence the JNK and p38 pathways. Further research will focus on the detailed mechanism of the regulatory function of PP2Cκ. For example, demonstrating the possible interaction between PP2Cκ and the aforementioned kinases or HSF1 *in vivo*.

We investigated the subcellular localization of PP2Cκ by transiently transfected GFP recombinant PP2Cκ into the AD293 cell line. PP2Cκ was observed only in the nucleus in the transfected cells. It is consistent with our results that PP2Cκ activates the heat shock factor in cell nucleus.

In conclusion, a novel human PP2C isoform: PP2Cκ was cloned and its tissue distribution and initial phosphatase activity were determined. PP2Cκ may function as an active regulator in the heat shock factor pathway. Further research is required to confirm our speculation and to reveal the functional model.

Acknowledgments

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