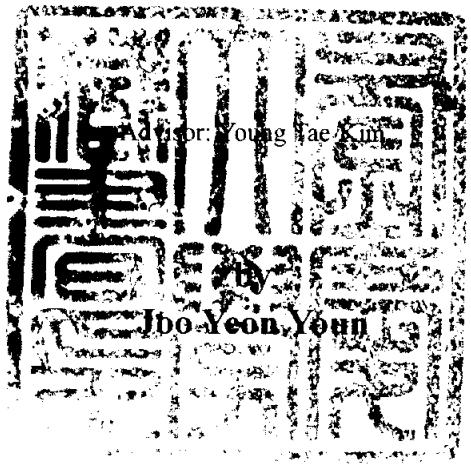
Cloning of Phosphoinositide 3-kinase γ cDNA from Flounder (*Paralichthys olivaceus*)

넙치 Phosphoinositiede 3-kinase γ 유전자에 대한 연구



A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Science

in the Department of Microbiology, Graduate School,

Pukyong National University

February 2004

윤주연의 이학석사 학위논문을 인준함

2003년 12월 26일

주 심 이학박사 이 훈 구 

위 원 이학박사 송 영 환 

위 원 이학박사 김 영 태 

Cloning of Phosphoinositide 3-kinase γ cDNA from Flounder (*Paralichthys olivaceus*)

A Dissertation

by

Joo Yeon Youn

Approved as to style and content by:

Dean of Graduate School	Chairman
Member	Member
Member	Member

December 2003

CONCENTS

LISTS OF FIGURES

Cloning of Phosphoinositide 3-kinase γ cDNA from Flounder (*Paralichthys olivaceus*)

Joo Yeon Youn

Department of Microbiology, The Graduate School,

Pukyong National University

ABSTRACT

Phosphoinositide 3-kinase (PI3K) plays a central role in cell signaling and leads to cell proliferation, survival, motility, exocytosis, and cytoskeletal rearrangements as wall as specialized cell responses, superoxide production, and cardiac myocyte growth. PI3K is made up with three classes and the type I PI3K is preferentially expressed in leukocytes and activated by βγ subunits of heterotrimeric G-proteins. In the present study, the cDNAs encoding PI3Kγ gene was isolated from a brain cDNA library constructed with the flounder (*Paralichthys olivaceus*). The

nucleotide sequence of the isolated PI3Kγ was 1341bp long, encoding 447 amino acids. The sequence of flounder PI3Kγ shares 89.6%, 84.7%, 84%, and 74.9% identities with that of zebrafish (*Danio rerio*), mouse (*Mus musculus*), Norway rat (*Rattus norvegicu*), and human (*Homo sapiens*), respectively. In order to determine the tissue distribution of PI3Kγ in flounder tissues, the reverse transcription polymerase chain reaction (RT-PCR) was used, and the expression was detected in the brain, liver, and kidney tissues.

INTRODUCTION

Phosphoinositides were recognized early as precursors for second messengers in cell surface receptor-coupled signal transduction pathways. Phosphoinositide 3-kinasse (PI3K) catalyzes the addition of a phosphate molecule to the three positions of the inositol ring of phosphoinositides (PtdIns), producing four different lipid products: the singly phosphorylated form PtdIns-3-P, the doubly phosphorylated forms PtdIns-3,4-P_2 and PtdIns-3,5-P_2, and the triply phosphorylated form PtdIns-3,4,5-P_3 (Foster *et al*, 2003).

There are multiple isoforms of PI3K in mammalian cells and these are subdivided into three main classes on the basis of their structures, in vitro substrate specificity, and mode of regulation (Vanhaesebroeck *et al.*, 1997; Rameh and Cantley, 1999). ClassI PI3Ks comprise a p110 catalytic subunit and a regulatory adapter subunit. ClassII PI3Ks are large (170-200 kDa) proteins that have a catalytic domain that is 45-50% homologous to classI PI3Ks. ClassIII PI3Ks are typified by the yeast protein (Ellson *et al.*, 2002). ClassI PI3Ks have been the major focus of PI3Ks studies because these isoforms are generally coupled to extracellular stimuli. ClassI PI3Ks are activaied by a variety of extracellular stimuli and have been linked to an incredibly diverse set of key cellular functions,

including cell cycle progression, cell growth, cell proliferation, cell motility, cell differentiation, cell survival and intracellular trafficking (Coelho and Leevers, 2000; Cantrell, 2001; see Figure 1). The emerging links between PI3-kinase activity and many human maladies, including allergy, inflammation, heart disease and cancer, has made them the focus of intense study, and inhibitors of these enzymes are considered potential therapeutic agents.

ClassI PI3K is a heterodimeric complex, comprising a p110 catalytic subunit, of which there are four characterized isoforms(α, β, γ, and δ). ClassI PI3K is subdivide into classIA and IB. TypeIA PI3Ks, p110α, p110β, and p110δ, shared with 42-58% amino acid sequence identity and associated with the p85 family of regulatory subunit, but typeIB PI3Ks, P110γ, binds to a p101 adaptor molecule. Whereas classIA PI3Ks are activated by interaction with tyrosine-phosphorylated molecules, classIB p110γ (PI3Kγ) is activated by engagement of heterotrimeric GTP-binding protein (G pretein)-coupled receptors (GPCR). PI3Kγ is preferentially expressed in leukocytes (Fry, 2001;Roymanns and Slegers, 2001). PI3Kγ is activated by βγ subunits of heterotrimeric G-proteins, which thus link seven transmembrane (7TM) helix receptor activation to phosphatidylinositol(3,4,5)-trisphosphate production (Hazeki *et al.*, 1998; Hirsch *et al.*, 2000). PI3Kγ controls thymocyte survial and

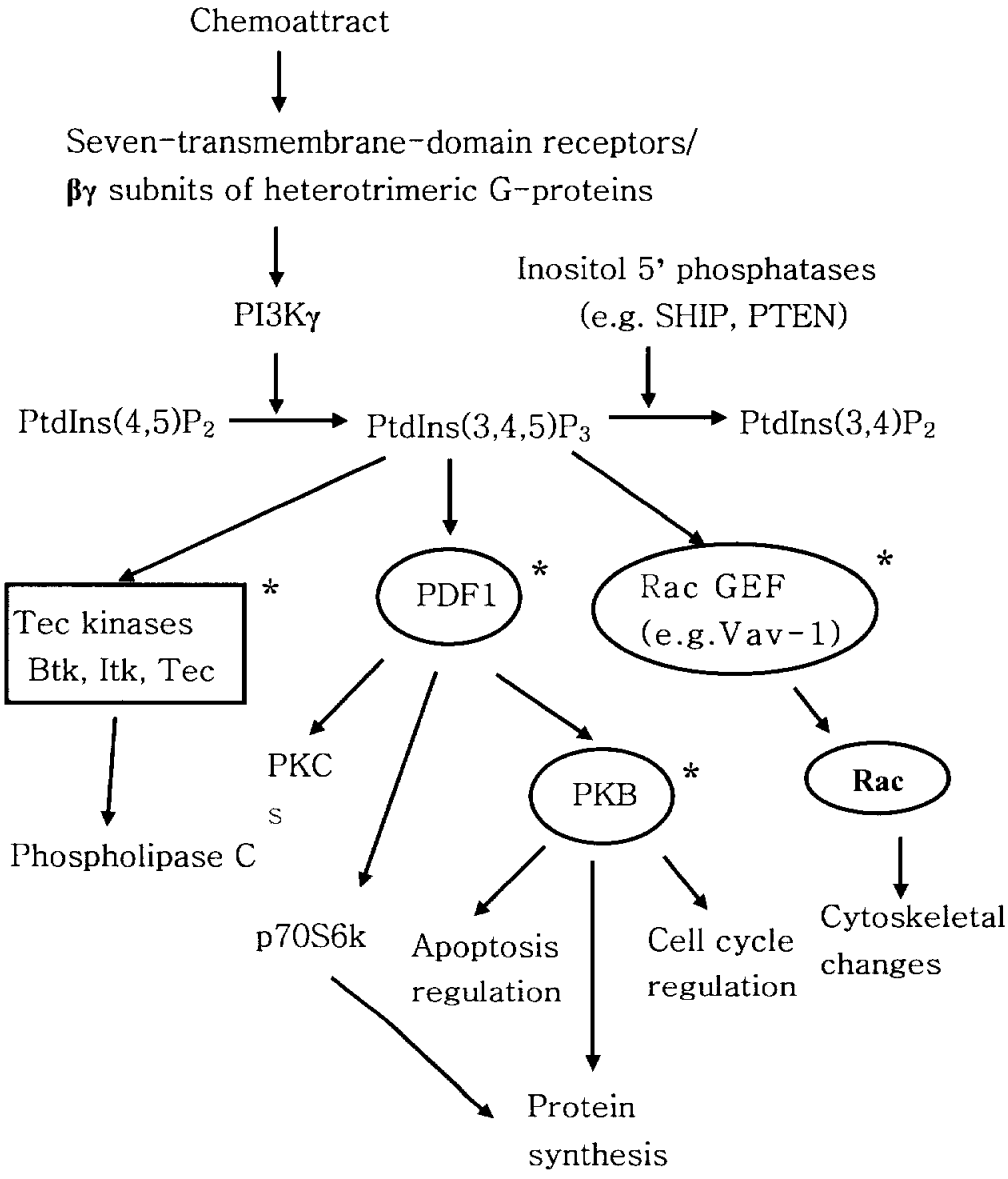


Figure 1. The major signalling pathways initiated by the major lipid product of PI3Kγ: PtdIns(3,4,5)P_3. Asterisked proteins have a pleckstrin homology (PH) domain that directly binds to PtdIns(3,4,5)P_3.

activation of mature T cells but has no role in the development or function of B cells. PI3Kγ links GPCR stimulation to the formation of phosphatidylinositol 3,4,5-triphosphate and the activation of protein kinase B, ribosomal protein S6 kinase, and extracellular signal-regulated kinase 1 and 2 (Pirola and Wymann, 1998; Sasaki *et al.*, 2000). Thus, PI3Kγ regulates thymocyte development, T cell activation, neutrophil migration, and the oxidative burst.

Recent studied in mice lacking functional PI3Kγ showed that PI3Kγ plays a key role as a modulator of inflammation (Bourne *et al.*, 2000) and allergy, and also in the regulation of cardiac contractility (Hawkins *et al.*, 2002; Hirsch *et al.*, 2000; Naga Prasad *et al.*, 2003;).

Elucidation of the structural diversity of PI3Kγ in recent years by molecular cloning of cDNAs and genes from various species has provided insight into their functions. PI3Kγ cDNA genes have been cloned from *Mus musculus* (Altruda, 2000), *Rattus norvegicu*(Aksoy, 1999), *Danio rerio* (Montero, 2003), and *Homo sapiens*(Stoyanov, 1995). However, knowledge of the molecular structure of PI3Kγ in marine fishes is extremely limited. In addition, the nature of PI3Kγ in these fish and their roles in the control of the PtdIns signaling pathways is still unclear.

The flounder (*Paralichthys olivaceus*), one of the most evolved teleosts, is a commercially important marine aquaculture species in Korea and has been the object of studies on various functional genes at the molecular level (Cho *et al.*, 1999; Kim and Kim 1999; Cho and Kim, 2002). In the present study, it is focused on the isolation of cDNA encoding the flounder PI3Kγ and characterization of the cloned gene. These data will provide a base of knowledge for the PI3Kγ gene at the molecular level and the functional diversity of PI3Kγ.

EXPERIMENTAL PROCEDURES

MATERIALS

Bacterial strains

The *E. coli* strain XL1-Blue (F'::*Tn10 proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ) M15/recA1 endA1 gyrA96 (Nal*^{r}*) thi hsdR17* (r_k^- m_k^+) *supE44 relA1 lac*) was used for transformation and color selection. The *E. coli* strain BL21 (DE3) codon plus [F$^-$ *ompT hsd*S(r_B^- m_B^-) *dcm gal* Tet^r λ(DE3) *end*A Hte (*argU ileY leuW Cam*^{r})] was used for the overexpression of flounder PI3Kγ gene.

Fish tissues

The brain, liver, and kidney tissues were obtained from mature flounder (n=10; size: 45±10 cm, body weight; 900±300 g; 3years old) and stored at -70℃ until use.

Enzymes

Restriction enzymes were purchased from Promega (U.S.A.). Taq polymerase and Reverse Transcriptase were obtained from Bioneer (Taejeon, Korea). Sequencing ready reaction kit (ABI Prism^{TM} dye terminator) was purchased from Perkin Elmer (U.S.A.).

Oligonucleotide primers

The primers for PI3Kγ cloning were designed from the base on the conserved sequences from known PI3Kγ sequences and synthesized from GenoTech (Tajeon, Korea). The oligonucleotide primers used for this study were summarized in Table 1.

Other Materials

pGEM-T Easy vector (Promega, U.S.A.) was used for ligation of the PCR fragments. pET-44a expression vector (Invitrogen, U.S.A.) was used for expression. DIG labeling and detection kit were purchased from Boehringer Mannheim. Nitro blue tetrazolium

chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for alkaline phosphatease color developing solutions were purchased from Bio-Rad. Protein low and prestained molecular weight markers were obtained from Fermentas (U.S.A.). TRIzol reagent for total RNA isolation was purchased from Invitrogen(U.S.A.). Other chemicals were purchased from Fluka (U.S.A.) and Sigma (U.S.A.).

Table 1. Oligonucleotide primers used for this study

Primer	Sequence	Remark
PI3KF	5'-GACCTTCCTGGTGCGGGACG-3'	Forward primer for RT-PCR
PI3KR	5'-CGTTCTTGATTCCCAGCCA-3'	Reverse primer for RT-PCR
PI3KN	5'-CATATGCAGCTAACGGAGAGCGC-3'	Forward primer for expression
PI3KX	5'-CTCGAGTCTGCGTCCGGAGG-3'	Reverse primer for expression
PI3KF1	5'- ACACTTGCAACACACACG - 3'	Forward primer for preparation of probe
PI3KR1	5'- TAATACGACTCATAGGGC - 3'	Reverse primer for preparation of probe

METHODS

Isolation of RNA

Total RNA from flounder brain, liver, and kidney tissues were isolated using total RNA isolation kit (Promega). Each of fresh flounder tissues (100mg) was homogenized in 1ml denaturation reagent using a power driven homogenizer (Pyrex). The sample was kept on room temperature for 5 min and 100μl of chloroform and 100μl of phenol was added and vigorous shaked for 15 sec. Then, the sample was kept on room temperature for 3min and centrifuged for 15 min at 12,000 rpm. The aqueous phase was carefully transferred to a new tube, and 0.5 volume of isopropanol was added to it and mixed by inverting several times. After that, RNA Track^{TM} resin (0.05v/v) was added, vortexed for 30 sec, and spun for 1 min. The pellet was washed twice with 300μl of 75% ethanol, dried briefly, resuspended in 0.1 volume for diethyl pyrocarbonate (DEPC) treated water, and centrifuged for 2 min at 13,000 rpm. Supernatant was transferred to a new tube. Poly(A) RNA was isolated using a Micro-Fast Track^{TM} 2.0 Kit (Invitrogen). Ten μl (500μg) of purified poly(A) RNA was aliquoted to a new

microcentrifuge tube and 1ml of Micro-Fast Track™ 2.0 lysis buffer was added.

Then, the mixture was heated up to 65℃ for 5min and placed immediately on ice for 5min. Sixty-three μl of 5M NaCl was added, and missed well with isolated with a method described in Micro-Fast Track™ 2.0 Kit manual. The quantity of total RNA and poly(A) RNA were determined spectrophotometrically.

Construction of flounder cDNA library

The complementary DNA (cDNA) library was constructed using a ZAP-cDNA Synthesis Kit (Stratagene). Conserved nucleotide sequences of PI3K among the vertebrate species were determined using NCBI (National Center for Biotechnology Information) nucleotide and protein sequence database and used for the designing of oligonucleotide primers for screening PI3K were synthesized from GenoTech (Taejeon, Korea). PCR was carried out using a pair of the 'PI3KF1' and 'PI3KR1' primers (see Table 1). The main PCR reaction was 94℃ for 30 sec, 52℃ for 30 sec, and 72℃ for 1min 30sec in a 30 cycle reaction. Probe for screening PI3K was labeled with a DIG (digoxigenin) oligonucleotide 3'-end

labeling kit (Roche). DIG labeled probes were quantified and used for the immnoscreening procedure. Approximately, 1×10^5 plaques from the cDNA library were screened with above probes and isolated several positive plaques. These plaques were recovered and further confirmed by the second screening. Positive plaques were recovered from the second screening and the phagemid containing the insert was excised according to the manufacturer's instructions (Stratagene).

Plasmid DNA purification and Sequencing

Plasmid DNA was purified using Wizard® Plus SV minipreps DNA purification system (Promega). Sequencing reaction was performed using an ABI Prism™ DNA sequencing kit and an ABI377 Genetic Analyzer (Perkin Elmer). Sequencing reaction mixture (4μl of big dye ready reaction mixture, 500ng of template DNA, 3.2 pmole of primer, and water to 20μl volume) was prepared and placed into a thermal cycler. Thermal cycling with 25 cycle of program (96℃ 10 sec, 50℃ 5 sec, 60℃ 4 min) was performed. After that, reaction mixture was precipitated with ethanol and dissolved in 20μl TSR (template suppressing reagent) following

with 2 min incubation at 95℃, cooled down on ice and DNA sequence was analyzed using ABI377 sequencing analyzer. Nucleotide and its deduced protein sequences were analyzed using the EMBL and Genbank databases.

Reverse transcription polymerase chain reaction (RT-PCR)

In order to perform RT-PCR, total RNA was isolated from brain, liver, and kidney from mature flounder (n=10; size: 45±10 cm, body weight; 900±300 g; 3years old). The RT-PCR was performed using Bioneer's RT-PCR system. The reaction components were set up for Master mix 1 and Master 2. Master mix 1 contained temple RNA, 50 pmol of primer, and DEPC-water. The sample was incubated for 10 min at 65℃ and cooled down on ice. Master mix 2 consisted of 5X RT-PCR buffer, 2.5 mM dNTP mixture, 100 mM DTT, RNase inhibitor, and MMLV RTase. Mix 1 and mix 2 were added to a 0.2μl tube. The sample was placed in a thermocycler (GeneAmp PCR system 2400, Perkin Elmer) and incubated for 1 hr at 42℃ for reverse transcription followed by thermocycling. The temperature profile of PI3Kγ was on prereaction at 94℃ for 5 min and 30 cycling reaction with 94℃

40 sec denaturation, 56℃ for 30 sec annealing, 72℃ for 1 min, and finally 7 min extension at 72℃. After reaction, 15μl of RT-PCR product was analyzed with 1% agarose gel electrophoresis.

Expression of flounder PI3Kγ gene in *E.coli*

PI3Kγ gene was amplified by PCR using a pair of oligonucleotide (Table 1). PCR product was ligated into pGEM-T vector and the resulting plasmid was digested with *NdeI* and *XhoI* restriction enzymes. Then, the excised fragment was ligated into pET44-a(+) vector. The resulting plasmid containing PI3Kγ gene was named pET-44a-PI3K. The Plasmid was transformed into the competent *E. coli* strain BL21 (DE3) codon plus. The cell harboring a plasmid that containing PI3Kγ gene was cultured overnight in 10ml of LB/amp (containing 50μg/μl ampicillin) broth at 37℃ in shaking incubator. The cell was induced by adding IPTG (Isopropyl-β-D-Thiogalactopyranoside) to a final concentration of 1mM at mid-log growth (OD_{600}=0.5).

Analysis of expressed protein on SDS-PAGE

At various induction periods, 1ml of the culture was microcentrifuged at 13,000 rpm for 1 min at room temperature and supernatant was removed by aspiration. Each pellet was resuspended in 100μl of 2XSDS-sample loading buffer (0.5M Tris-HCl pH 6.8, 10% Glycerol, 20% SDS, β-mercaptoethnol, distilled water, 0.05% (w/v) bromophenol blue) and heated at 100℃ for 3 min. The sample was centrifuged 13,000 rpm for 1 min at room temperature, and stored on ice until all of the samples collected and ready to load on a gel. Samples (20μl) were loaded on a 12% SDS-PAGE (10% seperating gel, pH 8.8 overlayed 4% stacking gel, pH 6.8). Electrophoresis was performed at 50V for 3h. The gel was stained with coomassie brilliant blue and destained with destaing solution (7% acetic acid, 15% methanol).

Western blot analysis

After total proteins containing PI3Kγ-His protein were seperated on a 12% SDS-acrylamide gel electrophoresis, the gel was transferred onto a nitrocellulose membrane by a semi-dry method.

The blot was incubated for 1h with 3% gelatin in TTBS (20 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 2.5 mM KCl, 0.1% Tween-20) and then rinsed with TTBS. Subsequently, a polyclonal antibody against goat anti-6-histidine (diluted 1:10000) was added and incubated for 1hr at room temperature. After three washes with TTBS, the membrane was incubated with anti-goat antibody conjugated with alkaline phosphatase (Sigma: diluted 1:10000 in TTBS containing 1% gelatin) at room temperature for 30min. The membrane was then rinsed three times with TTBS and developed at room temperature in a developing buffer (15mg of Nitro Blue Tetrazolium; 0.7% N, N-dimethylformamide; 30mg of 5-bromo-4-chloro-3indolyl phosphate per 100ml; 1mM $MgCl_2$ and 100mM $NaHCO_3$, pH 9.8).

RESULTS

Nucleotide sequences of flounder PI3Kγ

The PI3Kγ gene of flounder was isolated using polymerase chain reaction (PCR) from the flounder brain cDNA library. PCR products were cloned into T vector. Cloned DNA was purified and sequenced with automatic DNA sequencer using ABI Prism DNA sequencing kit.

Figure 2 shows the nucleotide sequenced of the complete cDNA encoding the flounder PI3Kγ gene (GeneBank accession number AY514674) and its deduced amino acid sequence. Sequence of cloned PI3Kγ was analyzed with Blast program of NCBI (National Center for Biotechnology Information). The flounder PI3Kγ gene contains a 1744 bp including an open reading frame and encoding a 447-amino-acid protein. The cDNA consists of a 86 bp of 5'-UTR (untranslated region), a 1341 bp of coding region, and a 314 bp of 3'-UTR, followed by a poly (A) sequence. As shown in Figure 2, the flounder PI3Kγ cDNA clone contains an in-frame termination codon (TGA) at bases 1431-1434.

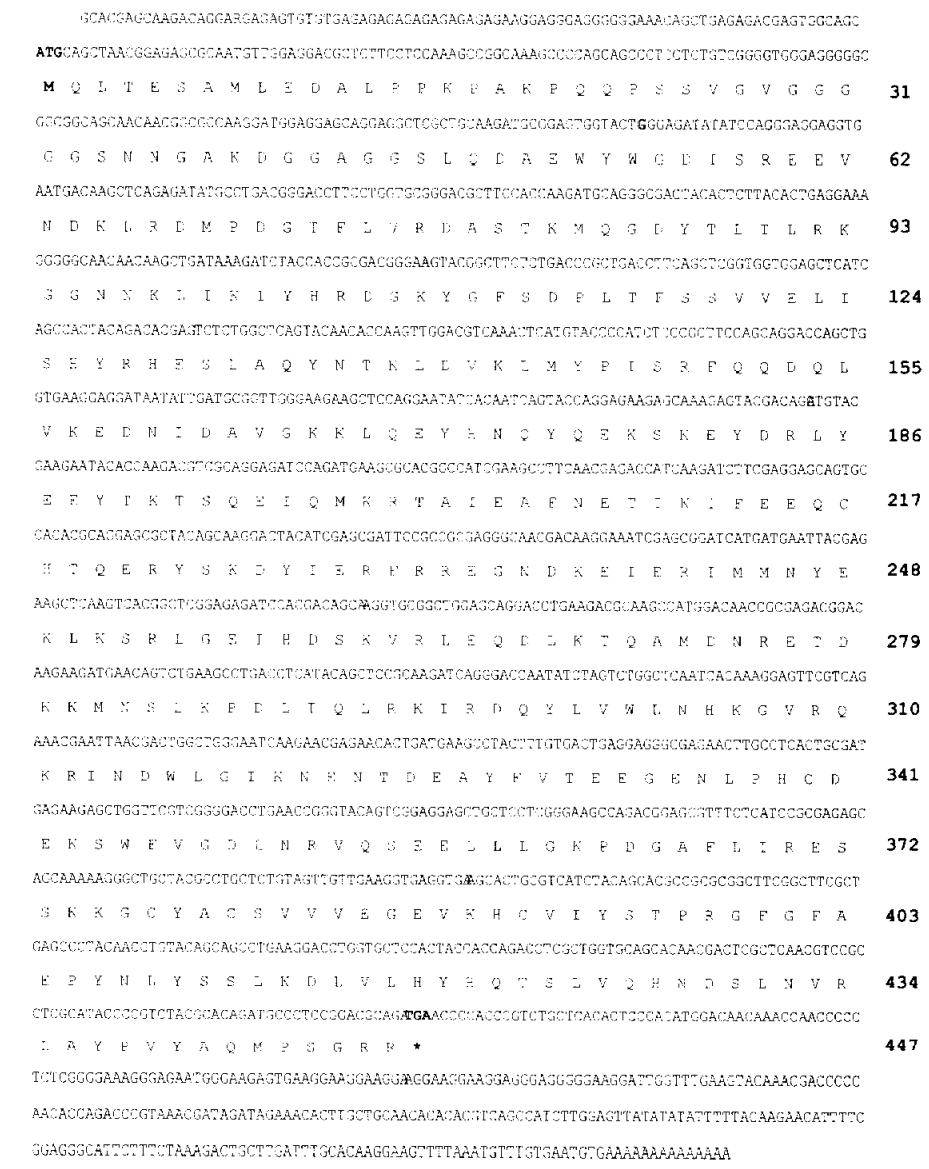


Figure 2. Nucleotide and deduced amino acid sequences of the cDNA encoding flounder PI3Kγ gene. The nucleotide sequence is numbered to the left and the amino acid to the right.

Sequence identity and Phylogenetic tree

Figure 3 shows an alignment of the amino acid sequences of the flounder and other PI3Kγ. The PI3Kγ proteins were compared using the Blast protein database (NCBI).

The flounder PI3Kγ have a high sequence similarity in amino acid residues with other species. The deduced flounder amino acid sequence was about 89.6%, 84.7%, 84%, and 74.9% identical with PI3Kγ of zebrafish (*Danio rerio*), mouse (*Mus musculus*), Norway rat (*Rattus norvegicu*), and human (*Homo sapiens*), respectively.

A molecular phylognentic tree was constructed to analyze the evolutionary relation ships of the PI3Kγ protein (Figure 4). It shows the evolutionary divergence of the PI3Kγ genes of zebrafish, flounder, mouse, Norway rat, and human. The flounder PI3Kγ protein was more closely related to the zebrafish PI3Kγ than to the human one as reflected in the sequence identity (89.6% vs 74.9%).

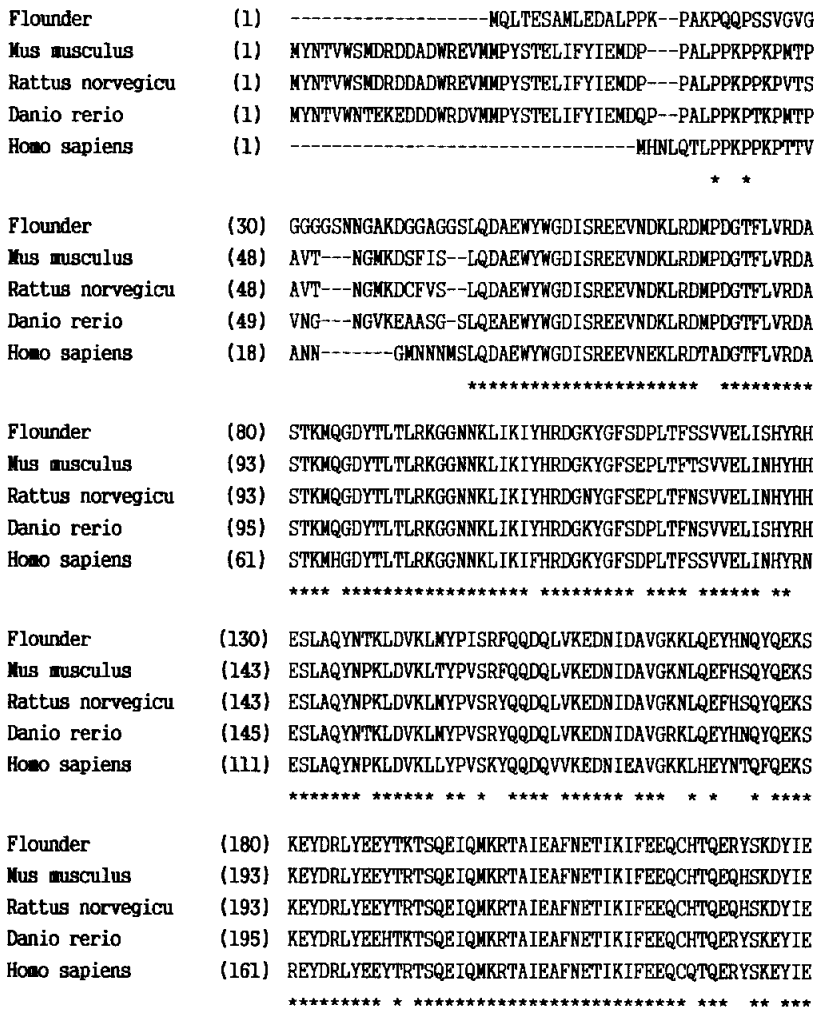


Figure 3. Multiple alignment of the predicted flounder PI3Kγ amino acid sequence with other species. The known PI3Kγ protein sequences were taken from GenBank. Amino acid residues that are identical in the PI3Kγ proteins are marked with shade.

```
Flounder            (230)  RFRREGNDKEIERIMMNYEKLKSRLGEIHDSKVRLEQDLKTQAMDNRETD
Mus musculus        (243)  RFRREGNEKEIERIMMNYDKLKSRLGEIHDSKLRLEQDLKKQALDNREID
Rattus norvegicu    (243)  RFRREGNEKEIERIMMNYDKLKSRLGEIHDSKVRLEQDLKKQALDNREID
Danio rerio         (245)  RFRREGNDKEIERIMMNYEKLKSRLGEIHDSKTRLEQDLKTQALDNRETD
Homo sapiens        (211)  KFKREGNEKEIQRIMHNYDKLKSRISEIIDSRRRLEEDLKKQAAEYREID
                            * **** *** *** ** *****  ** **  *** *** **   ** *

Flounder            (280)  KKMNSLKPDLIQLRKIRDQYLVWLNHKGVRQKRINDWLGIKNEN-TDEYF
Mus musculus        (293)  KKMNSIKPDLIQLRKIRDQHLVWLNHRGVRQRRLNAWLGIKNEDSDESYF
Rattus norvegicu    (293)  KKMNSIKPDLIQLRKIRDQHLVWLNHRGVRQRRLNAWLGIKSEDTDESYF
Danio rerio         (295)  KKMNSLKPDLIQLRKIRDQYLVWLNHKGVRQKRINDWLGLKNETTDDAYF
Homo sapiens        (261)  KRMNSIKPDLIQLRKTRDQYLMWLTQKGVRQKKLNEWLGN-ENTEDQYS
                           * *** ********* *** * **   ****  ** ***   *     *

Flounder            (329)  VTEEGENLPHCDEKSWFVGDLNRVQSEELLLGKPDGAFLIRESSKKGCYA
Mus musculus        (343)  INEEDENLPHYDEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYA
Rattus norvegicu    (343)  INEDDESLPHYDEKTWFVEDVNRVQAEDLLYGKPDGAFLIRESSKKGCYA
Danio rerio         (345)  VSEEDENLPHYDEKSWFVGDLNRTQSEDLLHGKPDGAFLIRESSKKGCYA
Homo sapiens        (309)  LVEDDEDLPHHDEKTWNVGSSNRNKAENLLRGKRDGTFLVRESSKQGCYA
                             *  * *** *** * *   **   * ** ** ** ** ***** ****

Flounder            (379)  CSVVVEGEVKHCVIYSTPRGFGFAEPYNLYSSLKDLVLHYHQTSLVQHND
Mus musculus        (393)  CSVVADGEVKHCVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHND
Rattus norvegicu    (393)  CSVVADGEVKPCVIYSPARGYGFAEPYNLYGSLKELVLHYQQTSLVQHND
Danio rerio         (395)  CSVVVEGEVKHCVIYSTPRGFGFAEPYNLYSTLKDLVLHYHQTSLVQHND
Homo sapiens        (359)  CSVVVDGEVKHCVINKTATGYGFAEPYNLYSSLKELVLHYQHTSLVQHND
                           ****  **** ***     * *********  ** *****  ********

Flounder            (429)  SLNVRLAYPVYAQMPSGRR-
Mus musculus        (443)  SLNVRLAYPVHAQMPTLCR-
Rattus norvegicu    (443)  SLNVTLAYPVHAQMPSLCR-
Danio rerio         (445)  SLNVRLAYPVYAQMPSGPRR
Homo sapiens        (409)  SLNVTLAYPVYAQQRR----
                           **** ***** **
```

Figure 3. Multiple alignment of the predicted flounder PI3Kγ amino acid sequence with other species (continued).

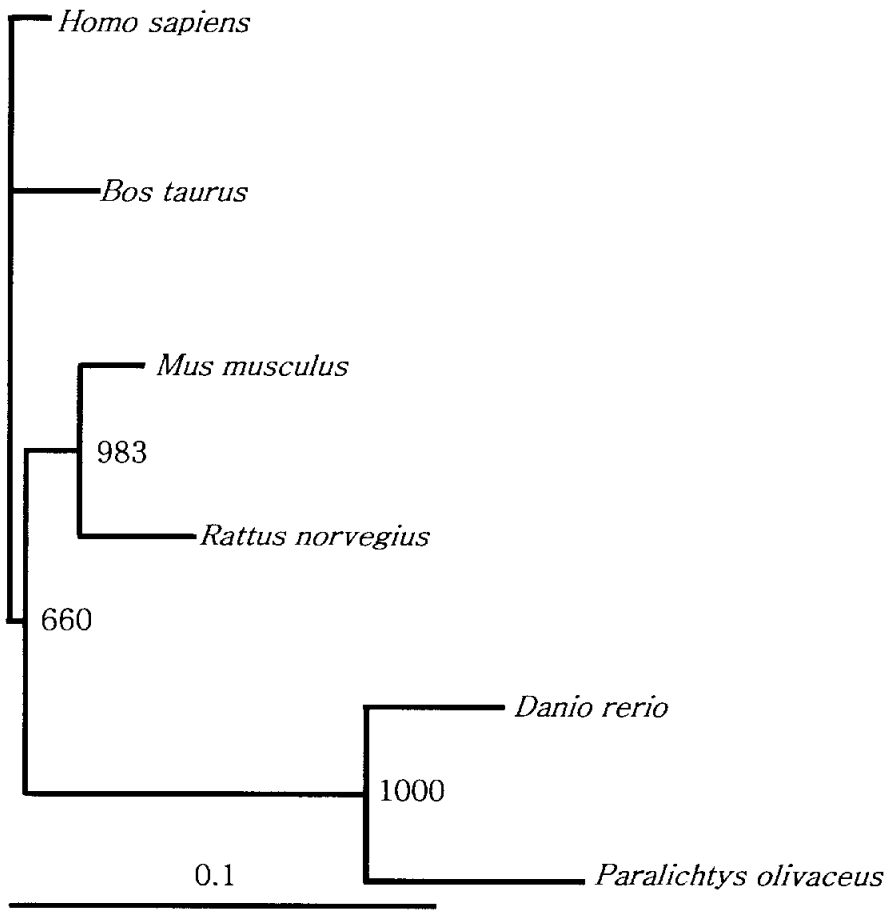


Figure 4. A molecular phylogenetic tree of PI3Kγ based on the sequence NJ method. A phylogenetic tree was constructed by the neighbor-joining (NJ) method using the program Treecon (Van de DPeer and De Wachter, 1994) for the amino acid sequences of PI3Kγ from *H. sapiens, B. Taurus, M. musculus, R. norvegius, and P. olivaceus.*

Tissues distribution of PI3Kγ

In order to determine the expression of the PI3Kγ gene, total RNA was isolated from flounder brain, liver, and kidney tissues using a TRIzol reagent and the quality of isolated RNAs was confirmed by formaldehyde RNA gel electrophoresis. Specific primers, PI3KF and PI3KR, were synthesized on the basis of the consensus sequence of PI3K and used for the detection of PI3Kγ mRNA using a RT-PCR. The products (10μl) of RT-PCR were analyzed on 1% agarose gel electrophoresis. As shown in Figure 5, approximately 750bp DNA fragment was amplified from all total RNAs extracted from the brain, liver, and kidney tissues (Figure 5). The resulting RT-PCR patterns provided evidence for the expression of PI3Kγ in tissues from the brain, liver and kidney, suggesting that the flounder PI3Kγ mRNA has a wide tissue distribution.

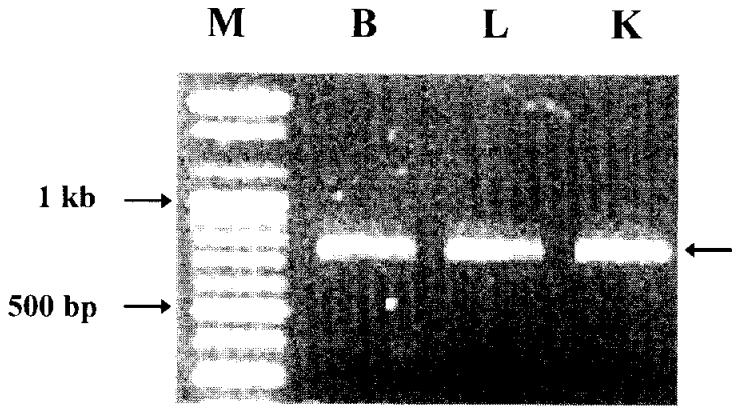


Figure 5. Pattern of the PI3Kγ expression detected by RT-PCR. Lane M, molecular maker; lane B, total RNA template for RT-PCR isolated from flounder brain; laneL, liver; lane K, kidney.

Expression of flounder PI3Kγ in *E.coli*

In order to subclone for the construction of expression vector of PI3Kγ gene, a pair of primers was designed on the basis of known PI3Kγ sequences. The resulting PCR fragment about 1.7kb was eluted and ligated into pGEM T-vector. Then, the flounder PI3Kγ gene was subcloned into the prokaryotic expression vector, pET-44a(+), which allows expression of recombinant protein with C-terminal fusion His-tag. The resulting pET-44a-PI3Kγ plasmid (Figure 6) was transformed into *E.coli* BL21 (DE3) codon plus strain and the expression of the recombinant protein by the addition of IPTG. The expression patterns of the PI3Kγ proteins were analyzed using 12% SDS-PAGE (Figure 7). The cloned PI3Kγ protein was strongly expressed with IPTG induction. The optimum induction time was approximately 1h after IPTG induction. The molecular weight of PI3Kγ fusion protein is approximately 49kDa, while the predicted PI3Kγ protein is approximately 46kDa, corresponding to a C-terminal fusion tag (3kDa).

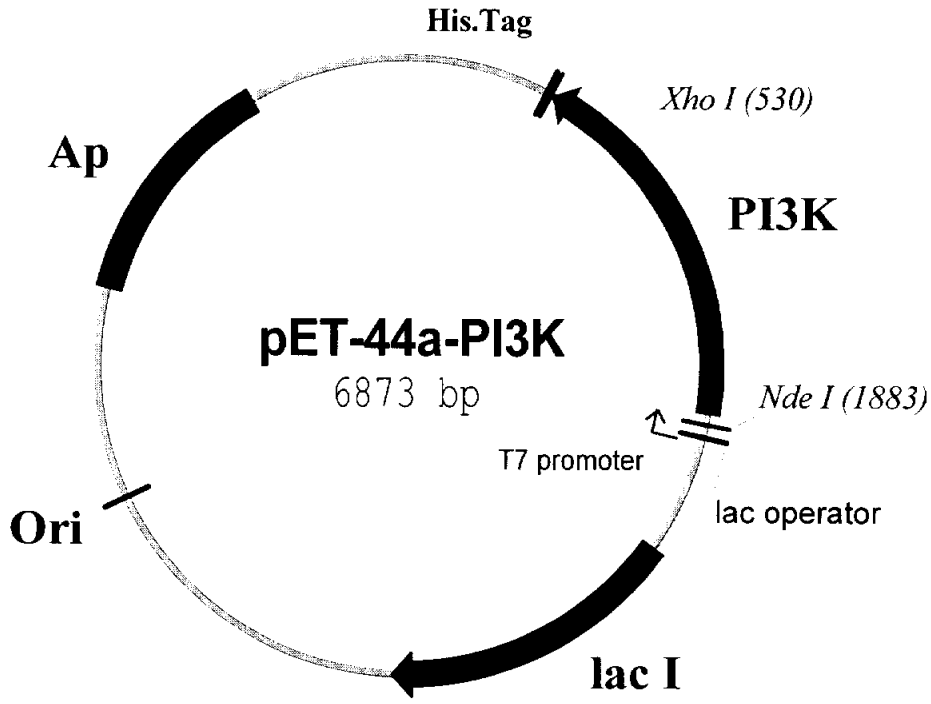


Figure 6. Construction of recombinant pET-44a-PI3Kγ plasmid. To express the PI3Kγ gene, the pET-44a-PI3Kγ plasmid was constructed by PCR using a set of primers, PI3KN and PI3KX. These primers, were generated the PI3Kγ sequence bearing both the N- and C-terminal ends of the flounder PI3Kγ coding sequence flanked by *Nde* I and *Xho* I site, respectively.

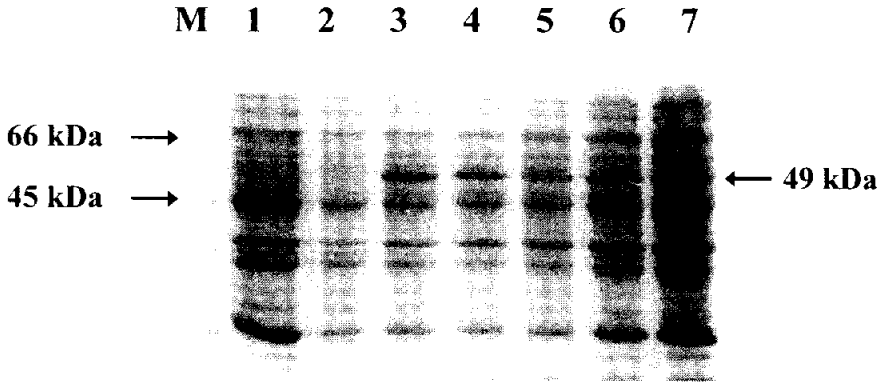


Figure 7. Analysis of the expressed proteins using SDS-PAGE. The expressed proteins of PI3Kγ gene in *E.coli* BL21 (DE3) codon plus were analyzed using 12% SDS-PAGE. Lane M, stained protein molecular weight marker; lane 1, proteins from uninduced cell extracts (control); lane 2-7, proteins from induced cell extracts 0, 1, 2, 3, 6, and 8h after IPTG induction, respectively.

Western blot analysis

In order to perform western blot, the induced cell was harvested by centrifugation at 0, 1, 3, 6hours, respectively. Proteins were electrophoretically transferred from an SDS-PAGE gel to nitrocellulose membrane, probed with goat antiserum aganist 6-His tag, and incubated with alkaline phosphatase coupled to goat antibody against goat IgG. The nitrocellulose membrane developed using NBT/BCIP. As shown in Figure 8, western blot was analyzed and confirmed.

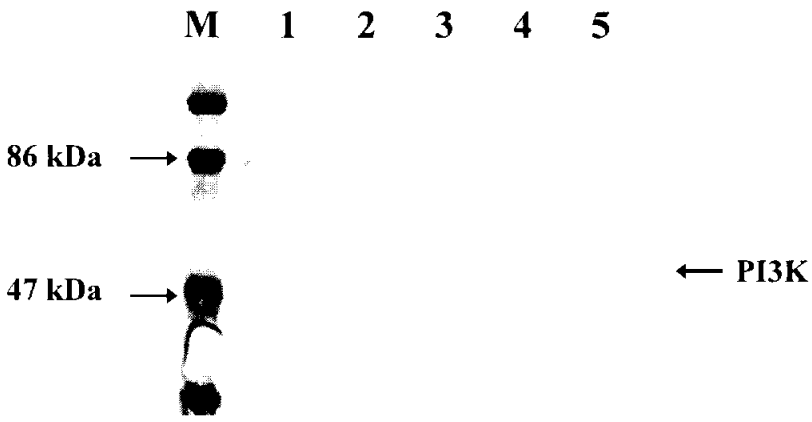


Figure 8. Analysis of the expressed proteins using western blotting.

The expressed protein was analyzed on 10% SDS-PAGE. Lane M, prestained protein molecular weight marker; lane 1, uninduced cell extracts as control; lane 2-5, induced cell extracts 0, 1, 3, and 6h after IPTG induction, respectively.

DISCUSSION

Fish are the largest and most diverse group of vertebrates. Their evolutionary position relative to other vertebrates and their ability to adapt to a wide variety of environments make them ideal for studying both organism and molecular evolution. Phosphorylated lipids are produced at cellular membranes during signaling events and contribute to the recruitment and activation of various signaling components. The role of phosphoinositide 3-kinase γ (PI3Kγ), which catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate, is involved in cell survival pathway, the regulation of gene expression, cell metabolism, and cytoskeletal rearrangements.

The cDNA encoding flounder phosphoinositide 3-kinase γ has been cloned. The nucleotide sequence of the PI3Kγ has an open reading frame (ORF) consisting 1341bp encoding 447 amino acid residues and has a 5'-noncoding region (86 nucleotides) and a 3'-noncoding region (314 nucleotide). A polyadenylation signal AATAAA is not found in the flounder PI3Kγ gene. However, the canonical AATAA signal appears to be its most common natural variant ATTAAA, which is found in 12% in vertebrate m RNA sequences. In a flounder PI3Kγ mRNA sequence, poly(A) tail was found to be 3'-noncoding sequence, suggesting that the variation in

polyadenylation signal many occur in this gene.

The deduced amino acid sequence of the flounder PI3Kγ protein shares 80% sequence identity with the human, mouse, rat, and zebrafish PI3Kγ molecules and even higher in functional domains. Thus, flounder PI3Kγ protein functions in an identical manner with their signaling partners. In fact, flounder PI3Kγ displays putative interaction sites for MAPK (mitogen activated protein kinase), Ras, G-protein βγ subunits, ATP, and lipids. We also provide phylogenic information on PI3Kγ that is essential for understanding the molecular evolution of this gene in vertebrates.

We have studied the tissue distribution of the cloned PI3Kγ. The resulting RT-PCR DNA banding patterns provided evidence for the expression of PI3Kγ in tissues from the brain, kidney, and liver. The flounder PI3Kγ mRNA has a wide tissue distribution. The recombinant flounder PI3Kγ was efficiently expressed in *E. coli*. The molecular weight of the expressed PI3K protein turned out to be approximately 49kDa. The present study will certainly be helpful for future studies in understanding the roles of PI3Kγ.

국문초록

Phosphoinositide 3-kinase (PI3K)는 항 산화 제어반응, 심근세포 성장, 및 세포내 특수반응 뿐만 아니라 세포분화, 생장, 운동, 식균 및 내항작용, 세포 골격유지에 관여하는 등 세포 신호체계에서 핵심 역할을 하는 효소이다. PI3K는 세 그룹으로 나누어지며 type I PI3K는 leukocyte에서 우선적으로 발현되고 G-proteins의 βγ subunits에 의해서 활성화 된다. 본 연구에서는 넙치(*Paralichthys olivaceus*)의 PI3Kγ를 암호화하는 cDNA를 클로닝 하였다. 넙치 PI3Kγ는 1341bp 염기로 구성되는 한 개의 ORF를 가지며 이 단백질은 447 아미노산으로 구성되어있다. PI3Kγ는 zebrafish의 PI3Kγ와 89.6%, mouse와는 84.7%, Norway rat와는 84%, human의 PI3Kγ와는 74.9%가 아미노산 상동성을 나타내었다. 넙치의 각 조직별로 PI3Kγ 유전자가 발현되는지 reverse transcription chain reaction (RT-PCR)을 실시한 결과 PI3Kγ mRNA는 넙치의 뇌, 간, 신장 조직에서 발현되었다.

ACKNOWLEDGMENTS

오랜 시간이 흘렀습니다. 부족한 저를 거두어 그 동안 부모와도 같은 사랑과 격려로 이끌어 주신 김영태 교수님과 세심하게 끝까지 논문을 읽어주신 이훈구 교수님, 송영환 교수님께 감사 드립니다. 염려과 관심을 아끼지 않으셨던 이원재 교수님과 최태진 교수님, 대학원 과정동안 학문적 충고로 부족한 제자를 이끌어 주신 이명숙 교수님, 윤철원 교수님께도 감사를 드립니다. 그리고 2년이 넘는 시간동안 저를 돌봐주셨던 김진상 교수님께도 감사 드리며 빠른 쾌유를 빕니다.

바쁘신데도 불평 한 마디 없이 많은 도움을 주셨던 재형 선배, 실험실에 잘 적응 할 수 있도록 도와준 용배 선배, 언제나 친동생처럼 부족한 언니를 잘 따르던 미영이, 항상 웃음을 주었던 재광 선배, 소연언니 그리고 귀여운 학부생 희진이, 지현이, 유진이, 가영이에게 고마움을 전하며, 짧은 시간이었지만 저에게 가족과도 같은 생화학 실험실의 무궁한 발전을 기원합니다.

대학원 생활동안 많은 조언과 도움을 주신 전정민, 이종규, 강지희, 이대성, 김종오, 그 외 선배님들, 동기라는 이유만으로 많은 의지가 되었던 지현이, 재경이, 경규, 동욱이, 그리고 선비, 혜영이, 윤숙이, 지선이, 그 외 후배들에게 고마움을 전하며, 2년이란 세월을 함께 보냈던 응용 미생물학 실험실의 성희, 여주, 성경이, 유경이, 동희, 호선이, 영아, 현민이에게 고마움을 전합니다.

마지막으로 저를 항상 믿어주시고 힘을 주셨던 사랑하는 부모님과 언니 오빠들에게 고마움을 전하며, 힘들고 지칠 때 힘이 되어주었던 사랑하는 친구 진한이, 희정이, 혜진이, 현주, 세정이, 영옥이에게 이 작은 결실을 바칩니다.

REFERENCES

Aksoy, I. A., S. Aksoy, L. C. Cantley, D. A. Fruman, M. J. Ramsey, T. M. Roberts, and J. D. Tucker. 1999. Mouse Phosphoinositide 3-kinase p110 Gnen: Cloning, structural Organization, and Localization to Chromosome 3 Band B. *Biochem. Biophys. Res. Comm.* 262, 438-442.

Altruda, F., G. Bulgarelli-Leva, E. Hirsch, S. Marengo, E. Patrucco, M. Rocchi, E. Tolosano, and M.P. Wymann. 2000. Analysis of the murine phosphoinositide 3-kinase gene. *Gene* 256, 69-81.

Bourne, H. R., P. Rickert, G. Servant, F. Wang, and O. D. Weiner. 2000. Leukocytes navigate by compass: roles of PI3K and its lipid products. *Trends in Cell Biology* 10, 466-473.

Cantrell, D. A. 2001. Phosphoinositide 3-kinase signalling pathways. *J. Cell Sci.* 114, 1439-1445.

Cho, J. J., J. H. Lee, S-K. Kim, T-J. Choi, and Y. T. Kim. 1999. Complementary DNA encoding *nm23*/NDP kinase gene from the Korean tiger shark *Scyliorhinus torazame*. *Mar. Biltechnol.* 1, 131-136.

Cho, J. J. and Y. T. Kim. 2002. Sharks and cancer: a potential source of antiangiogenic factors and tumor treatments. Mar. Biotech. 4, 521-525.

Coelho, C. M. and S. J. Leevers. 2000. Do growth and cell division rates determine cell size in multicellular organisms? *J. Cell Sci.* 113, 2927-2934.

Ellson, C., P. Hawkins, and L. Stephens. 2002. Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Current Opinion in Cell Biology* 14, 203-213.

Foster, F. M., M. J. Fry, M. Siemon, and A. C. J. Traer. 2003.phosphoinositide (PI) 3-kinase family. *J. Cell Sci.* 116, 3037-3040.

Fry, M. J. 2001. Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play? *Breast Cancer Res.* 3, 304-312.

Hawkins, P., L. Stephens, and S. Suire. 2002. Activation of phosphoinositide 3-kinase by Ras. *Current Biology* 12, 1068-1075.

Hazeki, O., T. Katada, H. Kurosu, T. Okada, T. Suzuki, and S. Takasuga. 1998. Activation of PI 3-Kinase by G protein βγsubunits. *Life Sciences.* 62, 1555-1559.

Hirsch, E., F. Altruda, A. Mantovani, S. Sozzani, and M. P. Wymann. 2000. Lipids on the move: phosphoinositide 3-kinases in leukocyte function. Trends Immunol. Today 21, 260-264.

Hirsch, E., V. L., Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, A. Mantovani, F. Altruda, and M. P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase γ in inflammation. *Science* 287, 1049-1053.

Kim, D. S. and Y. T. Kim. 1999. Identification of an embryonic growth factor IGF-II from the central nervous system of the teleost, flounder, and its expressions in adult tissues. *J. Microbiol. Biotech.* 9, 113-118.

Montero, J. A., B. Kilian, J. Chan, P. E. Bayliss, and C. P. Heiserberg. 2003. Phosphoinositide 3-kinase is required for process outgrowth and cell polarization of gastrulating mesendodermal cells. *Curr Biol.* 15,1279-89.

Naga Prasad, S. V., H. A. Rockman, and C. Perrino. 2003. Role of phosphoinositide 3-kinase in cardiac function and heart failure. *Trends Cardiovasc Med* 13(5), 206-12.

Pirola, L. and M. P. Wymann. 1998. Structure and function of phosphoinositide 3-kinase. *Biochimica et Biophysica Acta* 1436, 127-150.

Rameh, L. and L. Cantley. 1999. The role of phosphoinositide 3-kinase lipid products inn cell function. *J. Biol. Chem.* 274, 8347-8350.

Roymans, D. and H. Slegers. 2001. Phosphatidylinositol 3 kinases in tumor progression. *Eur. J. Biochem.* 268, 487-498.

Sasaki, T., R. G. Jones, W. A. Stanford, P. S. Ohashi, A. Suzuki, and J. M. Penninger. 2000. Function of PI3Kγ in Thymocyte development, T cell activation, and neutrophil migration. *Sci.* 287,1040-1046.

Stoyanov,B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nuernberg, P. Gierschik, K. Seedorf, J.J. Hsuan, M.D. Waterfield, and R.Wetzker. 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Sci.* 269, 690-693.

Vanhaesbroeck, B., S. J. Leevers, G. Panayaton, and M. D. Waterfield. 1997. Phosphoinositide 3-kinase: a conserved family of signal transducers. *Trends. Biochem. Sci.* 22, 267-272.