Cloning and Characterization of Phosphoinositide 3-Kinase γ cDNA from Flounder (Paralichthys olivaceus)

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Phosphoinositide 3-kinase (PI3K) plays a central role in cell signaling and leads to cell proliferation, survival, motility, exocytosis, and cytoskeletal rearrangements, as well as specialized cell responses, superoxide production, and cardiac myocyte growth. PI3K is divided into three classes; type I PI3K is preferentially expressed in leukocytes and activated by βγ subunits of heterotrimeric G-proteins. In this study, the cDNAs encoding the PI3Kγ gene were isolated from a brain cDNA library constructed using the flounder (Paralichthys olivaceus). The sequence of the isolated PI3Kγ was 1341 bp, encoding 447 amino acids. The nucleotide sequence of the PI3Kγ gene was analyzed with that of other species, including Oreochromis niloticus and Danio rerio and it turned out to be well conserved during evolution. The PI3Kγ gene was subcloned into the expression vector pET-44a(+) and expressed in the E.coli BL21 (DE3) codon plus cell. The resulting protein was expressed as a fusion protein of approximately 49 kDa containing a C-terminal six-histidine extension that was derived from the expression vector. The expressed protein was purified to homogeneity by His-tag affinity chromatography and showed enzymatic activity corresponding to PI3Kγ. The binding of wortmannin to PI3Kγ as detected by anti-wortmannin antiserum, closely followed the inhibition of the kinase activities. The results obtained from this study will provide a wider base of knowledge on the primary structure and characterization of the PI3Kγ at the molecular level.

Key words: Characterization, gene cloning, paralichthys olivaceus, phosphoinositide 3-kinase γ (PI3Kγ)

Introduction

Phosphoinositides were recognized early as precursors for second messengers in cell surface receptor–coupled signal transduction pathways. Phosphoinositide 3-kinase (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-P2 and PtdIns-3,5-P2, and the triply phosphorylated form PtdIns-3,4,5-P3 [9].

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 [19, 23]. Class I PI3Ks comprise a p110 catalytic subunit and a regulatory adapter subunit. Class II PI3Ks are large (170-200 kDa) proteins that have a catalytic domain 45-50% homologous to class I PI3Ks. Finally, class III PI3Ks are typified by the yeast protein [8]. Class I PI3Ks have been the major focus of PI3K studies because these isoforms are generally coupled to extracellular stimuli; these PI3Ks are activated 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 [4, 7]. 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.

A class I PI3K is a heterodimeric complex, comprising a p110 catalytic subunit, of which there are four characterized isoforms (α, β, γ, and δ). Class I PI3Ks are subdivided into class IA and IB. Type IA PI3Ks p110α, p110β, and p110δ share 42-58% amino acid sequence identity and are associated with the p85 family of regulatory subunits; on the other hand type IB PI3K P110γ binds to a p101 adaptor molecule. Whereas class IA PI3Ks are activated by interaction with tyrosine-phosphorylated molecules, class IB p110
γ (PBKγ) is activated by engagement of heterotrimeric GTP-binding protein (G protein)-coupled receptors (GPCR). PBKγ is preferentially expressed in leukocytes [10, 20]; furthermore, it is activated by γ4 subunits of heterotrimeric G-proteins, which thus link seven transmembrane (7TM) helix receptor activation to phosphatidylinositol (3, 4, 5)-trisphosphate production [11, 14]. PI3Kγ controls thymocyte survival, as well as the 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-trisphosphate and the activation of protein kinase B, ribosomal protein S6 kinase, and extracellular signal-regulated kinase 1 and 2 [18, 21]. Thus, PBKγ regulates thymocyte development, T cell activation, neutrophil migration, and the oxidative burst. Recent studies in mice lacking functional PBKγ showed that PBKγ plays a key role as a modulator of inflammation and allergy, as well as in the regulation of cardiac contractility [11, 13, 17].

Elucidation of the structural diversity of PBKγ in recent years by molecular cloning of cDNAs and genes from various species has provided insight into their functions. PBKγ cDNA genes have been cloned from Mus musculus [2], Rattus norvegicus [1], Danio rerio [16], and Homo sapiens [22]. Knowledge of the molecular structure of PBKγ in marine fishes is extremely limited. In addition, the nature of PI3Kγ in these fish and their roles in the control of the PI3K 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 [5, 6, 15]. The present study focuses on the isolation of cDNA encoding the flounder PBKγ and characterization of the cloned gene. These data will provide a base of knowledge for the PBKγ gene at the molecular level and the functional diversity of PI3Kγ.

### Materials and Methods

#### RNA isolation and construction of the flounder cDNA library

Total RNA from flounder (P. olivaceus) brain, liver, and kidney tissues were isolated using a total RNA isolation kit (Promega). The complementary DNA (cDNA) library was constructed using a ZAP-cDNA Synthesis Kit (Stratagen), as described in the manufacturer’s instructions. The resulting library contained approximately 1×10^5 clones/ml. The library was then amplified up to 3×10^5 clones/ml.

#### Screening PI3Kγ cDNA and DNA sequencing

Conserved nucleotide sequences of PBK among the vertebrate species were determined using the National Center for Biotechnology Information (NCBI) nucleotide and protein sequence database and used for the design of oligonucleotide primers for screening PI3K, which were synthesized from GenoTech (Taejon). PCR was carried out using a pair of the “PBKFI” and “PI3KRI” primers (Table 1). The probe for screening PBK was labeled with a digoxigenin (DIG) oligonucleotide 3'-end labeling kit (Roche). DIG-labeled probes were quantified and used for the immunoscreening procedure. Approximately 1×10^5 of plaques from the cDNA library was screened with the above probes and several positive plaques were isolated. 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 (Stratagen).

#### Comparative sequence analysis of flounder PI3Kγ

To examine the molecular evolution of PBKγ (AY514674) from Paralichthys olivaceus, the following PI3Kγ sequences were imported from the Swiss-Prot database / GenBank: *D. rerio* (BC164683), *O. niloticus* (XM003448849), *M. musculus* (NM008841), *B. taurus* (NM174796), and *H. sapiens* (NM001256045). The nucleotide sequences were analyzed using

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Remark</th>
</tr>
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<tbody>
<tr>
<td>PBKF</td>
<td>5'-GACCTTCTGGTGGGGGAGC-3'</td>
<td>Forward primer for RT-PCR</td>
</tr>
<tr>
<td>PBKR</td>
<td>5'-CTGTCCTGGATCCGAGCCA-3'</td>
<td>Reverse primer for RT-PCR</td>
</tr>
<tr>
<td>PBKN</td>
<td>5'-CATATGGCAGCTAACAGAGGGG-3'</td>
<td>Forward primer for expression</td>
</tr>
<tr>
<td>PBKX</td>
<td>5'-CTCAGATCTGGCTCGAGGAG-3'</td>
<td>Reverse primer for expression</td>
</tr>
<tr>
<td>PBKFI</td>
<td>5'-ACACTTGCACACACACAGC-3'</td>
<td>Forward primer for preparation of probe</td>
</tr>
<tr>
<td>PBKRI</td>
<td>5'-TAATACGACTCATATGAGGC-3'</td>
<td>Reverse primer for preparation of probe</td>
</tr>
</tbody>
</table>
the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment was conducted using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw), and sequence identities were calculated using GeneDoc (http://www.psc.edu/biomed/genedoc). A phylogenetic tree was constructed by the neighbor-joining (NJ) method using the Treecon program [9] for the amino acid sequences of PI3Ks from D. rerio, O. niloticus, M. musculus, B. taurus, and H. sapiens.

Reverse transcription polymerase chain reaction (RT-PCR)

In order to perform RT-PCR, total RNA was isolated from the brain, liver, and kidney from mature flounder (n=10; size: 45±10 cm, body weight: 900±300 g; 3 years old). The RT-PCR was performed using Bioneer's RT-PCR system. The reaction components were set up for Master mix 1 and Master mix 2. Master mix 1 contained template RNA, 50 pmol of primer, and DEPC-water. The sample was incubated for 10 min at 65°C 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 0.2 μl tube. The sample was placed in a thermocycler (GeneAmp PCR system 2,400, Perkin Elmer) and incubated for 1 hr at 42°C for reverse transcription followed by thermocycling. The temperature pro-

**Fig. 1.** 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.
file of PBKγ was on pre-reaction at 94°C for 5 min and 30
cycling reaction with 94°C 40 sec denaturation, 56°C for 30
sec annealing, 72°C for 1 min, and finally a 7 min extension
at 72°C. After reaction, 15 μl of RT-PCR product was ana-
yzed with 1% agarose gel electrophoresis.

Expression of flounder PI3Kγ gene in Escherichia coli

The PI3Kγ gene was amplified by PCR using a pair of
oligonucleotides (Table 1). The PCR product was ligated into
the pGEM-T vector and the resulting plasmid was digested
with NdeI and XhoI restriction enzymes. Then, the excised
fragment was ligated into the pET44-a(+) vector. The resulting
plasmid containing PI3Kγ gene was called pET-44a-PBK.
The plasmid was transformed into the competent E. coli
strain BL21 (DE3) codon plus. Cells harboring a plasmid that
contained the PI3Kγ gene were cultured overnight in 10 ml
of Luria-Bertani / ampicillin (LB/amp; containing 50 μg/μl
ampicillin) broth at 37°C in a shaking incubator. The cell
was induced by adding isopropyl-β-D-thiogalactopyranoside
(IPTG) to a final concentration of 1 mM at mid-log growth
(OD600=0.5).

Purification of recombinant PI3Kγ proteins

The pET-44a(+) PI3K plasmid contains PI3K-histidine
(PDK-His)-tagged DNA sequences. The PDK-His fusion pro-
etin was eluted using a His Trap Kit (Pharmacia). The pellet
from 1 L of induced culture was resuspended in 100 ml of
binding buffer containing 5 mM imidazole, 0.5 M NaCl, 50
mM Tris-HCl pH 7.6, 1 mg/ml lysozyme (Sigma-Aldrich),
and protease inhibitors (Sigma-Aldrich). The cells were dis-
rupted by sonication for 30 sec in VC130 (Sonics and
Materials Inc). Cell debris was pelleted by centrifugation
at 12,000 rpm in a Sorvall SA-600 rotor for 15 min. The super-
natant was filtered through a 0.22 μm pore membrane, di-
luted in binding buffer, and then loaded on a His Trap chr
omatography column. The supernatant was eluted with three
column volumes of 500 mM imidazole, 0.5 M NaCl, and 50
mM Tris-HCl pH 7.6 (elution buffer). Each 3 ml fraction
was collected and measured for its protein content on SDS-
PAGE.

Enzyme activity assay

PBKγ protein activity was measured by the spectrophoto-
metric method of Stoyanov et al. [22]. Protein kinase assays
using purified PBKγ proteins and GST-p110a / p84a protein
were carried out at 30°C. To assay protein phosphorylation,
immobilized PBKγ was washed twice with kinase buffer
without ATP [50 mM Hepes (pH 7.4) / 150 mM NaCl / 5
mM, EDTA / 5 mM dithiothreitol / 10 mM MgCl₂ / 0.01%
Triton X-100] and resuspended in the same buffer (MgCl₂
concentrations were varied where indicated). As indicated,
TPA (800 nM), BIM (100 nM), wortmannin (100 nM), or lip-
osomes were added to the reaction mixture. An equal vol-
ume of kinase buffer supplemented with ATP was added
to initialize the phosphorylation reaction. Incubation for 20
min at 30°C was followed by denaturation and autoradiog-
raphy.

Protein determination

Protein concentration was determined by the Bradford
method. The Bradford reagent was from Bio-Rad and bovine
serum albumin (BSA) served as a standard protein.

Results and Discussion

Nucleotide sequences of flounder PI3Kγ

The PI3Kγ gene of flounder was isolated using PCR from
the flounder brain cDNA library. PCR products were cloned
into T vector. Cloned DNA was purified and sequenced with
an automatic DNA sequencer using the ABI Prism DNA se-
quencing kit.

Fig. 2 shows the nucleotide sequence of the complete
cDNA encoding the flounder PI3Kγ gene (GeneBank ac-
cession number AY514674) and its deduced amino acid
sequence. The sequence of cloned PI3Kγ was analyzed with
the NCBI BLAST program. The flounder PI3Kγ gene contains
1,744 bp, including an open reading frame and encoding a
447 amino acid protein. The cDNA consists of 86 bp of a 5'
untranslated region (UTR), 1,341 bp of coding region, and
314 bp of 3'-UTR, followed by a poly (A) sequence. As
shown in Fig. 2, the flounder PI3Kγ cDNA clone contains
an in-frame termination codon (TGA) at bases 1431-1434.

Sequence identity and the phylogenetic tree

Fig. 3 shows an alignment of the amino acid sequences
of the flounder and other PIKγ. The PIKγ proteins were
compared using the BLAST protein database (NCBI).

The flounder PI3Kγ had a high sequence similarity with
other species in its amino acid residues. The deduced
flounder amino acid sequence was about 89.6%, 84.7%, 84%,
and 74.9% identical with the PIKγ of zebrafish (D. rerio),
mouse (M. musculus), Norway rat (R. norvegicus), and human (H. sapiens), respectively.

A molecular phylogenetic tree was constructed to analyze the evolutionary relationships of the PI3Kγ protein (Fig. 3). It shows the evolutionary divergence of the PI3Kγ genes of the 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%).

**Tissue 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 with RT-PCR. The products (10 μl) of RT-PCR were analyzed with 1% agarose gel electrophoresis. As shown in Fig. 4, an approximately 750 bp DNA fragment was amplified from all total RNAs extracted from the brain, liver, and kidney tissues (Fig. 4). 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.
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 based on known PI3Kγ sequences. The resulting PCR fragment of about 1.7 kb was eluted and ligated into the pGEM T-vector. Then, the flounder PI3Kγ gene was subcloned into the prokaryotic expression vector, pET-44a(+), which allows expression of recombinant protein with a C-terminal fusion His-tag. The resulting pET-44a-PI3Kγ plasmid (Fig. 5A) was transformed into the E. coli BL21 (DE3) codon plus strain and recombinant protein were expressed by the addition of IPTG. The expression patterns of the PI3Kγ proteins were analyzed using 12% SDS-PAGE (Fig. 5B). The donor PI3Kγ protein was strongly expressed with IPTG induction. The optimum induction time was approximately 1 hr after IPTG induction. The molecular weight of the PI3Kγ fusion protein is approximately 49 kDa, while the predicted PI3Kγ protein is approximately 46 kDa, corresponding to a C-terminal fusion tag (3 kDa).

Western blot analysis

In order to perform western blot, the induced cells were harvested by centrifugation at 0, 1, 3, and 6 hr. Proteins were electrophoretically transferred from an SDS-PAGE gel to nitrocellulose membrane, probed with goat antiserum against the 6-His tag, and incubated with alkaline phosphatase coupled with the goat antibody against goat IgG. The nitrocellulose membrane developed using NBT / BCIP. As shown in Fig. 5C, western blot was analyzed and confirmed.

Purification of the PI3Kγ protein

The expression and purification of the recombinant PI3Kγ protein was analyzed by 12% SDS-PAGE. The optimal induction of a recombinant PI3Kγ protein was achieved at 9 hr after induction. The recombinant PI3Kγ protein was purified using an affinity chromatography. Affinity chromatography was applied for the single-step purification in order to separate a particular protein using a specific interaction with a ligand that specifically binds to a target protein from the cellular total proteins. Using this technique, the PI3Kγ protein was purified to homogeneity and the purified protein was shown to be enzymatically active. The molecular mass of the purified protein was 49 kDa, which represents the value calculated from the gene sequence (Fig. 6).

Enzyme activity of PI3Kγ

In view of the potent inhibition of serpentine receptor-mediated PtdIns (3, 4, 5) P3 production and cell responses by wortmannin, the inactivation mechanism of PI3Kγ by this substance was investigated. When GST-p110a / p85a and
PI3Kγ were incubated with increasing concentrations of wortmannin under identical conditions, the inhibitor displayed similar IC₅₀ values (approx. 2 nM) for both lipid kinases, as measured by the formation of [³²P] PtdIns3P from PtdIns and [³²P] ATP. Cell lysates were incubated after IPTG induction and enzyme purification and PI3Kγ activity was measured (Table 2). Covalent binding of wortmannin to PI3Kγ was detected by anti-wortmannin antisera; this occurred in parallel with inhibition and was found to be saturated at 20 nM (Fig. 7). As the inhibition of PEKs by wortmannin is mediated by a covalent modification of the catalytic subunit, reaction time, pH, buffer composition, and temperature all influence the inhibitor's potency and might explain the observed differences. In addition, the pronounced phosphorylation of PI3Kγ was demonstrated (Fig. 7). The unaltered incorporation of [³²P] confirmed the PI3Kγ-mediated phosphorylation of the protein.

In the present study, we studied the tissue distribution of cloned PI3Kγ. The resulting RT-PCR DNA banding patterns provided evidence for the expression of PI3Kγ in tis-

Table 2. Purification of recombinant flounder PI3Kγ from E.coli BL21(DE3) codon plus cells

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Activity (nmol/min)</th>
<th>Yield (%)</th>
<th>Specific activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crud extract</td>
<td>132</td>
<td>90.2</td>
<td>100</td>
<td>0.68</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>5</td>
<td>23.7</td>
<td>26.3</td>
<td>4.74</td>
</tr>
</tbody>
</table>
From Fig. 6, SDS-PAGE analysis of purified PI3Kα; Lane M, standard protein molecular weight markers; lane 1, cell lysate; lanes 2, pellet; lanes 3, Column flow through; and lanes 4-5, purified enzyme fraction mixture.

From Fig. 7, immobilized, recombinant PI3Kα and GST-110α / p85 PIK complexes were exposed to the indicated concentrations of wortmannin as indicated. PI3K activity was assayed by the formation of [32P] PtdIns3-P after wortmannin incubation. Legend: ○, Concentration-dependent inhibition of GST-p110α / p85; ●, Results for PI3Kα.

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Acknowledgement

References

초록 : 넌치에서 분리된 phosphoinositide 3-kinase γ 유전자의 클로닝 및 특성 연구

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Phosphoinositide 3-kinase (PI3K)는 항산화제, 게이코움, 심근세포 성장, 및 세포 내 특수반응 뿐만 아니라 세포분화, 생장, 운동, 식품 및 대화작용, 세포 길격유지에 관여하는 등 세포 신호계계에서 핵심 역할을 하는 효소이다. PI3K는 세 그룹으로 나누어지며 type I PI3K는 leukocyte에서 유전적 방식으로 발견되고 G-proteins의 βγ subunits에 의해서 활성화된다. 본 연구에서는 넌치(*Paralichthys olivaceus*)의 PI3Kγ를 암호화하는 cDNA를 클로닝하였다. 넌치의 PI3Kγ는 1,341 bp 염기로 구성되는 한 개의 ORF를 가지며 이 단백질은 447 아미노산으로 구성되어있다. PI3K는 zebrafish의 PKDγ와 89.6%, mouse와는 84.7%, Norway rat와는 84%, human의 PKDγ와는 74.9%가 아미노산 상동성을 나타내었다. PI3Kγ 유전자와의 대장균에서 발현을 위하여 pET-44a(+) using 파인과 DNA를 구축하여 대장균에서 발현시킨 결과 49 kDa의 제조합 단백질이 과발현 됨을 확인 할 수 있었다. His-tag affinity chromatography를 이용하여 PI3Kγ 단백질을 순수 분리하였으며 wortmannin을 이용하여 PI3Kγ의 활성을 분석하였다.