Identification of phorbol ester-induced genes by suppression subtractive hybridization and characterization of the protein phosphatase 2C and a surface antigen in *Uronema marinum* (Ciliata: Scuticociliatida)

Phorbol ester에 의해 유도되는 *Uronema marinum* (Ciliata: Scuticociliatida)의 유전자 및 protein phosphatase 2C와 표면항원자의 특성

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in the Department of Fish Pathology, The Graduate School, Pukyong National University

February 2005

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February 25, 2005

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Abstract

A subtracted cDNA library of phorbol 12-myristate 13-acetate (PMA)-treated *Uronema marinum* was constructed by the suppression subtractive hybridization technique, a sensitive PCR-based subtraction approach, to screening PMA-induced genes that might be directly involved in parasite infection. As a result of SSH screening, hundreds of colonies were obtained which contained cDNA fragments representing PMA-inducible mRNAs and sequence analyses of randomly selected 243 clones were performed. Reverse transcription polymerase chain reaction was used to confirm these results. After sequence analyses and BLAST X searches of these clones, two clones - protein phosphatase 2C and a surface antigen - were selected as candidates for the vaccine development and further analysed. The full-length cDNA of UmPP2C was 1137 bp containing a 933 bp of open reading frame encoding 310 amino acids. The deduced amino acid sequence of UmPP2C had 56% identity with the membrane-bound protein phosphatase Type 2C of *Paramecium tetraurelia*. The predicted UmPP2C protein was 310 amino acids in length with a molecular weight of 35 kDa and an isoelectric point of 5.06. The full length cDNA of surface antigen

was 3213 bp containing a 2754 bp of open reading frame encoding 917 amino acids. The predicted surface antigen was 917 amino acids in length with a molecular weight of 104 kDa and an isoelectric point of 4.53. The deduced amono acid sequence had repeated patterns containing CXXC and CXC motif and had 25% identity with the putative surface antigen with EGF domains and furin-like repeats of *Paramecium tetraurelia*. The entire ORF sequence of UmPP2C and the partial sequence of surface antigen were mutated by site-directed mutagenesis for using in other expression system.

Introduction

Several scuticociliate species belonging to the genera *Uronema, Miamiensis* and *Philasterides* are facultative histophagous parasites in marine fish (Thompson & Moewus, 1964; Cheung *et al.*, 1980; Yoshinaga & Nakazoe, 1993; Dykov & Figueras, 1994; Dragesco *et al*, 1995; Gill & Callinan, 1997; Munday *et al.*, 1997; Sterud *et al.*, 2000; Iglesias *et al.*, 2001). These ciliates are characterized by their high potential for invading systemically and destroying fish tissues, leading to high mortalities in cultured fishes.

In Korea, scuticociliatosis is a serious problem in culturing olive flounder *Paralichthys olivaceus*, and the causative agent has been identified as *Uronema marinum* by morphological characteristics (Jee *et al.*, 2001) and homology of 18S rRNA sequence (Kim *et al.*, 2004). Although there have been some reports on the in vitro killing of the scuticociliates by several chemotherapeutics (Yoshimizu *et al.*, 1993; Novotny *et al.*, 1996; Cribb *et al.*, 1999), any effective control measures in vivo or in culturing fields have not been developed. Also, there are currently no vaccines available to control or decrease the prevalence of the parasite.

As scuticociliates are not obligate parasites but opportunistic parasites, certain factors would influence on the change of their behaviour from free-living, bacteriovorous to parasitic, histophagous. Until now, however, the parasite and the host factors that determine the onset of disease remain undetermined. Several researchers have assumed that immunosuppression of fish by various stressors including handling, environmental pollution, inappropriate diet, and wounds could make it possible for scuticociliates to

cross the immunological barriers of fish (Cheung *et al.*, 1980; Dragesco *et al.*, 1995; Munday *et al.*, 1997; Sterud *et al.*, 2000; Iglesias *et al.*, 2003). However, it cannot be excluded that strengthening of scuticociliates infection potential by certain factors present in fish culture tanks might be involved in the infection of scuticociliates.

Transcriptional regulation of gene expression mediated by signal transduction mechanisms is an important way of modulating cellular responses to extracellular stimuli. In mammals, protein kinase C (PKC) constitutes a family of serine-threonine kinases that catalyze numerous biochemical reactions critical to the function of many cellular constituents. Situated at the crossroads of many signal transduction pathways, PKCs are also crucial to the relay of a large diversity of signals from the cytoplasm to the nucleus. PKCs are reversibly activated by upstream signaling elements such as growth factor receptors, and are able to reversibly activate downstream signaling modules such as the Raf-1 and the bcl-2 cascade. The Raf-1 cascade is one of the main systems for the transduction of signals through the cytoplasm. Its overstimulation by hyperactive PKC may therefore contribute to the erroneous expression of many genes, including those that participate in cell proliferation and invasion. The involvement of PKC in the activation of the bcl-2 protein is thought to represent an important cytoprotective device against lethal stimuli. Similarly, ciliates have a well developed signal cascade mechanisms as in multi-cellular eukaryotes (Csaba 1985, 1994; Kovács & Csaba 1995; Christensen *et al.*, 1998). Straarup *et al.* (1997) reported increased survival and proliferation of *Tetrahymena thermophila*, a freshwater ciliated protozoan, by treatment with phorbol 12-myristate 13-actate (PMA), a direct activator of PKC, and Christensen *et al.* (1998) reported that staurosporine, a PKC inhibitor, blocked signal transduction associated with cell survival, proliferation and chemosensory

behaviour in *T. thermophila*. Therefore, in the present study, we postulated that genes of *U. marinum* expressed differentially by PKC activation would facilitate to find genes associated with invasion into host fish and resistance against host immune factors.

A PCR-based subtraction approach, termed suppression subtractive hybridization (SSH) have been reported to be highly efficient at detecting gene species expressed differentially. SSH technique is applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue specific, or other differentially expressed genes (Diatchenko *et al.*, 1996). In the present study, therefore, a subtracted cDNA library of phorbol 12-myristate 13-acetate (PMA) - treated *U. marinum* was constructed using this SSH technique to screening PMA-induced genes that might be directly involved in parasite infection. As a result of SSH screening, hundreds of colonies were obtained which contained cDNA fragments representing PMA-inducible mRNAs. After sequence analyses and BLAST X searches of these clones, two clones - protein phosphatase 2C and a surface antigen - were selected as candidates for the vaccine development and further analysed.

Materials and Methods

1. Parasites

U. marinum were isolated from the infected olive flounders (*Paralichthys olivaceus*), which were obtained from the fish farm located in Jeiju island. Polymerase chain reaction was performed to confirm *U. marinum* using *U. marinum* 18S rRNA gene-specific primers. *U. marinum* were cultured in 0.25% yeast medium, and were harvested at the logarithmic phase. *U. marinum* cells were harvested by centrifugation at 1000 x g for 5min and divided two groups or three groups and adjusted $5x10^5$ cells per ml in filtered sea water containing 0.05% yeast extract.

For RNA extraction, cells were incubated in the Phorbol 12-myristate 13-acetate(PMA)-contained medium for an hour and harvested by centrifugation at 1,000 x g for 2min at 0℃ and 1ml of RNA later was added to the pellets immediately. Cells in RNA *later* were stored at -20℃ until used. PMA was used at 0 and 10㎍ per ml for SSH or 0, 10, 50㎍ per ml for RT-PCR.

2. RNA sample preparation

2.1. Isolation of Total RNA from *U. marinum*

Total RNA and poly A$^+$ RNA was isolated from both PMA-treated (10㎍ per ml) and non-treated *U. marinum*. Total RNA was isolated using Tri

Reagent (Sigma). $1x10^7$ cells of *U. marinum* were lysed in 1.5ml tube by adding 1ml Tri Reagent and incubated for 5min at room temperature to permit complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added to the lysate and the tube was vigorously shaken by hand for 15 seconds. The sample was centrifuged at 12,000 x g for 15 minutes at 4℃. The aqueous phase was transferred to a new tube and the RNA was precipitated by adding same volume of isopropyl alcohol for 10 minutes at room temperature. RNA was precipitated by centrifugation at 12,000 x g for 10 minutes and washed with 70% ethanol. The pellet was air-dried for 5 minutes and dissolved in diethylpyrocarbonate (DEPC) treated water. Total RNAs were isolated several times and pooled.

2.2. Isolation of poly A$^+$ RNA from total RNA

poly A$^+$ RNA from total RNA was isolated using PolyATract mRNA isolation system IV (Promega). The 0.5mg of total RNA was dissolved in 500$\mu\ell$ of RNase-free water in a sterile, RNase-free 1.5ml tube and the tube was placed in a 65℃ heating block for 10 minutes. 3$\mu\ell$ of the Biotinylated-Oligo(dT) Probe and 13$\mu\ell$ of 20X SSC were added to the tube contained the total RNA and the mixture was incubated at room temperature until completely cooled. The entire contents of this annealing reaction were added to the tube containing the washed Streptavidin-Paramagnetic Particles (SA-PMPs) and incubated at room temperature for 10 minutes. The SA-PMPs were captured using th Magnetic Stand and the supernatant was carefully remove without disturbing the SA-PMP pellet. The particles were washed four times with 0.1X SSC (100$\mu\ell$ per wash) and after final wash, the supernatant was completely removed. The poly A$^+$ RNA was eluted in 250$\mu\ell$ of the RNase-free water and dissolved in 15$\mu\ell$ of RNase-free water

after precipitation. After total and poly A RNA isolation, RNA's integrity was determined by ultraviolet (UV) spectrophotometry (Ultrospec® 3100 *pro*, Amersham Phamacia Biotech) and formaldehyde gel electrophoresis.

3. Subtracted cDNA library construction by SSH (Suppression Subtractive Hybridization)

Suppression Subtractive Hybridization was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. The thermal cycler used in all procedures was iCycler™(BioRad, USA).

3.1. Preparation of PMA-treated and non-treated *U. marinum* cDNA

3.1.1. First- and Second- strand cDNA synthesis

2㎍ of poly A$^+$ RNA from each PMA-treated and non-treated *U. marinum* were reverse-transcribed into single-stranded cDNA in 10㎕ reaction containing 1μM cDNA synthesis primer, 1× First-strand buffer, 1mM dNTP, and 20 units AMV reverse transcriptase at 42℃ for 1.5 hr in an air incubator. The second-strand cDNAs were synthesized using Second-strand Enzyme cocktail containing DNA polymerase I, RNase H and *E. coli* DNA ligase and blunt-ending of DNA ends was performed by T4 DNA polymerase. The cDNAs were then phenol-extracted, ethanol precipitated, and resuspended in 50㎕ of H_2O and digested by 15 units of *Rsa* I for 1.5 hr. After phenol extraction and ethanol-precipitation, the resulting cDNA pellets were dissolved in 5.5㎕ of H_2O.

3.1.2. Adaptor ligation

PMA-treated *U. marinum* cDNA was separated into two pools that were ligated to different adaptors (1 and 2R), while the non-treated *U. marinum* cDNA was not ligated with an adaptor. 1㎕ of *Rsa* I-digested PMA-treated *U. marinum* cDNA was diluted in 5㎕ of H_2O. 2㎕ of diluted cDNA was then ligated to 2㎕ of adapter 1 and adapter 2R in separate ligation reactions in total volume of 10㎕ at 16℃ overnight, using 400 units of T4 DNA ligase. After ligation, 1㎕ of 20× EDTA/Glycogen mix was added and the samples were heated at 72℃ for 5 minutes to inactivate the ligase and stored at -20℃ until hybridization procedure.

3.2. Subtractive Hybridization

1.5㎕ of Non-treated *U. marinum* cDNA and 1㎕ of 4× Hybridization buffer were added to each of two tubes containing 1.5㎕ of adaptor 1- and 2R-ligated PMA-treated *U. marinum* cDNA. The samples were denatured at 98℃ for 1.5 minutes after overlaying with one drop of mineral oil, and then allowed to hybridize at 68℃ for 8 hr in a thermal cycler. After this first hybridization, the two samples were combined and 1㎕ of freshly heat-denatured non-treated *U. marinum* cDNA was added, and then the sample was allowed to hybridize at 68℃ overnight in a thermal cycler. Final hybridization was then diluted in 200㎕ of dilution buffer, heated at 68℃ for 7 minutes and stored at -20℃ until PCR amplification. After this second hybridization, PMA-treated *U. marinum* cDNA specific, double-stranded cDNAs with both adapters were generated.

3.3. PCR Amplification

Two PCR amplifications were performed in 25$\mu\ell$ reaction. The PCR primer 1, used in PCR amplifications, specifically binds to adapter 1 and 2R that allow specific amplification of PMA-treated *U. marinum* cDNAs. Primary PCR reaction contained 1$\mu\ell$ of diluted, subtracted cDNA and 24$\mu\ell$ of PCR master mixture prepared using the Advantage cDNA PCR kit and PCR primer 1. To extend the adaptors, the reaction mix was incubated at 75℃ for 5 minutes. This step created binding sites for the PCR primers. PCR was performed with the following parameters: 94℃ for 30 seconds; 27 cycles at (94℃ for 30 seconds; 66℃ for 30 seconds; 72℃ for 1.5 minutes). The primary PCR products were diluted 10-fold in H_2O and 1$\mu\ell$ of diluted primary PCR product was used as a template in secondary, nested PCR. PCR master mixture for the Secondary PCR amplification was prepared using nested PCR primer 1 and nested PCR primer 2R instead of PCR primer 1 in primary PCR. PCR parameters were as follows: 12 cycles at (94℃ for 30 seconds; 68℃ for 30 seconds; 72℃ for 1.5 minutes); and a final extension at 72℃ for 7 minutes. 8$\mu\ell$ from each primary and secondary PCR reaction were analyzed by 2% agarose gel electrophoresis.

4. Analysis of the Subtracted cDNAs

4.1. Cloning and Analysis of DNA sequences

The PCR product from the secondary PCR was purified with Wizard SV Gel and PCR clean-up system (Promega) and 3$\mu\ell$ of purified PCR product was directly ligated into pGEM-T easy plasmid vector (Promega) to make a

subtracted library and then transformed to *Escherichia. coli* strain DH5α competent cells. The transformed *E. coli* cells were plated onto Luria-Bertani (LB) agar plate containing ampicillin(50μg per ml), 5-bromo-4-chloro-3- indolyl-$_D$-galactoside (X-GAL) and Isopropyl 1-thio-β-$_D$-galactopyranoside (IPTG) and incubated at 37℃ overnight. Individual white colonies were then picked randomly and incubated in LB medium containing ampicillin overnight at 37℃. Plasmid DNAs were isolated from these cultures using GeneALL Plasmid miniprep kit (General Biosystem) according to the manufacturer's instruction. DNA sequencing was performed using Automatic sequencer ABI3730xl (96-capillary, Applied Biosystems) according to the protocol of ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kits v3.1 at the Macrogen. Homology searches of the translated amino acid sequences were performed using the BLAST X program of National Center for Biotechnology Information (NCBI).

4.2. Reverse-transcription polymerase chain reaction (RT-PCR)

To further investigate and quantify differential expression of the isolated genes, semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed. Total RNAs were isolated from the *U. marinum* treated with PMA at 0, 10, 50μg per ml of final concentration and RT-PCR was performed using AccuPower® RT/PCR PreMix (Bioneer). Primers used in the RT-PCR were shown in Table 1. 1μg of total RNA and the reverse primer were mixed and incubated at 70℃ for 5 minutes and placed on ice immediately. The incubated mixture and the forward primer were transfered to AccuPower® RT/PCR PreMix tube, then final reaction volume was adjusted to 20$\mu\ell$ by adding DEPC-dH$_2$O. The lyophilized blue pellet in the Premix was dissolved by pipetting and cDNA synthesis reaction and PCR

were performed as follows : 42℃ for 60 minutes, 94℃ for 5 minutes (cDNA synthesis and RTase inactivation) ; 94℃, 3 minutes; 30cycles at (94℃ for 30 seconds; 55℃ for 30 seconds; 72℃ for 30 seconds) ; and a final extention at 72℃ for 7 minutes. 4㎕ of the reaction products were electrophoresed on 1% agarose gel and stained with ethidium bromide. For normalization, 18S rRNA gene was used as an internal standard.

Table 1. The sequences of primers used in RT-PCR

Primers	Sequences
PP2C For-1	5′-GCTGGTGATAGTAGATCTGTTTTATGC-3′
PP2C Re	5′-TCCAACACCATTTGAAGTATCTGAGGC-3′
ScuGAL For-1	5′-GCTATGATGGATGGTATTATAAAGAAGATG-3′
ScuGAL Re-1	5′-ACCTACAGCTTCATTTTCACATTTAG-3′
UMF	5′-CTTCTGTACAGTCTCATTTC-3′
UMR	5′-AACGCCAATTAAAGATCAAC-3′

5. Isolation of full-length cDNAs of the selected clones by Rapid Amplification of cDNA ends(RACE)

In this study, 5′- and 3′- RACE were performed separately to determine full nucleotide sequences of the several selected clones. The RACE was performed using the Clontech SMART™ RACE cDNA Amplification Kit according to the manufacturer's instructions.

5.1. First-strand cDNA synthesis for 5′-RACE and 3′-RACE

For 5′-RACE, 1㎍ of total RNA isolated from PMA-treated *U. marinum* was reverse-transcribed into cDNA in 10㎕ reaction containing 1μM 5′-CDS Primer, 1μM SMART II A Oligo, 1X First-strand buffer, 2mM DTT, 1mM dNTP mix and 1㎕ of PowerScript Reverse Transcriptase at 42℃ for 1.5hr. For 3′-RACE, 1μM 3′-CDS primer A was contained in the reaction mixture instead of the 1μM 5′-CDS Primer and 1μM SMART II A Oligo. The reaction mixtures of first-strand cDNA synthesis were diluted with 100㎕ of Tricine-EDTA buffer and heated at 72℃ for 10 minutes. Samples were stored by making small aliquots at -20℃ until PCR.

5.2. PCR amplification

Primary and the Nested PCRs were performed to amplify the 5′- and 3′- cDNA ends of two selected genes using 1.5㎕ of the first-strand cDNAs, Universal primers and gene specific primers. Primers used in the RACE PCR were designed from partial sequences obtained by SSH and their nucleotide sequences were shown in Table 2.

Table 2. The sequences of primers used in 5′- and 3′- RACE PCR

Primers	Sequences
Universal primer A mix(UPM)	Long:5′-CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT-3′ Short : 5′-CTAATACGACTCACTATAGGGC-3′
Nested universal Primer A	5′-AAGCAGTGGTATCAACGCAGAGT-3′
PP2C For-1	5′-GCTGGTGATAGTAGATCTGTTTTATGC-3′
PP2C For-2	5′-CAGTGATGCTGGGGGATTCGTAAG-3′
PP2C Re	5′-TCCAACACCATTTGAAGTATCTGAGGC-3′
PP2C Re-1	5′-GTCCCGGTTATCAGTAGGATCACTTG-3′
ScuGAL For-1	5′-GCTATGATGGATGGTATTATAAAGAAGATG-3′
ScuGAL For-2	5′-TGCTAGATGCAAAAGTGAACAAGTAGG-3′
ScuGAL Re-1	5′-ACCTACAGCTTCATTTTCACATTTAG-3′
ScuGAL Re-2	5′-TCCTTCACTGTCGACCAAATAATAACC-3′
Gal-RACE-For-1	5′-TGCTGAAGTAGGATTATAATGTGCTGG-3′
Gal-RACE-For-2	5′-AGTTCCAGAATGCCCCTGTGAAGAAG-3′

5.3. Cloning and analysis of nucleotide sequences

Purified PCR products were cloned into pGEM-T easy vector (Promega). Ligation reactions of vector and insert (PCR products) were performed at 12℃ for overnight using 3 units of T4 DNA ligase (Promega) and the entire ligation mixtures were transformed to *E. coli* strain DH5α comptetent cells. Plasmid DNAs were isolated from positive white colonies on the ALB/X-Gal/IPTG agar plate using GeneALL Plasmid miniprep kit (GeneralBiosystem) according to the manufacturer's instruction. DNA sequencing was performed by automated means at the Macrogen.

6. Site-directed mutagenesis of selected genes

6.1. Site-directed mutagenesis of putative protein phosphatase 2C

Total RNA was isolated from *U. marinum* and the ORF of putative protein phosphatase 2C (UmPP2C) was amplified by RT-PCR. The 933 bp of purified PCR product was ligated into pGEM T-easy vector and transformed to *E. coli* strain DH5α comptetent cells. Plasmid DNAs were isolated from positive white colonies on a ALB/X-Gal/IPTG agar plate using GeneALL Plasmid miniprep kit (GeneralBiosystem) and DNA sequences were confirmed. The ORF of UmPP2C gene contained 16 TAA triplets coding for Q (Glutamine) which were conterted to CAA. Eight pairs of synthesized primers and thermal cycling based QuikChange® Site-Directed Mutagenesis kit (Stratagene) were used to convert all 16 TAA/Q triplets to CAA/Q triplets. DNA sequences of primers used in site directed mutagenesis of UmPP2C were shown in Table 3. Briefly, 1㎕ of pGEM T-easy vector

containg the UmPP2C ORF as template, 1X reaction buffer, 0.5㎕ (125ng per ㎕) of Primer 1, 0.5㎕(125ng per ㎕) of Primer 2, 0.5㎕ of dNTP mix, 1.25 units of *PfuTurbo* DNA polymerase and 20㎕ of ddH_2O were mixed in a 0.2ml thin wall tube. Thermal cycling was perfromed to synthesize mutant strand with the fallowing parameter: 95℃ for 30 seconds; 12 cycles at (94℃ for 30 seconds; 55℃ for 1 minutes; 68℃ for 5 minutes). 6㎕ of reaction product was transfer to a separate tube and 5 units of *Dpn* I restriction enzyme was added directly to the amplification reaction and the reaction mixture was immediately incubated at 37℃ for 1hr to digest the parental supercoiled dsDNA. After incubation, 6㎕ of *Dpn* I-digested reaction mixture and 6㎕ of thermal cycling reaction product transfered before were analysed on 1% agarose gel. 3㎕ of *Dpn* I-digestion reaction mixture was then transformed to *E. coli* strain XL-1 Blue supercompetent cells contained in the Kit. The transformed *E. coli* cells were plated onto Luria-Bertani(LB) agar plate containing ampicillin (50㎍ per ml) incubated at 37℃ overnight. Plasmid DNA isolated from the cultures of an individual colony was used the next mutagenesis reaction. Repeated mutagenesis reactions were performed using eight pairs of primers and the DNA sequence of isolated plasmid was confirmed after the final reaction.

Table 3. The sequences of primers used in site-directed mutagenesis of putative PP2C gene of *U. marinum* (UmPP2C).

Primer	Primer sequences
1-1	5′-CTTATTTACAAGCTTGTGCTAGTGAAATGCAAGGATGG-3′
1-2	5′-CCATCCTTGCATTTCACTAGCACAAGCTTGTAAATAAG-3′
2-1	5′-GGATGCGCATATTTTACAAATGAATATTAATGGAG-3′
2-2	5′-CTCCATTAATATTCATTTGTAAAATATGCGCATCC-3′
3-1	5′-GAGGTAAAGAAGTTGCCCAATTTGTAGAAAAACAT-3′
3-2	5′-ATGTTTTTCTACAAATTGGGCAACTTCTTTACCTC-3′
4-1	5′-CTGAAAGTGGTCAACAAGAATTGAACCAAATTAGAGCAG-3′
4-2	5′-CTGCTCTAATTTGGTTCAATTCTTGTTGACCACTTTCAG-3′
5-1	5′-CCTAATGAAGAACAATCAGGAGGACAATCTTATGCTGG-3′
5-2	5′-GGAGCATAAGATTGTCCTCCTGATTGTTCTTCATTAGG-3′
6-1	5′-AGATAGACCTAGAGACCAACAATTAATTATATCCAAC-3′
6-2	5′-GTTTGGATATAATTAATTGTTGGTCTCTAGGTCTATCT-3′
7-1	5′-ACTAATGAAGAACTTATCCAATATTGTAAAGAAAG-3′
7-2	5′-CTTTCTTTACAATATTGGATAAGTTCTTCATTAGT-3′
8-1	5′-AAGAAAGAATTGAAAAACAACAAGACTTAAATCAAATA-3′
8-2	5′-TATTGATTTTAAGTCTTGTTGTTTTTCAATTCTTTCTT-3′

6.2. Site-directed mutagenesis of putative surface antigen

Total RNA was isolated from *U. marinum* and the partial sequence of putative surface antigen gene ORF was amplified by RT-PCR. The 519 bp of purified PCR product was ligated into pGEM T-easy vector and transformed to *E. coli* strain DH5α comptetent cells. DNA sequences of isolated plasmids were confirmed. The partial sequence of surface antigen gene ORF contained 12 TAA triplets and 3 TAG triplets coding for Q (Glutamine) which were converted to CAA. Six pairs of synthesized primers and QuikChange® Site-Directed Mutagenesis kit were used to convert all 12 TAA/Q and 3 TAG/Q triplets to CAA/Q and CAG/Q triplets with the same procedure. Primer sequences used in Site-directed mutagenesis of surface protein is shown in Table4. 6 times of mutagenesis reactions were performed with same procedure of mutagenesis of UmPP2C. After sixth reaction was finished, DNA sequencing was perfomed to confirm mutated sequences.

Table 4. The sequences of primers used in site-directed mutagenesis of putative surface antigen.

Primer	Primer sequence
1-1	5′-GATAAGGTTAATGCTCAATGTCAGCAATGTTTAAATG-3′
1-2	5′-CATTTAAACATTGCTGACATTGAGCATTAACCTTATC-3′
2-1	5′-GTAGGTATAACATGCCAAGGACAAAATAGATCTAATG-3′
2-2	5′-CATTAGATCTATTTTGTCCTTGGCATGTTATACCTAC-3′
3-1	5′-GGATATTATGATGATCAACAAAACAATGCTGATTGTC-3′
3-2	5′-GACAATCAGCATTGTTTTGTTGATCATCATAATATCC-3′
4-1	5′-CTGTGCTCAGTGCACAAATAATTCAGATTGCCAAGTTTG-3′
4-2	5′-CAAACTTGGCAATCTGAATTATTTGTGCACTGAGCACAG-3′
5-1	5′-CAGTGAAGGAATTCAAACTTGTCAAAAATGTCCATTG-3′
5-2	5′-CAATGGACATTTTTGACAAGTTTGAATTCCTTCACTG-3′
6-1	5′-GAATGTCAAGGCCAAGGAAGAACTGTAACTCCTCAATG-3′
6-2	5′-CATTGAGGAGTTACAGTTCTTAATTGGCCTTGACATTC-3′

Results

1. Screening of PMA-inducible mRNAs by SSH

To identify mRNAs that are up-regulated in the PMA-treated *U. marinum*, SSH was performed using the PMA-treated *U. marinum* as the tester and the non-treated *U. marinum* as the driver. After two rounds of hybridization and two rounds of PCRs to selectively amplify the differentially expressed cDNA, a subtracted cDNA library was constructed. As a result of SSH screening, hundreds of colonies were obtained which contained cDNA fragments representing PMA-inducible mRNAs. From the subtracted library, 243 clones were randomly selected and sequenced. The DNA sequences were compared with those in the GenBank/NCBI database using the BLAST X searches. Characterization and functional classification of 243 SSH clones from BLAST X searches were shown in Table 5.

Table. 5. Characterization and functional classification of SSH clones.

	number of clones
with e-value < 1 ×10 ^{5}	**138**
Proteases	10
Cytoplasmic function	34
Hypothetical protein	4
Unknown	1
Others	89
with e-value ≥ 1 ×10 ^{5}	**105**
Hypothetical protein	28
No match	6
Other miscellaneous	71
total	243

2. Confirmation of SSH results by RT-PCR of selected genes

Semi-quantitative RT-PCR was performed to determine the patterns of expression of two selected gene mRNAs, putative protein phosphatase 2C (UmPP2C) and surface antigen. As shown in Fig.1, expression of UmPP2C mRNA was up-regulated in PMA-treated *U. marinum* at concentration of 10 μg per ml and expressions of surface antigen mRNA up-regulated by PMA treatment in a concentration-dependent manner.

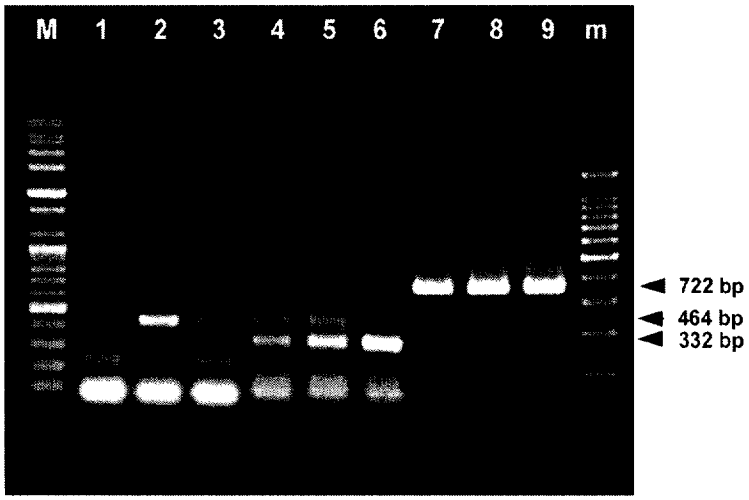

Fig. 1. Comparison of expression of UmPP2C and surface antigen between PMA-treated and non-treated *U. marinum* using RT-PCR. Total RNA samples were prepared from the PMA-treated *U. marinum*(at 0, 10, 50μg/ml of final concentrations). The one-step RT-PCR using AccuPower® RT/PCR PreMix(Bioneer) was performed equal amount of total RNAs as starting materials with specific primers. The PCR products were analysed on 1.5% agarose gel stained with ethidium bromide: 1-3, UmPP2C ; 4-6, surface antigen; 7-9, 18S rRNA gene; M, 1kb ladder molecular weight size marker (Bioneer)

3. Isolation and analysis of full-lenth cDNA of two selected clones

3.1 Full-length cDNA of putative protein phosphatase 2C in *U. marinum* (UmPP2C)

After sequencing the internal fragment through SSH procedure, 5′- and 3′- RACE PCR were used to capture the upward and downward fragments of UmPP2C. The RACE PCR procedure for cloning full-length cDNA of UmPP2C is shown in Fig. 2. The amplified upward, downward and internal fragments were then assembled to get the full-length cDNA of UmPP2C by alignment and deletion of overlapping fragments. The full-length cDNA of UmPP2C was 1137 bp, containing a 933-bp of open reading frame encoding 310 amino acids, a 4 bp of 5′-untranslated region(UTR), a 200 bp of 3′-untranslated region(UTR) including a poly(A) tail. The predicted UmPP2C protein was 310 amino acids in length with a molecular weight of 35028.12 Da and an isoelectric point of 5.06; these predictions were made based on a web analysis program/on web analysis software (http://us.expasy.org). The complete nucleotide sequence and the deduced amino acid sequence of UmPP2C are shown in Fig. 3. The deduced UmPP2C protein was used in alignment analysis using the ClustalW multiple alignment program. The results showed that, at the amino acid level, UmPP2C had 56% identity with the membrane-bound protein phosphatase Type 2C of *Paramecium tetraurelia*.

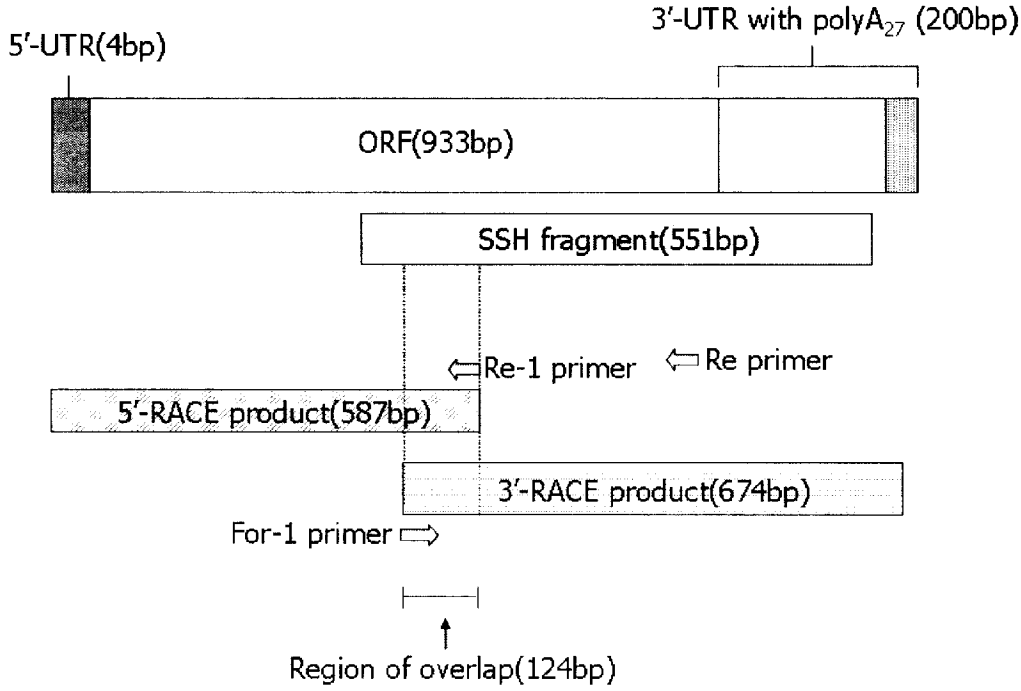

Fig. 2. The RACE PCR strategy for isolation of full-length cDNA of UmPP2C. Primers used in RACE PCR were designed from the sequences obtained SSH procedure. The full-length cDNA of UmPP2C was 1137 bp, containing a 933 bp of open reading frame encoding 310 amino acids, a 4 bp of 5′ UTR, a 200 bp of 3′-UTR including a poly(A)$^+$ tail.

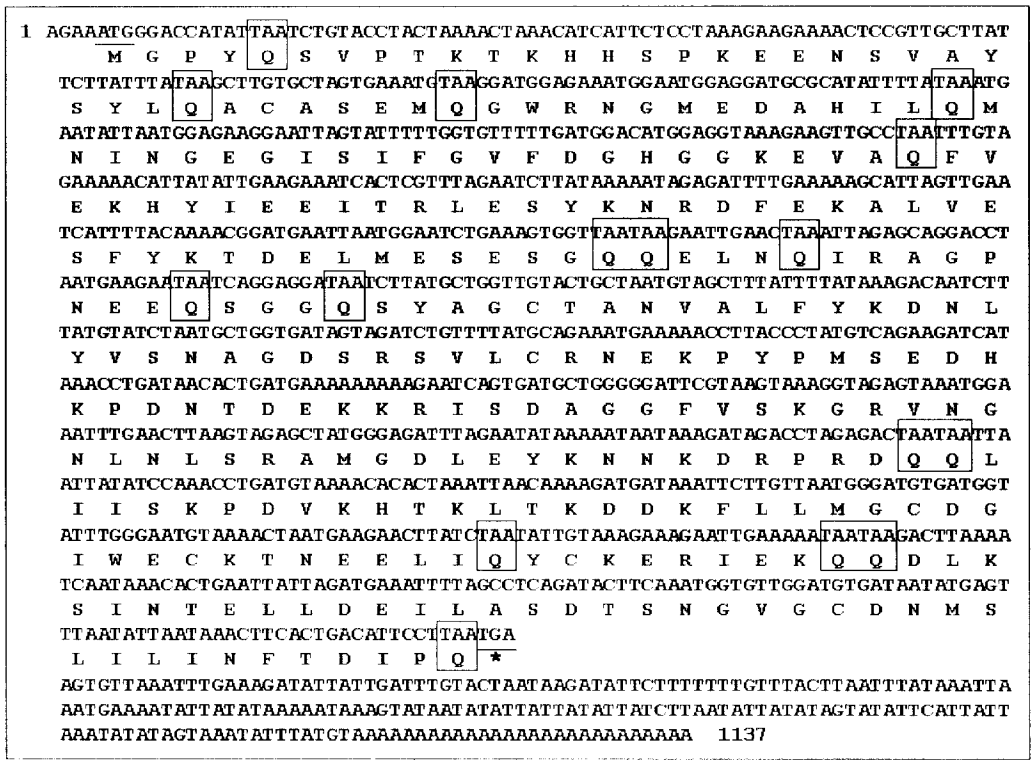

Fig. 3. The full-length cDNA sequence and deduced amino acid sequence of UmPP2C. The full-length cDNA of UmPP2C has a 933 bp ORF which has both the start codon (ATG) and the stop codon (TGA). The ATG and TGA codons are underlined. The TAA codons coding for Q were marked by the squares.

3.2 Full-length cDNA of putative surface antigen in *U. marinum*

Full-length cDNA of *U. marinum* surface antigen was obtained through SSH procedure, 5′- and 3′- RACE PCR with the same way to get the full-length cDNA of UmPP2C. The RACE PCR procedure for cloning full-length cDNA of surface antigen is shown in Fig. 4. The full-length cDNA of surface antigen was 3213 bp, containing a 2754-bp of open reading frame encoding 917 amino acids, a 368 bp of 5′-untranslated region, a 91 bp of 3′-untranslated region including a poly(A)$^{+}$ tail. The predicted surface antigen was 917 amino acids in length with a molecular weight of 104205.18 Da and an isoelectric point of 4.53. And the deduced amono acid sequence had repeated patterns containing CXXC and CXC motif. The results of homology searches using BLAST X program showed that, at the partial amino acid sequence level, *U. marinum* surface antigen had 25% identity with the Putative surface antigen with EGF domains and furin-like repeats of *Paramecium tetraurelia* and E-value was 1e-63.

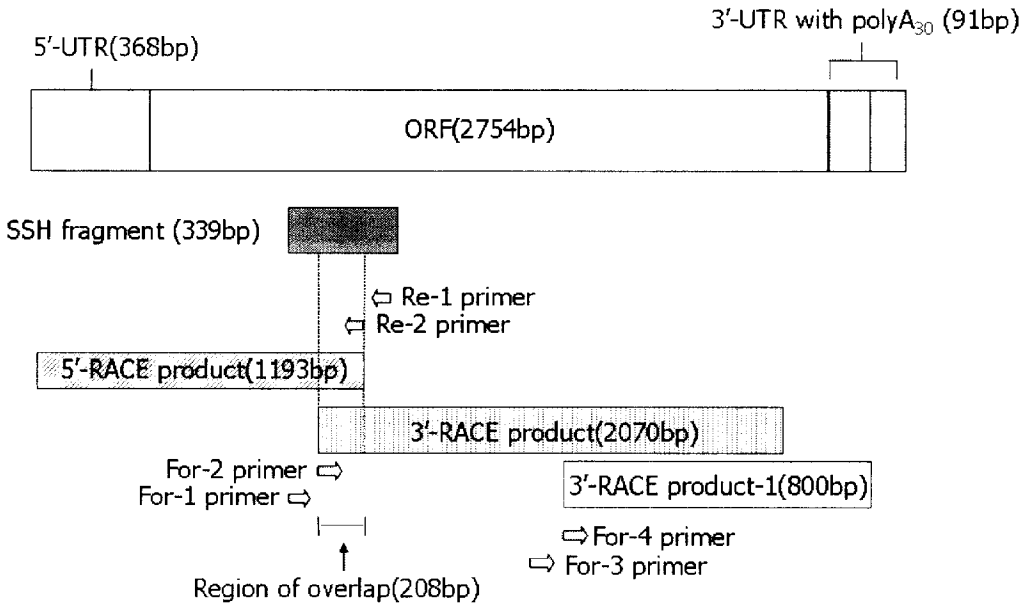

Fig. 4. The RACE PCR strategy for isolation of full-length cDNA of surface antigen. Primers used in RACE PCR were designed from the sequence obtained SSH procedure. Primers used to obtain the 3′-RACE product-1 fragment were designed from the sequence of the 3′-RACE product (2070bp). For-3 and For-4 primer indicate Gal-RACE-For-1 and Gal-RACE-For-2 primer in Table 2. The full-length cDNA of surface antigen was 3213 bp, containing a 2754 bp of open reading frame encoding 917 amino acids, a 368 bp of 5′-UTR, a 91 bp of 3′-UTR including a poly(A)$^+$ tail.

4. Site-directed mutagenesis

In this study, two genes (UmPP2C and surface antigen) were cloned from *U. marinum* by SSH and RACE. The heterologous expressions of these genes were impossible because in this protozoan the universal stop codons TAA and TAG code for Q. For this reason, site-directed mutagenesis was performed to convert TAA or TAG codon to CAA or CAG codon. 16 TAA/Q triplets of the UmPP2C cDNA and 12 TAA/Q triplets and 3 TAG/Q triplets of the partial sequence of surface antigen were mutated to universal TAA/Q or TAG/Q triplets successfully. Fig. 5 shows examples of site-directed mutagenesis reaction.

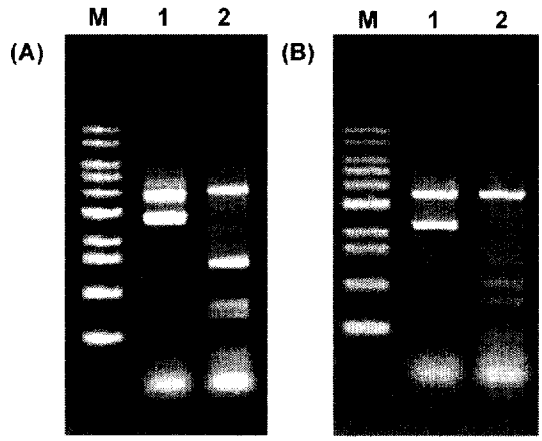

Fig. 5. Reaction examples of site-directed mutagensis of UmPP2C (A) and surface antigen (B). 5㎕ of reaction products were electrophoresed on the 1% agarose gel and stained ethidium bromide after thermal cycling (1) and *Dpn* I-digestion (2). M, 1kb ladder molecular weight size marker (Bioneer).

Discussion

In the preliminary experiment, increased protease activities were measured by adding Phorbol 12-myristate 13-acetate (PMA) to the *U. marinum* at a concentration of 10μg per ml using the fluorescence polarization method described previously (Lee *et al.*, 2003). And PMA-treated (at a concentration of 50μg per ml) *U. marinum* was more resistant to serum of olive flounder *Paralichthys olivaceus*(data not shown). Straarup *et al.* (1997) showed *Tetrahymena thermophila* cells died at 250 cells per ml in PMA-unsupplemented medium, whereas they survived and proliferated at 250 cells in PMA-supplemented medium at a concentration of 10μg per ml.

Recently, many researchers have used this SSH technique to identify differentially expressed gene families since it enables the rapid and sensitive comparison of mRNA expression between two cell populations (Lee *et al.*, 2002; O'Farrell *et al.*, 2002). *Leishmania* parasites alternate between two distinct lifestages, the promastigote and the amastigote. Amastigotes, the intracellular life stage in *Leishmania*, have the abilities to evade killing by host macrophage cells and to persist for long periods of time in the host, resulting in the chronic symptoms of disease (Aebischer, 1994). Bellatin *et al.* (2002) identified genes that are preferentially expressed in *Leishmania mexicana* amastigotes using SSH technique. The authors suggested that amastigote-specific proteins are responsible for intracellular parasite survival and the host response to these proteins should explain *Leishmania* pathogenesis.

In this study, a sensitive PCR based subtraction approach, suppression subtractive hybridization (SSH) was adopted to isolate genes induced in *U.*

marinum by adding PMA to the medium at a concentration of 10μg per ml and identify their expression profile. Using this technique, in this study, many kinds of PMA-inducible genes, such as protein kinases, proteases and surface antigens were identified and semi-quantitative RT-PCR was used to evaluate the relative expression of selected genes and confirm the differential expression of the genes identified in SSH libraries. Among these genes which were identified by SSH, two genes - protein phosphatase 2C (UmPP2C) and surface antigen - were firstly selected as candidates for the vaccine target antigens because these genes may induce protective immunity in the host. The full-length cDNAs of these two selected genes were isolated by 5'- and 3'-RACE and the ORF of UmPP2C and partial sequence of surface antigen were mutated by site-directed mutagenesis for using in other expression system since heterologous expressions of proteins were impossible. Generally, two of the three universal stop codons, TAA and TAG, code for glutamine in protozoa.

UmPP2C had 56% identity with the membrane-bound Protein Phosphatase Type 2C of *Paramecium tetraurelia* (PtPP2C). PtPP2C was first isolated by Klumpp *et al.* (1994) and futher characterization and localization were performed by Grothe *et al.* (1998). In an attempt to reveal clues to a physiological function, they established the subcellular localization of PtPP2C in the ciliate. The most distinct subcellular localization of PtPP2C was found in the cilia, where it was associated with microtubules and dynein, *i.e.* it was clearly targeted to the ciliary motor. A family of cytoplasmic dyneins exists, which in association with dynactin and microtubules are major players in the transport of cellular cargo such as membraneous organelles, possibly large protein complexes and mRNA. This dynein-motor complex can be regulated by phosphorylation and dephosphorylation. Although the localization and protein activity of UmPP2C were not demonstrated, UmPP2C

may have the similar properties with PtPP2C. In fact, in the present study, UmPP2C mRNA was expressed through signal pathway by PMA. Therefore, the presence of PP2C in *U. marinum* suggested that UmPP2C can be used as a target antigen protein because the movement of *U. marinum* using cilia is very important part of survival and infection.

Recently, many new protein molecules have been discovered on the surface of the protozoan parasites, such as *Plasmodium, Trypanosoma* and *Leishmania*, most belonging to the merozoite surface antigen (MSP) family. Due to their surface exposure, they are accessible to antibodies and are therefore considered possible vaccine candidates (Mello *et al.*, 2004). Many attempts revealed that protective immunity was obtained by immunization with recombinant surface antigen (Musoke *et al.*, 1992; Mello *et al.*, 2004) or plasmid DNA encoding surface antigen (Nielsen *et al.*, 1999; Dumonteil *et al.*, 2004). *Plasmodium falciparum* sporozoites invade liver cells in humans and set the stage for malaria infection. Circumsporozoite protein (CSP), a predominant surface antigen on sporozoite surface, has been associated with the binding and invasion of liver cells by the sporozoites. CSP is an important molecule for the parasite, because it is involved in the development of infectious sporozoites in mosquitoes, plays a role in the invasion of salivary gland, and is essential to the binding and invasion of liver cells in the vertebrate host (Rathore *et al.*, 2003). The Gal-lectin is the major surface molecule of *Entamoeba histolytica* that allows the parasite to adhere to colonic mucin and target cells, and is one of the parasite's most immunogenic molecules. It is the major antigen recognized by sera from patients with invasive amebiasis, and immunization of gerbils with the native protein or some of its portions confers protection against an intrahepatic challenge with live trophozoites. Moreover, monoclonal antibodies (MAbs), antiserum and secretory IgA (sIgA) against the Gal-lectin have

been shown to inhibit adherence of trophozoites to colonic mucin and target cells. The Gal-lectin, or some of its selected portions, would thus be excellent candidate molecules to be included in a subunit vaccine (Gaucher *et al.*, 2002). Several researchers demonstrated that ciliates such as *Ichthyophthyrius multifiliis, Philasterides dicentrarchi* and *Tetrahymena* spp. expressed surface immobilization antigens(i-antigens) on its surface by immobilization/agglutination of ciliates in the presence of immune sera and these results suggest that the response induced by these surface antigens may play an important role in protction against infection by this ciliates.

Surface antigen identified in the present study, play a similar role with other protozoan surface antigens. As *U. marinum* are characterized by their high potential for invading systemically and destroying fish tissues, surface antigen plays an important role in attachment to the host cells and destroy the host's tissues. Therefore, inhibition of *U. marinum* surface antigen by vaccination might induce protective immunity.

To develop an efficient controlling measure on scuticociliatosis by *U. marinum*, lots of the basic biological and genetic information was needed. This study offered some information of expressed genes in *U. marinum* and proposed two candidate genes for the vaccine development. To use these two genes as vaccine target antigens, further analyses of expressed protein and antibody production by immunization should be performed.

Phorbol ester에 의해 유도되는 *Uronema marinum* (Ciliata: scuticociliatida)의 유전자 및 protein phosphatase 2C와 표면항원 유전자의 특성

이은혜

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요약

우리나라 양식 넙치의 스쿠티카증 원인체인 *Uronema marinum*에서 phorbol 12-myristate 13-acetate (PMA)에 의해 유도되는 유전자들을 PCR을 이용한 subtraction 기법인 suppression subtractive hybridization를 사용하여 조사하였다. SSH screening을 통하여 PMA에 의해 유도되는 mRNA로부터 합성된 cDNA의 단편이 포함된 clone들을 다수 얻을 수 있었으며, 그 중 243개의 clone을 선별하여 염기서열을 분석하였고, NCBI의 BLAST X program을 사용하여 단백질에 대한 상동성 조사를 실시하였다. 분석된 유전자들 중에서 protein phosphatase 2C와 표면 항원으로 추정된 두 가지 유전자에 대하여, 5'-과 3'- RACE를 실시하여 full-length cDNA를 얻었다. UmPP2C의 cDNA full-sequence는 그 길이가 1137 bp 였으며, 310 아미노산을 coding 하는 933 bp의 open reading frame을 포함하였다. UmPP2C 단백질의 분자량은 약 35 kDa, isoelectric point는 5.06으로 예상되었으며, *Paramecium tetraurelia*의 membrane-bound protein phosphatase Type 2C 와 56%의 상동성을 나타내었다. 표면 항원 유전자의 cDNA full sequence는 길이가 3213 bp 였으며, 917 아미노산을 coding하는 2754 bp의 open reading frame을 포함하였다. DNA 염기서열로부터 추정되는 단백질은 CXXC와 CXC motif가 포함된 반복된 서열을 포함하고 있으며, 예상되는 표면 항원 단백질의 분자량은 약 104 kDa 이며,

isoelectric point는 4.53 이었다. *Paramecium tetraurelia*의 putative surface antigen with EGF domains and furin-like repeats와 25%의 상동성을 나타내었고, 이 때의 E-value는 1e-63으로 나타났다. 이후의 다른 발현 시스템에서의 사용을 위해서, UmPP2C의 전체 ORF와 표면 항원 유전자의 일부 sequence내에 있는 TAA codon을 site-directed mutagenesis법을 사용하여 CAA codon 으로 치환하였다.

감사의 글

수산생명의학과에 들어와서 대학생활을 한지 벌써 7년입니다. 그 동안 많은 일이 있었고 아쉬운 일도 많았지만, 부족하나마 작은 결실을 하나 완성하게 되었습니다. 논문이 완성되기까지는 많은 분들의 도움이 있었기에 이 작은 지면을 빌어 감사의 마음을 전하고자 합니다. 먼저 제가 살아오는 동안 언제나 나의 중심에 계시며 힘이 되어 주시고 바른길로 갈수 있도록 마음을 다스려 주시는 하나님께 감사를 드립니다.

학부 2학년 때부터 지금까지 실험실 생활을 하는 동안 부모님의 마음으로 제자들을 사랑해주시고 언제나 변함없는 모습으로 학자로서의 모범을 보여주시고 공부할 수 있게 힘과 용기를 주시는 김기홍 교수님께 진심으로 감사를 드립니다. 부디 선생님과 사모님께서 앞으로 더욱 건강하시고 행복하시길 빌겠습니다. 또한 바쁘신 와중에도 논문심사로 시간을 쪼개어 주시고 관심을 가져주신 정현도 교수님과 정준기 교수님께도 감사를 드리며, 새로운 학문을 접하면서 많은 것을 알게 해주신 박수일 교수님, 허민도 교수님, 강주찬 교수님 감사를 드립니다.

그 동안 집에 돌아가 잠자는 시간외에는 대부분의 시간을 실험실에서 보냈탓에 실험실 식구들과의 인연이 남다릅니다. 저에게는 까마득하기만한 일을 당당히 해내신 실험실 최고 선배 조재범 박사님, 우리실험실 식구가 된지 이제 1년이 넘었고 큰언니처럼 편한 선정이 언니, 무슨 일이든 야무지게 잘 해내는 세련이 언니, 우리 실험실 기둥 천수 선배, 이분들 때문에 실험실 생활을 할 때 정말 든든합니다. 또한 후배이지만 석사과정을 함께하며 친구처럼 고민을 같이 나누어준 성미에게 고맙다는 말을 하고 싶고 마지막까지 같이 잘 마무리했으면 좋겠습니다. 무슨일이든 열심히 최선을 다하는 형준이 오빠, 불평하지 않고 실험을 많이 도와준, 이제 곧 대학원생이 될 01학번 민선, 수경, 승혁이, 혼자 요즘 너무 힘든 막내 동진이, 얼마전까지 실험실원 이면서 즐거움을 많이 준 성택이와 성현이에게도 감사의 마음을 전합니다. 졸업을 하신 후에도 실험실에 관심을 가지고 실험에 필요한 도움을 주시는 맹덕 선배와 벌써 두 아이의 엄마로서 또한 직장인으로서 열심히 일하시는 윤정이 언니, 실험실 생활을 오래 같이 했고 졸업해서 다른 곳에 있는 찬희 선배, 재혁 선배, 경진 선배와 통영 시청에 있는 지영이, 건강 때문에 같이 대학원 생활을 하지 못한 도선 선배, 실험실에서 짧은 시간이라도 함께 지냈던 모든 선, 후배님들과 멀리서 응원해주는 재연이 언

니에게도 감사를 드립니다. 또한 김성구 교수님을 비롯한 생물공학과 생물고분자공학 실험실 식구들, 남윤권 교수님 이하 양식학과 유전자원공학 실험실 식구들께도 감사의 말을 전합니다.

지금 멋진 해병의 아내로 또한 춤꾼으로의 인생을 살아가는 멋진 친구 수현이, 1년 먼저 졸업했지만 대학원 생활을 같이한 영화, 대심이, 미주, 현정이, 승엽이, 이번에 같이 졸업하는 혜정이와 지금은 여러 곳에서 각자 열심히 생활하고 있는 수산생명의학과 98학번 동기들 모두에게도 감사의 말을 하고 싶습니다. 또한 일일이 이름을 적지는 못했지만 이번에 같이 졸업할 모든 분들과 필요할 때 도움을 주는 9호관 2층 모든 실험실 식구들에게도 감사를 드리며 우리 수산생명의학과의 무궁한 발전을 기원합니다.

어렸을 때부터 서로 기도해주며 지내온 수영로교회 친구들 희수, 예지, 정혜, 현주, 일선, 정민, 선희와 98기 동기들 에게도 감사하며 바쁘다는 핑계로 자주 만나지 못해 미안할 따름입니다. 그리고 가까운 곳에 있어서 언제나 든든하며 필요할 때 기댈 수 있고 함께 공부할 수 있는 사랑하는 승호선배에게도 감사의 말을 전합니다.

마지막으로 지금까지 길러주시고, 어려운 형편에 건강도 좋지 않으시지만, 공부할 수 있도록 믿어주시고 조용히 응원해주시는 부모님께 감사를 드리며 더 열심히 해야 하지만 그러지 못해 너무 죄송합니다. 또한 먼 미국땅에서 고생하면서 항상 동생을 걱정하는 오빠와 어린 나이에 힘들게 사회 생활하는 동생 은정이에게도 미안한 마음과 고마운 마음을 전합니다. 미처 이름을 다 기록하지는 못했지만, 부족하나마 이렇게 논문을 마칠 수 있게 도와주신 모든 분들에게 다시 한 번 감사를 드립니다.

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