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Molecular Cloning and Functional Characterization of Protein Phosphatase 2C of Two Scuticociliates – *Uronema marinum* and *Miamiensis avidus* (Ciliophora: Scuticociliatia)

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Summary. Complementary DNAs (cDNAs) of protein phosphatase 2C (PP2C) were cloned from two marine scuticociliates *Uronema marinum* and *Miamiensis avidus*. Both PP2C proteins showed structural characteristics of typical PP2C, such as highly conserved amino acid residues predicted for binding to phosphate and metal ions, 11 conserved PP2C motifs and 10 invariant residues. The phosphatase activity of recombinantly produced *U. marinum* PP2C (UmPP2C) was in proportion to the PP2C protein and Mg^{2+} concentrations, and was not sensitive to okadaic acid, but was inhibited by sodium fluoride, EDTA or Ca^{2+}. The expression of UmPP2C was significantly up-regulated by exposure the ciliates with PMA suggesting that UmPP2C dephosphrylates proteins phosphorylated by protein kinases as in other eukaryotes and has a regulatory function against abrupt increase of protein phosphorylation triggered by strong stimulations.

Key words: Protein phosphatase 2C (PP2C), Scuticociliates, *Uronema marinum*, *Miamiensis avidus.*

INTRODUCTION

Cellular signaling mediated through phosphorylation and dephosphorylation of proteins is crucial in the regulation of cellular functions in eukaryotes (Hunter 2000). Protein kinases and protein phosphatases are two main enzyme families responsible for controlling protein phosphorylation state. Phosphatases dephosphorylate proteins which have a phosphate by action of protein kinases at serine, thereonine, or tyrosine residues.

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Based on substrate specificity, metal ion requirements and sensitivity to inhibitors, serine/threonine (Ser/Thr)-specific phosphatases are divided into two classes – type I-phosphatases (PP1) and type II-phosphatases (PP2) which are further subdivided into PP2A, PP2B, and PP2C classes (Shenolikar and Ingebritsen 1984, Cohen 1989, Mumby and Walter 1993). Among them, PP2C proteins are monomeric enzymes, require Mg^{2+} or Mn^{2+} for dephosphorylation of proteins, insensitive to okadaic acid, and structurally distinct from other phosphatases (Cohen 1989, McGowan and Cohen 1998).

PP2C proteins have been reported in various organisms ranging from yeasts to mammals, and various functions of PP2C proteins have been investigated,

which are predominantly related to regulation of cellular stress signaling and cell growth (Robinson *et al*. 1994, Shiozaki *et al*. 1994, Huang *et al*. 1999, Lammers and Lavi 2007). Investigation of protozoan PP2C proteins has been conducted mainly on mammalian parasites such as *Plasmodium* spp. (Mamoun *et al*. 1998, Kumar *et al*. 2007), *Toxoplasma gondi* (Delorme *et al*. 2003, Gilbert *et al*. 2007, Jan *et al*. 2007) and *Leishmania chagasi* (Burns *et al*. 1993). In ciliates, PP2C has been reported from two species in the genus *Paramecium – P. tetraurelia* and *P. caudatum*, and the function of PP2C had been linked to ciliary movement regulation (Klumpp *et al*. 1994, Grothe *et al*. 1998, Noguchi *et al*. 2003).

Uronema marinum and *Miamiensis avidus,* which is the synonym of *Philasterides dicentrarchi* reported by Kim *et al*. (2004) (Jung *et al*. 2007, Song *et al*. 2009), are well known culprits of scuticociliatosis in cultured marine fish, and, in Korea, occurrences of scuticociliatosis have been deeply related with high cumulative mortalities in cultured olive flounder (*Paralichthys olivaceus*) (Dragesco *et al*. 1995, Iglesias *et al*. 2001, Kim *et al*. 2004). In the present study, we have cloned PP2C cDNA from these two ciliate species, characterized enzymatic property using recombinantly produced PP2C protein, and analyzed transcriptional changes in response to phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator.

MATERIALS AND METHODS

Cultivation of ciliates

Ciliates *U. marinum* and *M. avidus* were isolated from diseased olive flounders obtained from local fish farms in South Korea. Identification of ciliates species was done by PCR using species specific oligonucleotide primer pairs (Kim *et al.* 2004). *U. marinum* were cultured in 0.25% yeast extract-containing sea water medium and *M. avidus* were grown using Chinook salmon embryo (CHSE)-214 cells incubated at 20°C in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco).

Cloning of *U. marinum* PP2C cDNA (UmPP2C) and *M. avidus* PP2C cDNA (MaPP2C)

A subtracted cDNA library of the phorbol 12-myristate 13-acetate (PMA) treated *U. marinum* was constructed using suppression subtractive hybridization (SSH) technique to identify PMA-induced genes. Briefly, cultured *U. marinum* were harvested at the logarithmic phase by centrifugation at 1,000 × *g* for 5 min., divided into two groups, and adjusted to 5×10^5 cells per ml in filtered sea water containing 0.05% yeast extract. One group of ciliates were incubated in a medium containing 10 μg/ml of PMA for 1 h and harvested by centrifugation at 1,000 × *g* for 2 min. at 0°C. Total RNA and poly A^+ RNA were isolated from both PMA-treated and non-treated *U. marinum*. Total RNA was isolated using Tri Reagent (Sigma) and the poly A^+ RNA from total RNA was isolated using PolyATract mRNA isolation system IV (Promega). After total and poly A^+ RNA isolation, the integrity of RNA was determined by ultraviolet (UV) spectrophotometry and formaldehyde gel electrophoresis. SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. The partial sequence of UmPP2C gene was obtained by random sequencing of clones prepared using final PCR product. The full open reading frame (ORF) of UmPP2C gene was obtained by 5'- and 3'- RACE PCR using Clontech SMART™ cDNA amplification kit according to the manufacturer's instructions. A partial sequence (327-bp) of MaPP2C gene was obtained by PCR amplification of *M. avidus* cDNA using a primer set of UroPP2C-For-2 and UroPP2C-Re primers. The full length cDNA sequence of MaPP2C gene was obtained by 5'- and 3'- RACE PCR using Clontech SMART™ cDNA amplification kit according to the manual using MaPP2C-R primer and MaPP2C-F primer as gene-specific primers. The nucleotide sequences of primers used in the cloning were shown in Table 1. Multiple sequence alignments were generated using the CLUSTALW 1.8 program.

Expression and purification of recombinant UmPP2C protein

The ORF of UmPP2C gene was re-amplified by PCR using primers containing restriction enzyme sites at both 5'- and 3'-ends for recombinant expression. For heterologous expression of recombinant UmPP2C using *Escherichia coli* system, 16 universal TAA stop codons (ciliates use TAA as a codon encoding glutamine) in the UmPP2C full ORF sequence were converted CAA codons by site-directed mutagenesis using QuikChange® Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The nucleotide sequences of primers used in the site-directed mutagenesis were shown in Table 1. For expression of Glutathione S-transferase (GST) fusion protein, pGEX 4T-1 (GE Healthcare Life Sciences) vector was used. The T-vector harboring UmPP2C ORF with restriction enzyme recognition sites was digested with restriction enzyme *Sal*I and *Not*I, and ligated into pGEX 4T-1 vector digested with the same enzymes. Ligation reaction mixture was transformed into *E. coli* DH5α. Plasmid DNA from positive clones was isolated and transformed into the *E. coli* BL21 (DE3) codonplus RIPL competent cells (Stratagene) for expression of recombinant protein. GST-fused UmPP2C protein was purified from the culture of IPTG induced *E. coli* BL21 (DE3) codonplus RIPL cells harboring pGEX-UmPP2C using GSTrap FF column filled with Glutathione Sepharose 4 Fast Flow (GE Healthcare Bioscience) according to the manufacturer's instructions. The purified protein was dialyzed with 10 mM Tris-Cl (pH 7.2) for at least 4 hours for use in the further experiment. Protein concentration and purity was confirmed by SDS-PAGE and BCA method using BSA as a standard.

Measurement of protein phosphatase activity

Phosphatase activity of recombinant UmPP2C protein was measured using the non-radioactive Serine/Threonine Phosphatase As-

Table 1. The nucleotide sequences of primers used in the present study.

Primers	Sequences
UroPP2C For-1	5'-GCTGGTGATAGTAGATCTGTTTTATGC-3'
UroPP2C For-2	5'-CAGTGATGCTGGGGGATTCGTAAG-3'
UroPP2C Re	5'-TCCAACACCATTTGAAGTATCTGAGGC-3'
UroPP2C Re-1	5'-GTCCCGGTTATCAGTAGGATCACTTG-3'
Um18SF	5'-CTTCTGTACAGTCTCATTTC-3'
Um18SR	5'-AACGCCAATTAAAGATCAAC-3'
SDM 1-F	5'-CTTATTTACAAGCTTGTGCTAGTGAAATGCAAGGATGG-3'
SDM 1-R	5'-CCATCCTTGCATTTCACTAGCACAAGCTTGTAAATAAG-3'
SDM 2-F	5'-GGATGCGCATATTTTACAAATGAATATTAATGGAG-3'
SDM 2-R	5'-CTCCATTAATATTCATTTGTAAAATATGCGCATCC-3'
SDM 3-F	5'-GAGGTAAAGAAGTTGCCCAATTTGTAGAAAAACAT-3'
SDM 3-R	5'-ATGTTTTTCTACAAATTGGGCAACTTCTTTACCTC-3'
SDM 4-F	5'-CTGAAAGTGGTCAACAAGAATTGAACCAAATTAGAGCAG-3'
SDM 4-R	5'-CTGCTCTAATTTGGTTCAATTCTTGTTGACCACTTTCAG-3'
SDM 5-F	5'-CCTAATGAAGAACAATCAGGAGGACAATCTTATGCTGG-3'
SDM 5-R	5'-GGAGCATAAGATTGTCCTCCTGATTGTTCTTCATTAGG-3'
SDM 6-F	5'-AGATAGACCTAGAGACCAACAATTAATTATATCCAAC-3'
SDM 6-R	5'-GTTTGGATATAATTAATTGTTGGTCTCTAGGTCTATCT-3'
SDM 7-F	5'-ACTAATGAAGAACTTATCCAATATTGTAAAGAAAG-3'
SDM 7-R	5'-CTTTCTTTACAATATTGGATAAGTTCTTCATTAGT-3'
SDM 8-F	5'-AAGAAAGAATTGAAAAACAACAAGACTTAAATCAAATA-3'
SDM 8-R	5'-TATTGATTTTAAGTCTTGTTGTTTTTCAATTCTTTCTT-3'
MaPP2C-F	5'-CAACGGAAACTTAAATTTATCCAGAGCTATCG-3'
MaPP2C-R	5'-ATCGAGCATATTTTCAATAATTTCAGTGAGG-3'

say System (Promega) according to the manufacturer's instructions. Phosphatase activity was determined by measuring dephosphorylation of chemically synthesized phosphopeptide, RRA(pT)VA, a peptide substrate that is compatible with several serine/threonine phosphatases such as the protein phosphatase 2A, 2B and 2C. The assay was performed in 50 μl of reaction buffer (pH 7.2) containing 500 ng of purified UmPP2C protein, 0.1 mM phosphopeptide substrate, 50 mM imidazole, 0.2 mM EGTA, 5 mM $MgCl_2$, 0.02% β-mercaptoethanol and 0.1 mg/ml BSA. After incubation for 10 min. at room temperature, the reaction was terminated by addition of 50 μl of molybdate dye/additive mixture and optical density was measured at 595 nm using a microplate reader (Hewlett-Packard, SpectraCount™). The effect of bivalent cations, Mg^{2+}, Mn^{2+} and Ca^{2+}, on the phosphatase activity was measured in the same assay buffer with various concentrations of the ions. To distinguish PP2C from PP2A or PP2B, the effect of various protein phosphatase inhibitors such as okadaic acid (Sigma), sodium floride (NaF, Fluka), $CaCl_2$ and EDTA were examined using same assay buffer. The phosphatase activity was presented as pmol per min. calculated from the standard curve of KH_2PO_4.

Transcription of UmPP2C in response to PMA

U. marinum maintained in the culture medium were harvested by centrifugation and washed three times with HBSS containing 3% NaCl. Before stimulation, ciliates were stabilized for at least 2 hours. To examine whether transcriptional response of UmPP2C to PMA is dose-dependent, *U. marinum* were stimulated with PMA at concentration of 0, 0.5, 1, 5, 10, and 50 μg/ml for 90 min. After exposure of ciliates to PMA, total RNAs from harvested ciliates were isolated. The cDNAs were synthesized from 500 ng of total RNA using M-MLV reverse transcriptase and random hexamer (Promega). Semi-quantitative RT-PCR was carried out to determine the relative mRNA expression level of PP2C. PCR in a reaction volume of 10 μl was performed with 2 × Prime Taq Premix (Genet Bio) and 1 μl of 10^{-1} diluted cDNA template. Oligonucleotide primer pairs for PP2C and external control (18S ribosomal RNA) genes are provided in Table 1 (UroPP2C For-1 and UroPP2C Re; Um18SF and Um18SR). Thermal cycling conditions were 1 cycle of 3 min. at 95°C (initial denaturation) followed by 25 cycles (for PP2C) or 23 cycles (for 18S rRNA) of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C.

The length of PCR products of UmPP2C and 18S rRNA genes were 423- and 722-bp, respectively. The PCR cycle number of each gene was determined by preliminary analysis of PCR products amplified at every 3 PCR cycles from 15 to 33 cycles. PCR samples to be compared were electrophoresed on the 1.1% agarose gel and visualized with ethidium bromide (Et-Br) staining. Images of the Et-Br stained bands were imported into the Quantity-One image analysis software implemented in Gel Doc XR imaging device (Biorad). The band intensity of PP2C gene was normalized against that of 18S rRNA gene in order to estimate its relative mRNA levels.

Statistical analysis

Data were analyzed by the Student's *t*-test. Significant differences were determined at $P < 0.05$.

RESULTS

Protein phosphatase 2C (PP2C) cDNA sequence of *U. marinum* (UmPP2C) and *M. avidus* (MaPP2C)

The cloned cDNA of UmPP2C (GenBank accession no. GQ465984) was 1137-bp, containing a 933-bp of ORF encoding 310 amino acids, a 4-bp of 5'-untranslated region (UTR) and a 200-bp of 3'-UTR including a poly(A)+ tail. The full-length cDNA of MaPP2C (GenBank accession no. GQ465985) was 1245-bp, containing a 903-bp of open reading frame encoding 300 amino acids, a 65-bp of 5'-UTR and a 277 bp of 3'-UTR including a poly(A)+ tail. The predicted molecular weight of UmPP2C and MaPP2C proteins calculated using a web analysis program (http://us.expasy.org) was 35 kDa and 33.7 kDa, respectively. Sequence identity between UmPP2C and MaPP2C was 61.2% at the nucleotide level and 51.9% at the amino acid level. PP2C proteins of both two ciliate species had amino acid residues for binding phosphate and metal ions, 11 conserved PP2C motifs, and 10 invariant amino acid residues (Fig. 1). When the amino acid sequences of both UmPP2C and MaPP2C were compared with other PP2C sequences (Table 2), PP2C of *P. tetraurelia* (PtPP2C) showed the highest sequence identity with both UmPP2C and MaPP2C, which was higher than identity between UmPP2C and MaPP2C.

Enzyme activity of the recombinant UmPP2C

Phosphatase activity of recombinant UmPP2C in a standard PP2C buffer was in proportion to the protein concentration (Fig. 2). To examine the effect of bivalent cations such as Mg^{2+}, Mn^{2+} and Ca^{2+}, phosphatase activity was measured using various concentrations of $MgCl_2$, $MnCl_2$, and $CaCl_2$. The phosphatase activity was clearly observed in the presence of Mg^{2+} in a concentration dependent manner, whereas much lower activity or no activity was measured in the presence of Mn^{2+} or Ca^{2+} (Fig. 3). To distinguish PP2C from PP2A or PP2B, the effect of various protein phosphatase inhibitors such as okadaic acid, sodium floride, $CaCl_2$ and EDTA, was examined. As a result, the phosphatase activity of recombinant UmPP2C was not sensitive to okadaic acid, but was inhibited by sodium fluoride, EDTA or Ca^{2+} (Table 3).

mRNA expression of UmPP2C gene in response to PMA

The mRNA expression of UmPP2C was slightly higher but not significantly different from control when the concentrations of PMA were low (0.5–5 μg/ml), but the expression was significantly increased when the ciliates were exposed to more than 10 μg/ml of PMA (Fig. 4).

DISCUSSION

In the present study, we have cloned cDNA of PP2C from two marine scuticociliates *U. marinum* and *M. avidus*. Both PP2C proteins showed structural characteristics of typical PP2C, such as highly conserved amino acid residues predicted for binding to phosphate and metal ions, 11 conserved PP2C motifs and 10 invariant residues. The amino acid sequence of both PP2C ORFs showed more than 50% identity with each other and *P. tetraurelia* PP2C, while showed 20–30% identity with other PP2Cs, suggesting that PP2C of ciliates has been uniquely diversified.

In this study, recombinantly produced UmPP2C protein showed the characteristics of canonical PP2C enzymes, such as strict requirement of divalent cations for activity, complete inhibition of the activity by a serine/threonine inhibitor NaF, and insensitivity to okadaic acid. The clearly higher phosphatase activity of recombinant UmPP2C in the presence of Mg^{2+} than in the presence of Mn^{2+} suggests that Mg^{2+} is a more favorable cofactor for UmPP2C activity than Mn^{2+}. Although the intracellular free Mg^{2+} concentration of the ciliates is not known, highly elevated phosphatase activity of the recombinant UmPP2C by addition of just 1 mM Mg^{2+} in combination with the constitutive transcription of the gene in the ciliates suggest that active PP2C may always be necessary for ciliates to rapidly dephosphorylate target proteins.

Fig. 1. Multiple alignment of deduced amino acid sequences corresponding to the eleven conserved motifs of UmPP2C, MaPP2C and other PP2C members. As in other PP2Cs, UmPP2C and MaPP2C have amino acid residues binding phosphate ions (empty triangle) and metal ions (filled triangles). The 10 invariant amino acid residues within the PP2C sequences are presented as bold characters. (D – *Danio*, L – *Leishmania*, Pa – *Paramecium*, Pl – *Plasmodium*, S – *Schizosaccharomyces*, T – *Toxoplasma*). The numbers in square brackets mean the number of amino acids residues before or between motifs.

Table 2. Identity of amino acids sequence of UmPP2C and MaPP2C with other PP2C sequences.

	Accession No.	Identity to UmPP2C (%)	Identity to MaPP2C (%)
Uronema marinum	GQ465984	100	51.9
Miamiensis avidus	GQ465985	51.9	100
Paramecium tetraurelia	P49444	54.5	59.5
Plasmodium falciparum	XP_001351416	22.7	23.9
Plasmodium falciparum -1	O43966	13.8	15.5
Leishmania chagasi	P36982	20.3	17.2
Toxoplasma gondii	CAC86553	18.5	18.9
Toxoplasma gondii-1	ABV44288	15.2	16
Entamoeba histolytica	BAA89274	27.7	31.3
Giardia intestinalis	AAA74895	19.5	21.1
Caenorhabditis elegans	P49595	23.7	28.3
Schizosaccharomyces pombe	A56058	19.7	20.6
Saccharomyces cerevisiae	AAB64644	21.4	26.6
Homo sapiens PP2C alpha	IA6Q	27.6	26.5
Arabidopsis thaliana	NP_194914	35.3	37.8
Rattus norvegicus	AAC97497	22.3	22.1
Mus musculus	BAA05662	27.3	25.8
Danio rerio	NP_571504	25.6	26.6

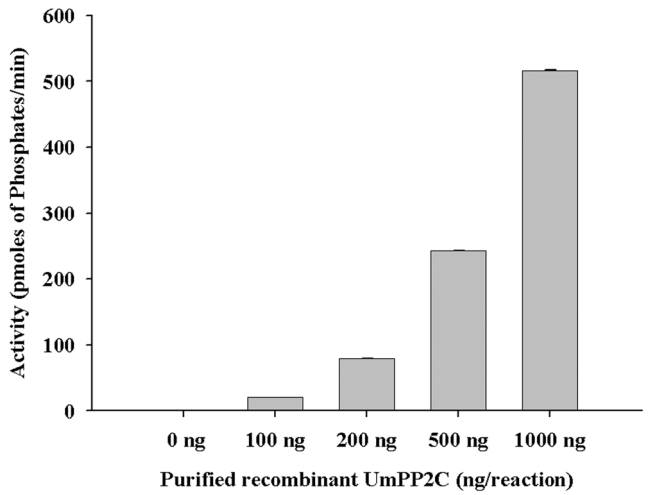

Fig. 2. Phosphatase activity of recombinant UmPP2C protein according to added concentrations. Phosphatase activity of purified recombinant UmPP2C was measured using a non-radioactive assay system, and phosphatase activity was determined by measuring dephosphorylation of chemically synthesized phosphopeptide, RRA(pT)VA. The phosphatase activity was presented as pmol per min. calculated from the standard curve of KH_2PO_4.

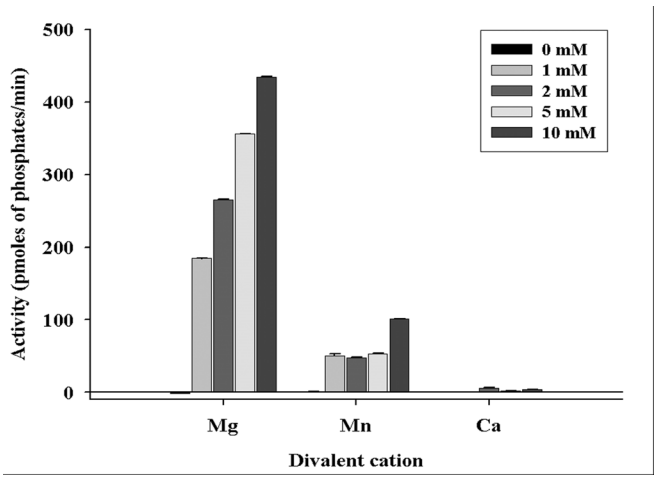

Fig. 3. Requirement of divalent cations for the protein phosphatase activity of purified recombinant UmPP2C protein. Effects of divalent cations on the phosphatase activity were determined by assays using various concentrations of cations such as Mg^{2+}, Mn^{2+} and Ca^{2+}. The highest phosphatase activity was observed in the presence of Mg^{2+}.

Table 3. Inhibition of phosphatase activity with various phosphatase inhibitors.

Inhibitors	Working concentration	% activity	% inhibition
Okadaic acid (μM)	0	100	
	1	100	0
	5	102	–2
	10	96	4
	20	95	5
NaF (mM)	0	100	
	10	29	71
	50	9	91
	100	10	90
	200	-6	106
Ca (mM)	0	100	
	1	0	100
	5	0	100
	10	0	100
	20	0	100
EDTA (mM)	0	100	
	10	7	93
	20	7	93
	40	8	92

It has been well demonstrated that PP2C acts to reverse protein kinase cascades triggered by stress (Shiozaki and Russell 1995, Gaits *et al.* 1997). In the present study, PP2C of *Uronema marinum* (UmPP2C) was detected as a gene upregulated its expression by exposure to PMA, a strong protein kinase C (PKC) activator. The expression of UmPP2C was not significantly different from control when the concentrations of PMA were low (0.5–5 μg/ml), but the expression was significantly increased when the ciliates were exposed to more than 10 μg/ml of PMA. These results suggest that UmPP2C dephosphrylates proteins phosphorylated by protein kinases as in other eukaryotes and has a regulatory function against abrupt increase of protein phosphorylation triggered by strong stimulations.

The regulatory roles of PP2C in response to stress have been demonstrated in *Arabidopsis* (Sheen 1998, Tahtiharju and Palva 2001), in yeast (Maeda *et al*. 1994, Shiozaki *et al.* 1994), and in mammalian cells (Hanada *et al.* 1998, Tong *et al.* 1998, Hanada *et al.* 2001, Tamura *et al.* 2002, Meskiene *et al.* 2003). However, there is little information with regard to functional aspects of parasitic protozoan PP2C in relation to stress or infection. As the present two ciliate species are facultative parasites, they can infect fish, and during the course of

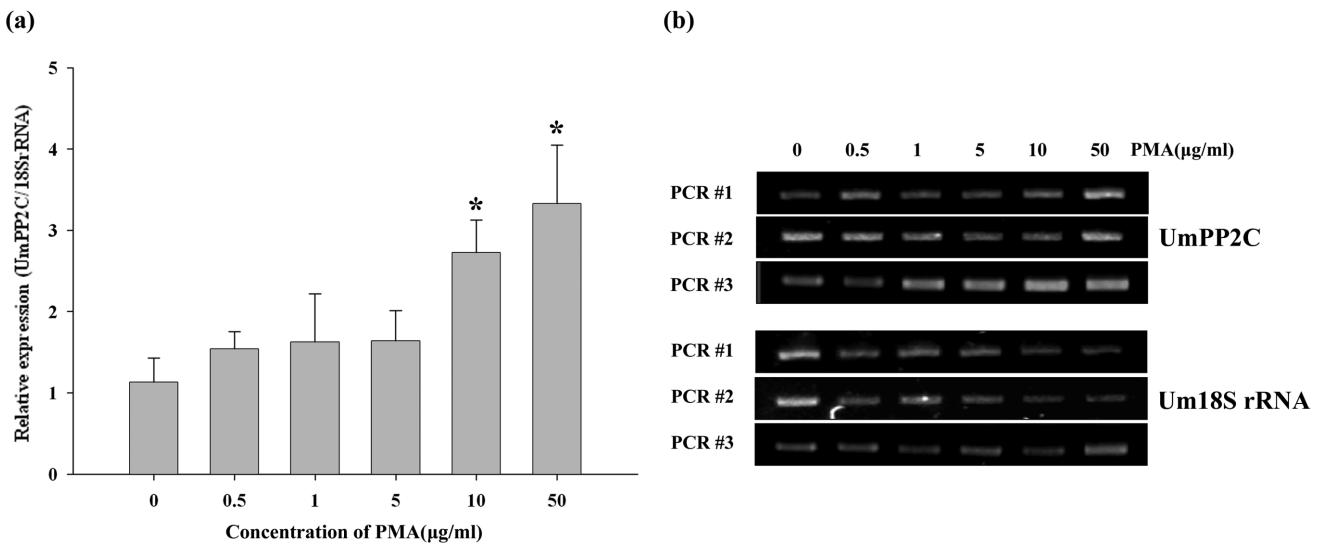

Fig. 4. Change of mRNA expression of UmPP2C gene by PMA treatment Based on semi-quantitative RT-PCR. Total RNA was isolated from *U. marinum* treated with PMA at various concentrations. *U. marinum* 18S ribosomal RNA gene was included as an external control. (**a**) – representative Et-Br stained gels showing the RT-PCR bands of *U. marinum* PP2C and 18S rRNA; (**b**) – scanning densitometry analysis to determine the relative mRNA levels of *U. marinum* PP2C based on the normalization against 18S rRNA control. Histograms indicate the means of triplicate examinations and standard errors were indicated by T bars. * – significantly different from the control group ($p < 0.05$).

infection, they shall be underwent dramatic changes in their surrounding environment. Therefore, further researches focused on the role of PP2C in the adaptation of ciliates to infection of fish should be conducted.

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