学位論文

A novel phosphatidylinositol 5-phosphate 4-kinase (PIPKIIγ) is phosphorylated in the endoplasmic reticulum in response to mitogenic signals

新規ホスファチジルイノシトール5-リン酸4-キナーゼは小胞体内に局在しリン酸化を受けて機能する

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A novel phosphatidylinositol 5-phosphate 4-kinase (PIPKIIγ) is phosphorylated in the endoplasmic reticulum in response to mitogenic signals

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Summary

In this study, I identify a novel rat PI5P 4-kinase(PIPKIIγ). PIPKIIγ comprises 420 amino acids with a molecular mass of 47,048 Da, showing greater homology to the type IIα and IIβ isoforms(64.4% and 63.3% amino acid identity, respectively) of PIP kinase than to the type I isoforms. It is predominantly expressed in kidney, with low expression in almost all other tissues. The PIPKIIγ is found to have PI5P 4-kinase activity as demonstrated in other type II kinases such as PIPKIIα. The PIPKIIγ that is present endogenously in rat fibroblasts, PC12 cells, and rat whole brain lysate or that is exogenously overexpressed in COS-7 cells shows a doublet-migrating pattern on SDS-PAGE. Alkaline phosphatase treatment and metabolical labeling in ^{32}P-orthophosphate experiments revealed that PIPKIIγ is phosphorylated *in vivo*, resulting in a shift in its electrophoretic mobility. The phosphorylation is induced by the treatment of mitogens such as serum and EGF. Immunostaining experiments and subcellular fractionation revealed that PIPKIIγ localizes dominantly in the endoplasmic reticulum (ER). The phosphorylation also occurs in the ER. Thus, PIPKIIγ may have an important role in the synthesis of PIP_2 in the ER.

Introduction

Phosphatidylinositol 4,5-bisphosphate($PI(4,5)P_2$) is a phospholipid with a variety of functions *in vivo* including not only the production of second messengers such as diacylglycerol and inositol 1,4,5-trisphosphate, but also the regulation of actin regulatory proteins, and the activation of phospholipase D and ADP-ribosylation factor. The latter two functions for $PI(4,5)P_2$ indicate that it is also involved in regulation of actin cytoskeleton or vesicle trafficking such as exocytosis and endocytosis. It has also been reported that $PI(4,5)P_2$ synthesis is potentiated by various stimuli including GTPγS(1,2,3), phorbol esters(4), tyrosine kinases(5), and integrins(6). The variations in its function and the regulation of its synthesis indicate that enzymes responsible for the production of PIP_2, such as PI kinase and PIP kinase, also show large diversities. Among them, PIP kinase, that catalyze the final step of $PI(4,5)P_2$ synthesis, seems to be important to accomplish the large diversity of intracellular roles for $PI(4,5)P_2$.

Two major subtypes (type I and II) comprising five isoforms (Iα, Iβ, Iγ, IIα, and IIβ) of mammalian PIP kinase have been identified to date(13, 16, 17, 18, 19). In budding yeast, two PIPK homologs (MSS4 and FAB1) are also known. In data base, sequences that seem to belong to this lipid kinase family are found in fission yeast, nematode, fruit fly, as well as in plants (Fig.1A). All members of this lipid kinase family have highly conserved "kinase domain" separated by a variable region called "insert domain" (Fig.1A).

Two subtypes of mammalian PIP kinase have different substrate specificity. Recently, Rameh *et al.* showed that type II PIPK is a PI5P 4-kinase while type I isoform is exactly a PI4P 5-kinase(22). They also showed the existence of PI5P *in vivo*, a novel phosphoinositide that had not been identified. These data shows that there are two different pathways for $PI(4,5)P_2$ synthesis (Fig.1B). Although the exact role for each pathway is not known, possible

intracellular functions for each subtype have been reported.

Type I isozyme has been reported to be activated by phosphatidic acid(7), to bind physically to small GTPase Rho(8) and Rac(9), and to be involved in Ca^{2+}-dependent exocytosis in PC12 cells(10). Human PIPKIβ has been shown to be identical to the STM7 gene, the putative gene responsible for Friedrich's Ataxia, suggesting that this isozyme plays roles in vesicular trafficking such as neurotransmitter release(11). On the other hand, type II isozymes have also been reported to have several functions *in vivo*. In platelets, PIPKIIα was shown to translocate to the cytoskeletal fraction after stimulation by thrombin(12). PIPKIIβ was identified by its specific interaction with a cytoplasmic region of the p55 tumor necrosis factor-α receptor and a role for PIPK in TNF-α signaling has been suggested(13).

From these data, it seems that synthesis of phosphoinositides are controlled by various mechanisms to accomplish their multiple functions in appropriate time and appropriate location within the cell.

Here, I identify a novel PIPKII isozyme (PIPKIIγ) by an RT-PCR method using degenerate primers designed from highly conserved primary sequences in PIPK family members. PIPKIIγ is phosphorylated on serine residues *in vivo* resulting in a mobility shift in SDS-PAGE. Mitogenic stimulation, such as by serum-, EGF- or PDGF-treatment, results in phosphorylation of PIPKIIγ. The results of the immunofluorescence experiments and subcellular fractionation suggest PIPKIIγ has important roles in the production of PIP_2 in the ER.

Experimental Procedures

Materials

PIP was purified by neomycin column chromatography from crude phospholipids extracted from bovine spinal cord as described(14). [α-^{32}P]dCTP, [γ-^{32}P]ATP, [^{32}P]orthophosphate and [^{3}H]PI(4,5)P_2 were from DuPont/NEN Research Products(Boston, U.S.A.). The Colony/PlaqueScreen used to screen the cDNA library were from NEN Life Science Products(Boston, U.S.A.). The PVDF membranes used for Western blot analysis were from Nihon Eido(Tokyo, Japan). Ni-NTA agarose was from QIAGEN(Chatsworth, U.S.A.). Partisphere SAX column was from Whatman International Ltd.(Maidstone, England). The thin layer chromatography silica plates and cellulose plate used to separate phospholipids and phosphoamino acids, respectively, were from Merck(Darmstadt, Germany). Monoclonal anti-Myc antibody was purchased from Santa Cruz Biotechnology(Santa Cruz, U.S.A.). Monoclonal anti-BiP antibody was from StressGen Biotechnologies Corp. Monoclonal anti-β-tubulin antibody was from Chemicon International Inc. (Temecula, U.S.A.) Rhodamine-, and fluorescein-conjugated anti-rabbit IgG antibodies and fluorescein-conjugated anti-mouse IgG antibody were from Organon Teknika Corp (West Chester, U.S.A.). Rhodamine-conjugated wheat germ agglutinin was from Molecular Probes, Inc. (Eugene, U.S.A.).

Cell Culture

COS-7 and 3Y1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. PC12 cells were grown in DMEM containing 10% horse serum and 5% fetal bovine serum.

Reverse Transcription - Polymerase Chain Reaction

Total RNA isolated from rat brain was reverse transcribed into cDNA by MuLV reverse transcriptase, and used as a template for PCR using degenerate primers (5'-GAITAYTGYCCIRWIGTITTYMG-3', 5'-ATICYIABIAIIARRCTRTARTCCAT-3' and 5'-ATICYIABIAIIARIGARTARTCCAT-3') corresponding to two highly conserved sequences in mammalian and yeast PIP kinases (D/EYCPXVFR, MDYSLLLGI/M) (see Fig.2A). The polymerase chain reaction was carried out as follows; 95℃ for 1 min, 43℃ for 1 min, and 72℃ for 2 min, for 40 cycles. The PCR product, about 500 bp long, was subcloned into the SmaI site of pBluescript SK(-) vector and sequenced.

cDNA cloning of PIPKIIγ

The PCR product encoding a novel sequence was cut out from the vector with EcoRI and BamHI, labeled with [α-^{32}P]dCTP, and used as a probe for screening a rat brain cDNA library. The longest clone obtained (about 2.4kb) encoded an ORF as long as about 400 amino acids, but did not include a potent start codon. On the other hand, another partial clone was obtained that included a potent start codon preceded by a sequence consistent with Kozak's consensus(15), but did not include a stop codon. From the sequences of these two clones, I could determine the complete sequence for this novel PIP kinase.

Northern Blot Analysis

A partial fragment corresponding to 418-1500bp of cDNA was labeled and used as a probe for Northern blot analysis. Hybridization was carried out against Mouse Multiple Tissue Northern (MTN™) Blot membrane

(CLONETECH Laboratories, Inc.).

Production of Polyclonal Antibody

A partial cDNA fragment encoding amino acids 130-420 was ligated into the PstI-HindIII site of a pQE32 His-tag expression vector(QIAGEN). The His-tagged protein was expressed in *E.coli* and purified with Ni-NTA agarose(QIAGEN) as described by the manufacturer. The purified protein was injected as an antigen into rabbits to raise polyclonal antiserum. The resulting antibody was affinity purified with the antigen protein transferred onto a PVDF membrane or immobilized on Hi-Trap NHS-activated column (Pharmacia).

Transfection into COS-7 Cells

The full-length cDNA of mouse PIPKIβ, rat PIPKIIβ and PIPKIIγ was ligated into the SalI-BamHI site of pCMV-Myc or the XhoI-BamHI site of pSRαXEBNeo mammalian expression vectors. Twenty micrograms of each plasmid was mixed with 1 X 10^7 cells and the mixtures were subjected to electroporation with a Gene Pulser (Bio-Rad). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Measurement of PIP Kinase Activity

Forty-eight hours after electroporation, the expression vector-transfected COS-7 cells were lysed with lysis buffer (20 mM Hepes, pH7.2, 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Nonident P-40, 1 mM EGTA, 25 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF). The expressed enzyme was

immunoprecipitated with monoclonal anti-Myc antibody and washed three times with lysis buffer and once with reaction buffer (50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$, 1 mM EGTA). The reaction was started by adding 50 μM PIP and 50 μM ATP, 10 μCi [γ-^{32}P]ATP in 50μl. After incubating for 10 min at room temperature, the lipids were extracted with 1N HCl and chloroform/methanol(2/1 in vol.) and spotted on TLC plates. The plates were developed in chloroform/methanol/ammonia/water(= 14/20/3/5 in vol.) and the products were observed by autoradiography or quantified by an image analyzer BAS2000 (Fuji).

Analysis of Phosphoinositides by SAX HPLC

Phosphoinositides separated by TLC were scraped out, deacylated and analyzed by SAX HPLC as described(20).

Dephosphorylation of Phosphoinositides by SHIP

A partial fragment corresponding to 1084-3947bp of the cDNA of human Src homology containing inositol polyphosphate phosphatase (SHIP) was cut out with SalI and BamHI, and ligated into the SalI-BamHI site of pCMV-Myc. The resulting expression vector was transfected into COS-7 cells as described above. Myc-SHIP was immunoprecipitated and the dephosphorylation of lipids was carried out in 50 mM Tris-HCl pH7.5, 10 mM $MgCl_2$ at 37℃ for 60 min. The lipids were extracted and separated by TLC (chloroform/methanol/acetic acid/water = 43/38/5/7 in vol.)

Dephosphorylation of PIPKIIγ by Alkaline Phosphatase

Myc-tagged PIPKIIγ was immunoprecipitated from the lysate of overexpressing COS-7 cells. The immunoprecipitates were washed first with

lysis buffer, then with alkaline phosphatase buffer (50 mM Tris-HCl, pH8.2, 50 mM NaCl, 1mM $MgCl_2$, 1 mM DTT, 1 mM PMSF), after which 2U of calf intestine alkaline phosphatase (Takara Shuzo Co., Ltd.) or storage buffer for CIAP (10 mM Tris-HCl, pH8.0, 1 mM $MgCl_2$, 50 mM KCl, 0.1 mM $ZnCl_2$, 50% glycerol) was added. The reaction was carried out at 30℃ for 60 min.

Metabolic ^{32}P-Labeling of PC12 cells and Phosphoamino Acid Analysis

The culture medium was changed to phosphate-free DMEM, and the PC12 cells were cultured for 30 min. [^{32}P]orthophosphate (0.2mCi/ml) was then added and the cells were incubated for 24h. Labeled cells were lysed in lysis buffer (20 mM Hepes, pH7.2, 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Nonident P-40, 1 mM EGTA, 25 mM NaF, 0.1 mM sodium vanadate, 1 mM PMSF), and the PIPKIIγ was immunoprecipitated with anti-PIPKIIγ antibody and transferred to a PVDF membrane. The band corresponding to PIPKIIγ was cut out and hydrolysed in 6N HCl for 1h at 110℃. The resulting amino acids, together with standard phosphoamino acids, were spotted on TLC plates and separated by electrophoresis in pH3.5 buffer(5% acetic acid, 0.5% pyridine). The labeled phosphoamino acids were detected by autoradiography. The positions of the standard phosphoamino acids were detected by ninhydrin staining.

Immunofluorescence of PIPKIIγ

Cells growing on glass coverslips were fixed with 3.7% formaldehyde in PBS for 15 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Incubation with the first antibody (polyclonal anti-PIPKIIγ and monoclonal anti-BiP) was carried out for 1 h and incubation with the second antibody or rhodamine-conjugated WGA for 30 min. The cells were observed with a

confocal fluorescence microscope (Bio-Rad).

Subcellular Fractionation

The subcellular fractionation was performed as described (21) with some modifications. Rat liver or 3Y1 fibroblasts were homogenized in 0.25M sucrose, 50 mM triethanolamine-HCl, pH7.5, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF 10μg/ml aprotinin. After centrifugation at 800 X g for 10 min and 10,000 X g for 10 min to devoid "nuclear" pellet and "mitochondrial" pellet respectively, "post-mitochondrial" supernatant was obtained. The supernatant was layered over a cushion of 1.3M sucrose in the same buffer and centrifuged at 202,000 X g for 2.5 h to yield three distinct fractions; "post-microsomal" supernatant (representing cytosol), interfacial "smooth microsomes" (representing the smooth ER and the Golgi apparatus) and "rough microsomal" pellet (representing the rough ER).

Result

Identification of a Novel PIP kinase

To identify novel PIP kinases, I applied an RT-PCR method using degenerate-primers corresponding to amino acid sequences highly conserved among mammalian PIP kinases and their yeast homologs, MSS4 and FAB1 (Fig.2A). Total RNA isolated from rat brain was reverse-transcribed and used as a template for further PCR. The PCR product was subcloned into pBluescript vector and sequenced. Among several sequences corresponding to known PIP kinases, one novel sequence homologous to the type II isoform of PIP kinase was obtained. I then tried to isolate a full length cDNA using this fragment as a probe. By screening a rat brain cDNA library including random-primed clones, I obtained two clones encoding overlapping sequences. Between these clones, I found an ORF of 1260 bp encoding 420 amino acids (Fig.2B). The calculated molecular mass is 47,048 Da.

The Novel PIP kinase Belongs to the Type II Subfamily

The whole amino acid sequence of the novel PIP kinase was revealed to be homologous to the types IIα and IIβ isoforms of PIP kinase rather than type I isoforms (Table 1) indicating that this PIP kinase is a third member of the type II isoform subgroup (Fig.2C). Thus I named this novel PIP kinase type IIγ (PIPKIIγ). PIPKIIγ has a highly conserved kinase homology domain separated by an insert domain showing no similarity to other PIP kinase family members.

Tissue Distribution of PIP kinase IIγ

To study the tissue distribution of PIPKIIγ, Northern hybridization was carried out against mRNA from various mouse tissues. An mRNA of about 3.5kb was detected in almost all tissues with the most abundant expression in kidney (Fig.3). Tissue distributions for other PIPK isoforms have also been reported. PIPKIα is expressed mainly in brain and testis, PIPKIβ is abundant in brain, lung, skeletal muscle and testis. PIPKIγ is expressed in kidney, but is also rich in brain and lung. For PIPKIIα, restricted expression in brain and heart has been reported, and an abundant expression in skeletal muscle for PIPKIIβ has been reported(13, 16, 17, 18, 19). The pattern of distribution for PIPKIIγ is different from that of any type I isoform or other type II isoform, suggesting specific functions for this isoform.

The PIP kinase IIγ is a Phosphatidylinositol 5-phosphate 4-kinase

I transfected a Myc-tagged version of the full length cDNA of PIPKIIγ (Myc–PIPKIIγ) into COS-7 cells. The protein expressed in the whole cell lysate and anti-Myc immunoprecipitate was detected as a doublet form by Western blotting with anti-Myc antibody (Fig.4A and studied further below). Next, Myc-PIPKIIγ was immunoprecipitated and the PIP kinase activity was measured. The immunoprecipitate phosphorylated PIP purified from bovine spinal cord (see *Materials and Methods*), whereas anti-Myc immunoprecipitates from cells transfected with vector alone failed to do so (Fig.4B). By using SAX HPLC, the resulting PIP_2 was confirmed to be $PI(4,5)P_2$ (Fig.4C).

Recently, Rameh *et al.* showed that the PIPKIIα is a PI5P 4-kinase, and slightly different from PI4P 5-kinase (22). To examine whether PIPKIIγ is a PIP 4- or 5-kinase, $[^{32}P]PI(4,5)P_2$ produced from the mixture of PI4P and PI5P (i.e. PIP purified from bovine spinal cord) by PIPKIIγ was treated with

SHIP, which was reported to dephosphorylate preferentially position 5 of PI(4,5)P_2(22). By SHIP-treatment, [^{32}P]PI(4,5)P_2 spot was decreased while a [^{32}P]PIP spot appeared (Fig.4D). This [^{32}P]PIP was confirmed to be [^{32}P]PI4P by SAX HPLC (Fig.4E). The same [^{32}P]PI4P spot was also detected when [^{32}P]PI(4,5)P_2 produced by PIPKIIβ was used(Fig.4D). On the other hand, [^{32}P]PI(4,5)P_2 produced by PIPKIβ did not show any [^{32}P]PI4P spot visible in a short exposure. But the long exposure showed a [^{32}P]PIP spot (Fig.4D) which might represent [^{32}P]PI5P produced by a weak 4-phosphatase activity of SHIP(22). The identification of [^{32}P]PI5P spot was also carried out by SAX HPLC (Fig.4E). These results indicate that PIPKIIγ is a PI5P 4-kinase like other type II isoforms.

PIP kinase IIγ is a Phosphoprotein

A polyclonal antibody was produced with a partial His-tagged protein expressed in *E.coli* as an antigen. With this polyclonal antibody, endogenous PIPKIIγ was detected as doublet bands at 47 kD in lysates from rat brain, PC12 cells, and 3Y1 fibroblasts by Western blotting (Fig.5A). When the full length cDNA (without Myc-tag) was transfected into COS-7 cells, the same doublet band was detected (Fig.5A), suggesting that this doublet corresponds to some modification of PIPKIIγ such as proteolysis or phosphorylation and is not due to crossreactivity of the antibody to another protein. To examine the possibility that these doublet bands correspond to phosphorylated PIPKIIγ, I treated the Myc-tagged version of PIPKIIγ with alkaline phosphatase. The Myc-PIPKIIγ that immunoprecipitated from overexpressing COS-7 cells showed a doublet banding pattern with the upper band predominant. When the immunoprecipitated Myc-PIPKIIγ was incubated with calf intestine alkaline phosphatase, the upper band disappeared completely(Fig.5B) while the lower

band increased in intensity. This indicates that the doublet migrating pattern is due to the phosphorylation of PIPKIIγ. To confirm this conclusion, I next labeled PC12 cells metabolically with [^{32}P]orthophosphate. After labelling, the cells were lysed and PIPKIIγ was immunoprecipitated, showing that PIPKIIγ was phosphorylated (Fig.5C). Together with the result of Western blotting, it was confirmed that this phosphorylated protein corresponds to the upper band of PIPKIIγ (Fig.5C). Next, the phosphorylated band was cut out from membrane and phosphoamino acid analysis was carried out. The results showed PIPKIIγ phosphorylation occurs predominantly on serine residues (Fig.5D). To determine whether enzymatic activity of PIPKIIγ is affected by its phosphorylation, I measured the activity of Myc-PIPKIIγ after alkaline phosphatase treatment. Myc-PIPKIIγ retained considerable activity even after alkaline phosphatase treatment (Fig.5E) indicating that the phosphorylation of PIPKIIγ does not affect its enzymatic activity.

PIP kinase IIγ is Phosphorylated in Response to Extracellular Stimuli

In response to extracellular stimuli such as growth factors or hormones, intracellular protein kinases are activated and phosphorylate their physiological substrates. Since PIPKIIγ was found to be phosphorylated on serine residues *in vivo*, I examined whether the level of PIPKIIγ phosphorylation is potentiated by extracellular stimuli. First, I treated rat 3Y1 fibroblasts with 10% serum for various periods. The upper band of PIPKIIγ increased in a time dependent manner, suggesting PIPKIIγ is phosphorylated in response to serum (Fig.6A). Then, I examined other extracellular stimuli including EGF, PDGF, bradykinin, and lysophosphatidic acid (LPA) for their abilities to induce the phosphorylation of PIPKIIγ. Among them, EGF and PDGF enhanced the phosphorylation as well as serum (Fig.6B, C). LPA and

bradykinin also induced phosphorylation to a lesser extent. Fig.5D clearly shows that PIPKIIγ phosphorylation does not take place on tyrosine residues. Moreover, PIPKIIγ was not recognized by an anti-phosphotyrosine antibody, PY20 (data not shown). Therefore, it seems likely that the phosphorylation is mediated by a serine/threonine kinase downstream of mitogenic signals mediated by receptor tyrosine kinases. PKC does not seem to be involved, since PMA did not potentiate phosphorylation (Fig.6B, C). In addition, a specific PKC inhibitor, H7, did not suppress phosphorylation in 3Y1 cells (data not shown).

Intracellular Localization of PIP kinase IIγ

Using a polyclonal antibody, I next examined the intracellular localization of PIPKIIγ. The polyclonal antibody used was confirmed to recognize specifically the doublet band corresponding to PIPKIIγ in 3Y1 cell lysates by Western blotting (Fig.5A). When rat 3Y1 fibroblasts were stained, PIPKIIγ was seen to predominate in the perinuclear regions, suggesting that it is localized in microsomal organelles such as endoplasmic reticulum (ER). To confirm this possibility, I double-stained rat 3Y1 fibroblasts with anti-PIPKIIγ antibody and with anti-BiP, an ER-retaining protein. Both staining patterns (Fig.7A) clearly indicate the localization of this enzyme in the ER. This staining pattern does not overlap with that by wheat germ agglutinin, a *trans*-Golgi staining reagent (Fig.7A).

The intracellular localization of PIPKIIγ was studied further by a subcellular fractionation method using 1.3M sucrose cushion (see *Materials and Methods*). PIPKIIγ was detected in smooth and rough microsome fractions of rat liver the same as ER-marker BiP, whereas a cytosolic protein β-tubulin was detected only in the top of the gradient (Fig.7B). The phosphorylated form of PIPKIIγ was also detected in these fractions

suggesting phosphorylation of PIPKIIγ occurred in microsomes. To confirm this possibility, post-mitochondrial supernatants of 3Y1 fibroblasts were subjected to the same subcellular fractionation after mitogenic stimulations. PIPKIIγ, together with BiP, was detected predominantly in the smooth microsomal fraction (Fig.7C). On stimulation by EGF, the phosphorylated form increased in this fraction indicating that the phosphorylation of PIPKIIγ occurred within the ER (Fig.7C). In addition, immunofluorescence staining also showed that the localization of PIPKIIγ in the ER was not affected by stimulation of the cells with serum or EGF (data not shown).

These results indicate that PIPKIIγ is phosphorylated in the ER in response to mitogenic signals, thus suggesting it has important roles in the synthesis of PIP_2 in the ER.

Discussion

Purification and cDNA cloning of the 53kD PIP kinase IIα from erythrocytes revealed the lipid kinase to belong to a distinct kinase family different from those of PI 3-, PI 4-kinases or protein kinases(16). This family also seems to include yeast homologs such as MSS4p and FAB1p. Furthermore, cDNA cloning of types Iα and Iβ, members of another subtype of mammalian PIP kinase, also showed them to belong to this same distinct lipid kinase family(17,18). Members of this novel lipid kinase family have several conserved regions within their primary sequences. Using an RT-PCR method involving degenerate primers corresponding to these highly conserved sequences, I succeeded in identifying a novel PIP kinase isoform and named it PIPKIIγ.

Although PIPKIIγ seems to belong to the type II subtype, the similarity between PIPKIIγ and other members of the type II PIP kinase family is not very high (64.4% for IIα, 63.3% for IIβ) compared with the homology between PIPKIIα and PIPKIIβ (78.7%) (Table 1). This together with the difference in its expression pattern from that of other PIP kinases, suggests PIPKIIγ has some distinct functions *in vivo*.

PIPKIIγ was detected as a doublet-migrating protein by Western blotting with a specific polyclonal antibody not only in rat brain lysates but also in 3Y1 fibroblasts and PC12 cells. The same doublet patterns were also observed when PIPKIIγ was overexpressed in COS-7 cells. The evidence presented in this study shows that PIPKIIγ is phosphorylated *in vivo* and that the upper band represents the phosphorylated form. Furthermore, phosphoamino acid analysis revealed that phosphorylation occurs predominantly on serine residues. I also observed that mitogens such as serum and growth factors immediately induced phosphorylation of PIPKIIγ. The total cellular amount of PIP_2, as well as the PIP kinase activity, have been reported to increase in

response to various extracellular stimuli including EGF(5), formyl-methionyl-leucyl-phenylalanine, platelet activating factor(1), thrombin(23), phorbol ester(4), and adhesion to fibronectin(6). Some of these extracellular stimuli have been reported to increase PIPK activity, especially in the cytoskeleton. In addition, the involvement of G-proteins, including small GTPases such as Rac and Rho, has also been suggested by data showing that the PIP kinase activity is potentiated by non-hydrolysable GTP or is associated with recombinant Rho and Rac protein. Despite the above observations, the exact molecular mechanism by which PIPK is regulated has not been made clear. Here I provide evidence for the phosphorylation of PIPKIIγ. It is possible that PIPK is regulated by a protein kinase downstream of extracellular stimuli. At present I do not know what kinase is responsible for the phosphorylation. The phosphorylation was found to be enhanced by tyrosine kinase activators such as EGF and PDGF rather than activators related to heterotrimeric G protein-coupled signalings, such as bradykinin and lysophosphatidic acid. Moreover, dibutyryl cAMP (not shown) and PMA do not increase the phosphorylation markedly. Considering that phosphorylation occurs on serine residues rather than on tyrosine residues, a serine kinase, other than PKA or PKC, downstream of a tyrosine kinase must phosphorylate PIPKIIγ. Although the exact roles of the phosphorylation remain unclear, it is possible that the phosphorylation of PIPKIIγ regulates its localization. Hinchliffe et al.(12) reported that the translocation of PIPKIIα to the cytoskeletal fraction of platelets in response to thrombin is inhibited by okadaic acid treatment, suggesting the importance of dephosphorylation for translocation. Although they also showed that the activity of PIPKIIα is regulated by its phosphorylation state(24), I did not observe any change in the activity of PIPKIIγ after phosphorylation by mitogenic stimulation nor dephosphorylation by alkaline phosphatase (data not shown and Fig.5E).

In this study, I demonstrated that PIPKIIγ is specifically localized in the ER in rat 3Y1 fibroblasts. Although most PIP kinase activity is found in the

plasma membrane and cytosol, Helms *et al.*(25) reported that $PI(4,5)P_2$ synthesis occurs in the ER. Several phosphoinositide metabolizing enzymes have been reported to be localized in microsomal fraction. Wong *et al.* reported that PI 4-kinase α is localized in the ER whereas PI 4-kinase β is localized in the Golgi apparatus in HeLa cells(26). Most PI-synthase activity is also detected in the ER(25, 27, 28). It is conceivable that $PI(4,5)P_2$ synthesis occurs efficiently in microsomes because of the relay of substrates between PI-synthase, PI kinase, and PIP kinase. In addition, PI5P, the preferential substrate for type II isozymes in $PI(4,5)P_2$ synthesis, is rare in NIH3T3 cells(22), compared with PI4P which exits abundantly in the cell. It may be important for this minor phosphoinositide to be localized at a restricted area such as in microsomes with its metabolizing enzyme, PI5P4K, for efficient $PI(4,5)P_2$ synthesis. Many of the characteristics of PI5P have yet to be elucidated, including its synthetic pathway as well as the identity of PI 5-kinase and its exact intracellular localization. However, together with the observation that PIPKIIγ is localized in the ER after phosphorylation by mitogenic signals, my results suggest that PIPKIIγ is involved in the synthesis of $PI(4,5)P_2$ in the ER.

Shibasaki *et al.* reported the type I PI4P 5-kinase overexpressed in COS-7 cells by an adenovirus expression system is localized mainly at plasma membranes and cytosol. They further reported that type I PIP kinases induce a pine needle-like structure of the actin cytoskeleton(29). In contrast, I observed no change in the actin cytoskeleton when type IIβ and IIγ isozymes were transiently overexpressed in COS-7 cells (data not shown). From these results, it is possible to conclude that each subfamily of PIP kinase has a distinct localization and function and is also responsible for the synthesis of distinct intracellular PIP_2 sources.

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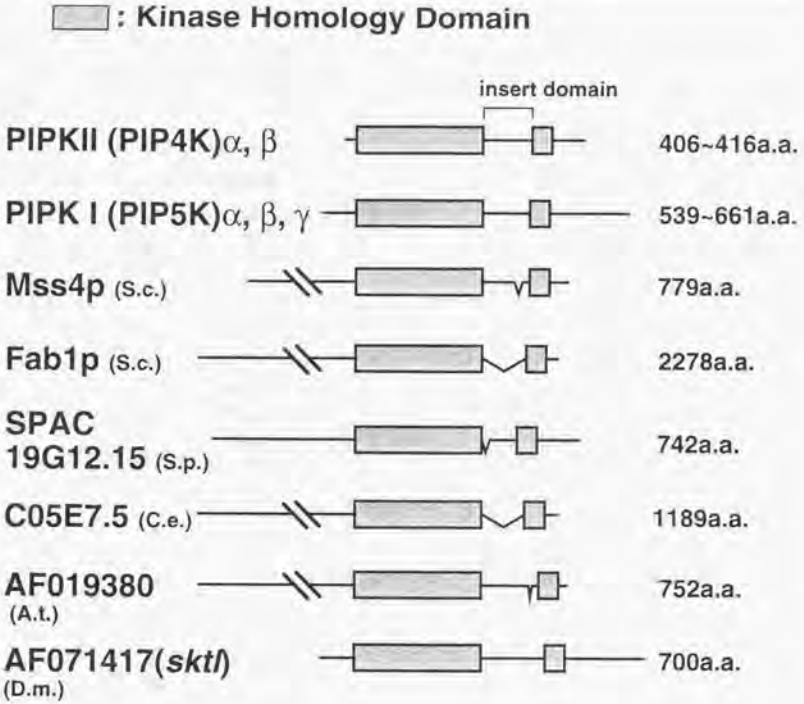

Fig.1 Members of PIP kinase family
(A) A schematic representation of PIP kinase family members in various species. Box represents "kinase homology domain". S.c. : Saccharomyces cerevisiae, S.p. : Schizosaccharomyces pombe, C.e. : Caenorhabditis elegans, D.m. : Drosophila melanogaster, A.t. : Arabidopsis thaliana.

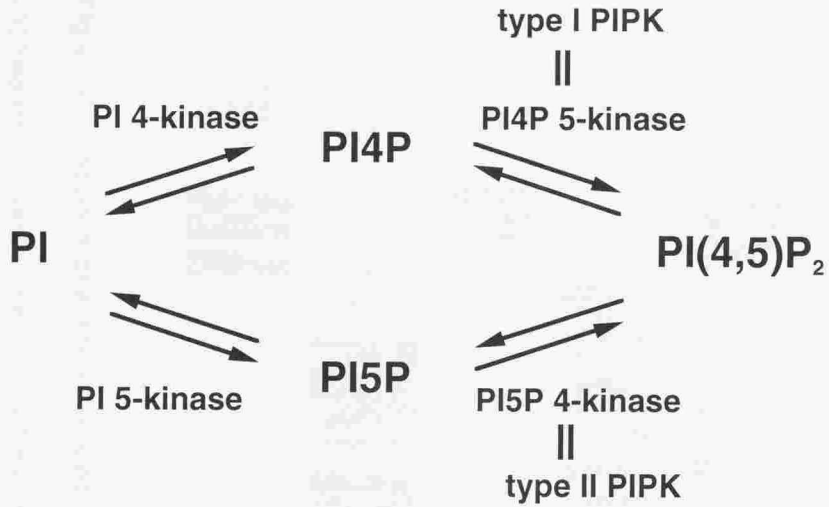

Fig.1

(B) Two pathways for $PI(4,5)P_2$ synthesis

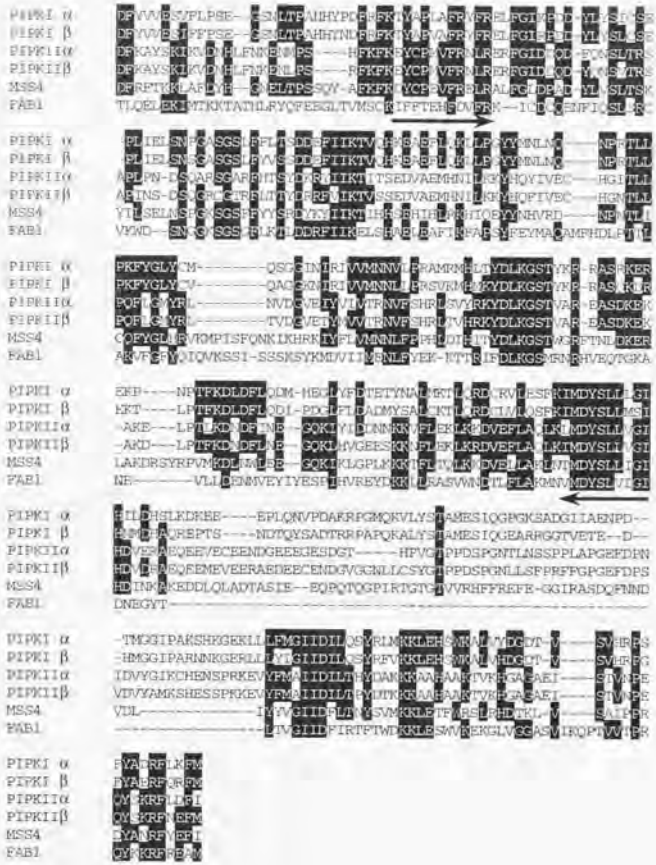

Fig.2 Identification of a novel PIP kinase

(A) Sequence alignment of PIP kinase family members from yeast (MSS4 and FAB1) to mammal (PIPKIα-IIβ). Highly conserved residues are shown in black. Selected regions ((D/E)YCPXVFR, MDYSLLLG(I/M)) for designing degenerative primers used in RT-PCR are indicated by allows.

```
                                                        TGAGCACCGCTTCCG  -60

GGTCGGGCTTCGGAGTCGCGGCCGGCTCCCGCTTCCACTCGCTTGGGTGCGCGGGAGATT   -1

ATGGCGTCTTCCTCCGTCCCTCCCGCCACCGCACCCGCGGCAGCTGGAGGCCCCGGCCCG   60
M  A  S  S  S  V  P  P  A  T  A  P  A  A  A  G  G  P  G  P     20

GGATTCGGCTTCGCCTCCAAAACCAAGAAGAAGCATTTCGTGCAGCAGAAAGTGAAGGTG  120
G  F  G  F  A  S  K  T  K  K  K  H  F  V  Q  Q  K  V  K  V     40

TTCCGGGCCGCCGACCCACTGGTGGGCGTGTTCCTGTGGGGCGTCGCCCACTCGATCAAC  180
F  R  A  A  D  P  L  V  G  V  F  L  W  G  V  A  H  S  I  N     60

GAGCTCAGTCAGGTGCCACCCCCAGTGATGCTGCTGCCAGACGACTTTAAAGCCAGCTCC  240
E  L  S  Q  V  P  P  P  V  M  L  L  P  D  D  F  K  A  S  S     80

AAGATCAAGGTCAACAATCACCTTTTCCATAGAGAAAATCTTCCCAGTCATTTCAAGTTC  300
K  I  K  V  N  N  H  L  F  H  R  E  N  L  P  S  H  F  K  F    100

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K  E  Y  C  P  Q  V  F  R  N  L  R  D  R  F  A  I  D  D  H    120

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L  L  P  Q  F  L  G  M  Y  R  V  S  V  E  N  E  D  S  Y  M    200

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P  V  R  E  E  E  S  E  W  D  G  D  C  N  L  T  G  P  P  A    320

CTGGTAGGCTCCTATGGCACCTCCCCTGAGGGCATTGGAGGCTACATTCATTCCCACCGG 1020
L  V  G  S  Y  G  T  S  P  E  G  I  G  G  Y  I  H  S  H  R    340

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GGGGCCCCAGAAGGAGGTGTATTTCATGGGCTCATTGACATTCTAACACAGTATGATGCC 1140
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ACTGTCCATCCTGAGCAGTACGCTAAGCGATTCCTGGATTTTATTTCCAACATCTTTGCC 1260
T  V  H  P  E  Q  Y  A  K  R  F  L  D  F  I  S  N  I  F  A    420

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*
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AAGCCCTGGAACGGAGGGCTTTCAGGACACTCCAGTGTGGGTGCAGGCATTCACGTAGGG 1800

ACAGTCTGGCCTGGAGGTCAGGGGAATGCCAGCAGGCTGGTACCTACCTCACAAGGCAGG 1860

ACTAAGTGGACTTTTGCGATTATGCAGAGGATGGAGAAGGTCCTTGTCAAGCATTGGCTG 1920

TTCCCATTCTCCTTCCCCACCCTACTTTCACCTCTTCCCATGAGTCCTGGGTGCCAGAGC 1980

CTTGAGGCAGAGGTGCCCAAGCTTAGGTGCAGGGAGAGCATAGAGGCTGGGAGGACAGGT 2040

CGGAGTGCAGGGTTCATTCCCTGTGTGACACCCTTTCTTCCCGTCTACTTACTCCCAGGA 2100

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CTGCCTGTAAATCCAGCACTCAGGAGGCTGAGTCAGAATTACTCAAGGGAGTTCAAGGCC 2400

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CAGTTACTGGCAGG

Fig.2

(B) cDNA and deduced amino acid sequences of novel PIPKIIγ

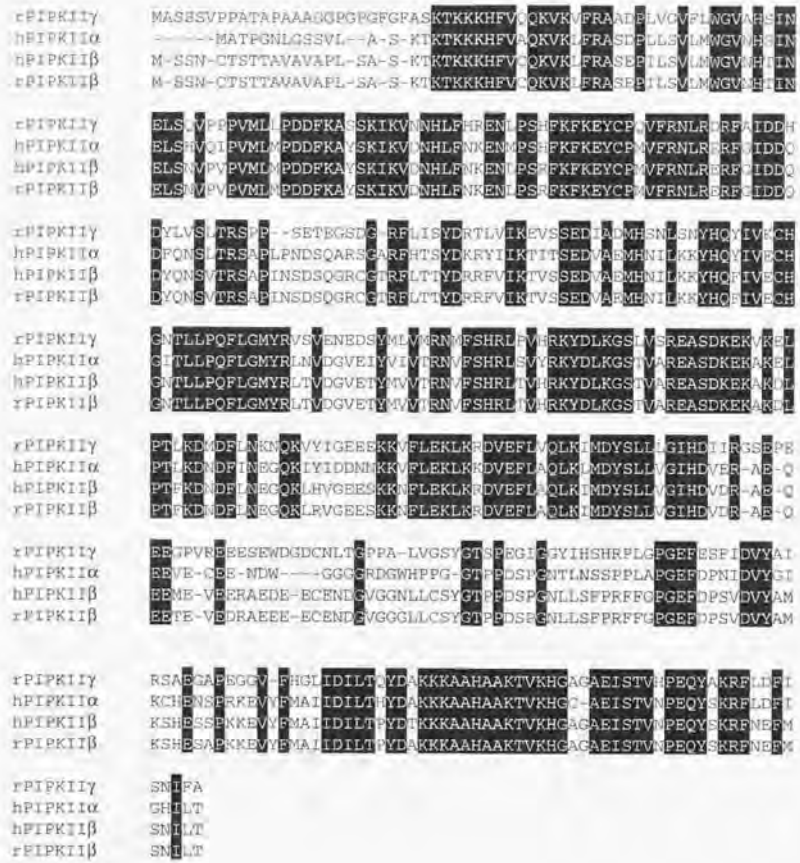


Fig.2

(C) Sequence alignment of rat PIPKIIγ with known type II isoforms from humans (h) and rats (r). Identical amino acids are shaded black. Rat PIPKIIβ was cloned simultaneously from the same library.

	Iα	Iβ	Iγ	IIα	IIβ	IIγ
Iα	100					
Iβ	65.7	100				
Iγ	68.0	66.7	100			
IIα	28.5	27.7	29.0	100		
IIβ	32.3	31.1	31.1	78.7	100	
IIγ	30.0	30.5	30.3	64.4	63.3	100

(%)

Table 1

Identity between amino acid sequences of mammalian PIP kinase isoforms including novel PIPKIIγ

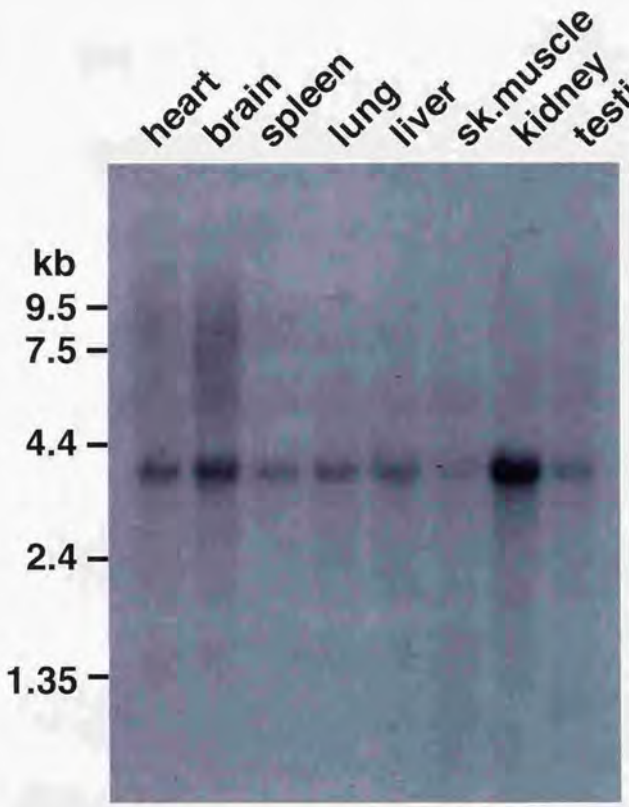


Fig.3
Northern blot analysis of PIPKIIγ Northern blot analysis of PIPKIIγ against mRNA from various mouse tissues. 2μg of mRNA was used in each lane. According to the size of the cDNA cloned, the 3.5kb transcript may represent the full length mRNA.

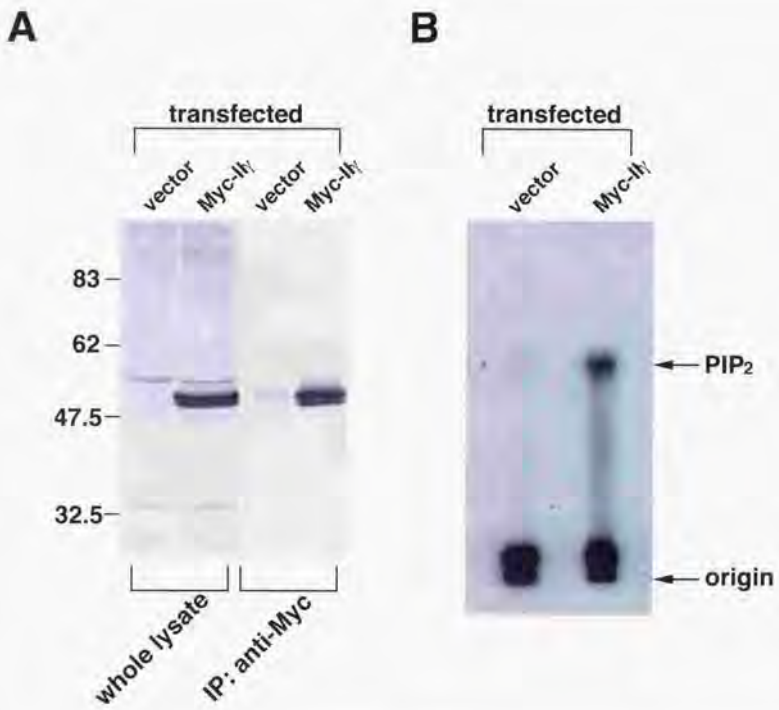


Fig.4

PIPKIIγ is a PI5P 4-kinase

(A) Expression of Myc-tagged PIPKIIγ in COS-7 cells. Whole lysates (left) and anti-Myc immunoprecipitates (right) from vector alone (vector) and Myc-PIPKIIγ transfected (Myc-IIγ) COS-7 cells were immunoblotted with anti-Myc antibody.

(B) PIP kinase activity of immunoprecipitated Myc-PIPKIIγ. Myc-PIPKIIγ was immunoprecipitated, and washed with lysate buffer and then with reaction buffer for PIP kinase. The reaction was carried out for 10 min at room temperature. The resulting lipid was extracted and subjected to TLC. The position of PIP_2 is indicated.

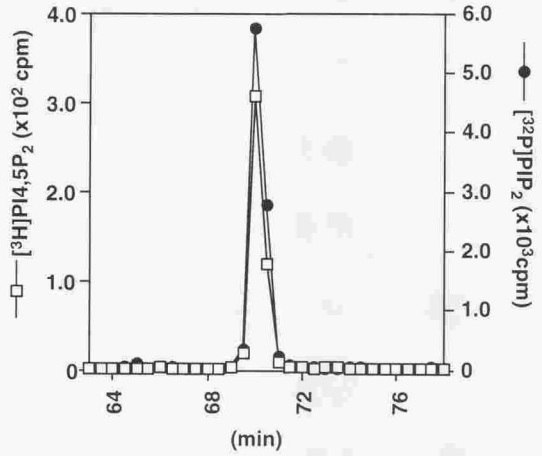


Fig.4

(C) The PIP_2 produced in (B) was scraped out from the plate, deacylated and separated by SAX HPLC. (open squares : $[^3H]PI(4,5)P_2$; closed circles : $[^{32}P]PIP_2$ produced by PIPKIIγ)

D

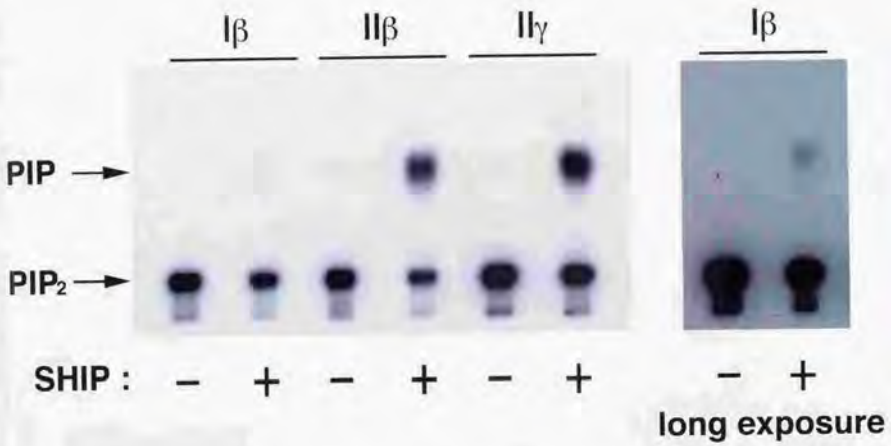


Fig.4

(D) Dephosphorylation of [^{32}P]PI(4,5)P_2 products with SHIP. [^{32}P]PI(4,5)P_2 produced by PIPKIβ, PIPKIIβ and PIPKIIγ were treated with immunoprecipitated Myc-SHIP at 37℃ for 60 min. The lipids were extracted and separated by TLC (left). PI5P was visualized by a long exposure (right).

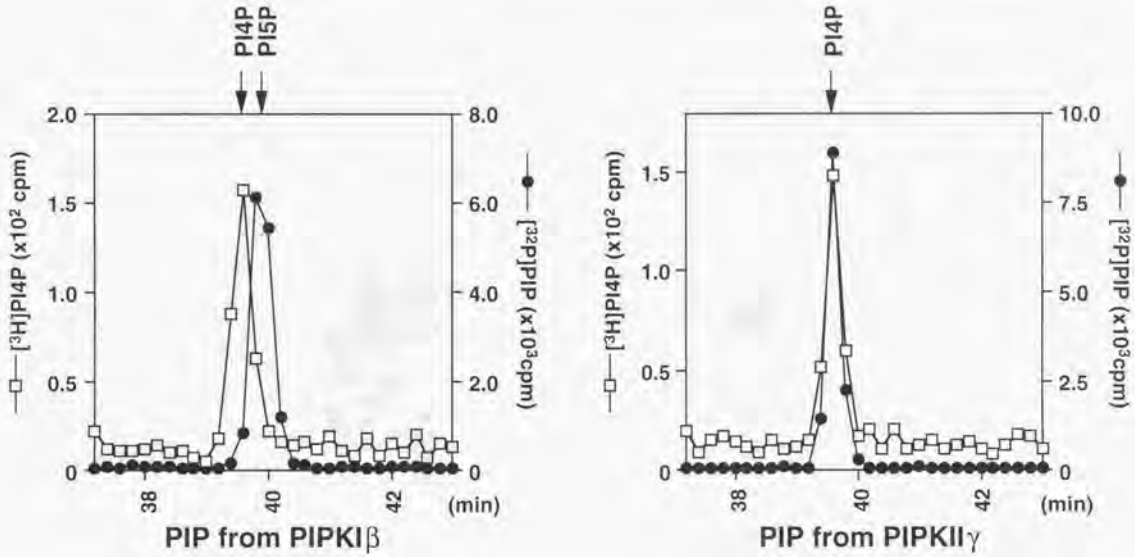


Fig.4

(E) The identification of PIP product. [^{32}P]PIP produced by SHIP treatment in (D) was analyzed by SAX HPLC as in (C). (open squares : [^{3}H]PI4P ; closed circles : [^{32}P]PIP from PIPKIβ (left panel) or PIPKIIγ (right panel))

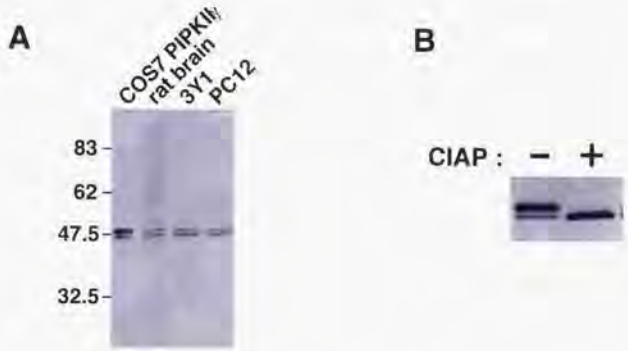


Fig.5
PIPKIIγ is a phosphoprotein
(A) Western blot analysis against lysates of rat brain, PC12 cells, 3Y1 cells and PIPKII γ-overexpressing COS-7 cells. The PIPKIIγ overexpressed in COS-7 cells used here was not the tagged form. The 47kD PIPKIIγ doublets are indicated by an arrow.

(B) Alkaline phosphatase treatment of immunoprecipitated Myc-tagged PIPKII γ. Immunoprecipitated Myc-PIPKIIγ was incubated with 2U of CIAP at 30℃ for 60 min. The resulting products were detected by Western blot with anti-Myc antibody.

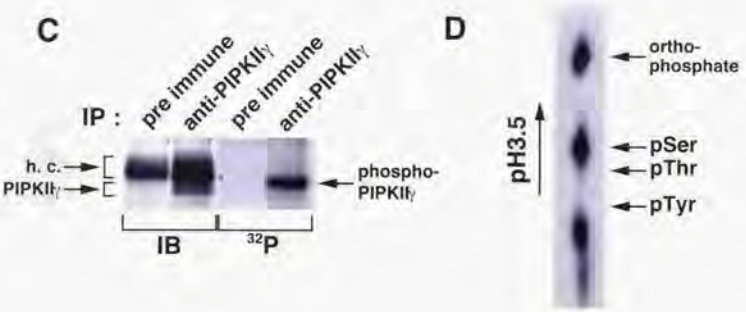


Fig.5

(C) ^{32}P-labeling experiment. PC12 cells were metabolically labeled with [^{32}P]orthophosphate. PIPKIIγ was immunoprecipitated from the labeled cell lysates and detected by autoradiography or Western blot with anti-PIPKIIγ. (**h.c.** : "heavy chain")

(D) Phosphoamino acid analysis of PIPKIIγ. The immunoprecipitated PIPKIIγ used in (B) was hydrolysed with 6N HCl for 1h at 110℃. The resulting amino acids were separated by TLC electrophoresis and detected by autoradiography. The positions of standard phosphoamino acids and free orthophosphate are also indicated.

E

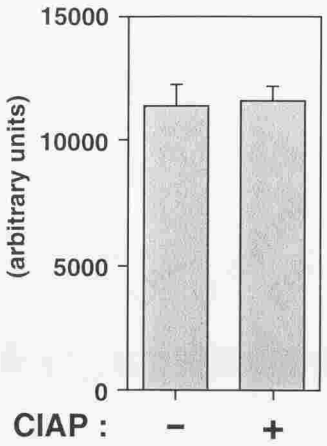


Fig.5

(E) PIP kinase activity of Myc-PIPKIIγ after CIAP treatment. Immunoprecipitate treated with CIAP as in (B), were washed with the reaction buffer for PIP kinase and the reaction was carried out. Results are representative of three independent experiments.

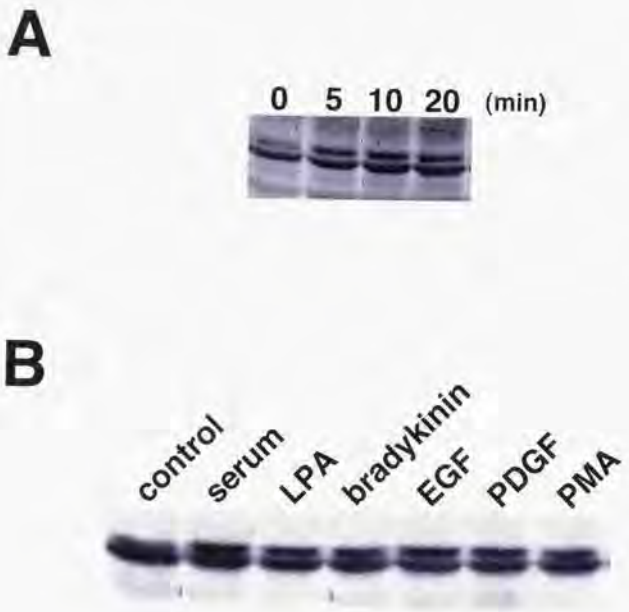


Fig.6
Phosphorylation of PIPKIIγ induced by mitogenic stimulation of 3Y1 fibroblasts

(A) PIPKIIγ is phosphorylated in response to stimulation by serum. Rat 3Y1 fibroblasts were serum-starved for 48h before treatment with 10% fetal bovine serum for the indicated times. Cells were lysed with SDS-sample buffer(125mM Tris-HCl, pH6.2, 2% SDS, 0.2% 2-mercaptoethanol) and immunoblotted with anti-PIPKIIγ.

(B) Rat 3Y1 fibroblasts were serum-starved (control), then stimulated with 10% fetal bovine serum (serum), 2μM lysophosphatidic acid (LPA), 1μM bradykinin, 100ng/ml EGF, 20U/ml PDGF and 1μM PMA for 10 min respectively. The phosphorylation level of PIPKIIγ was evaluated by Western blotting as in (A).

C

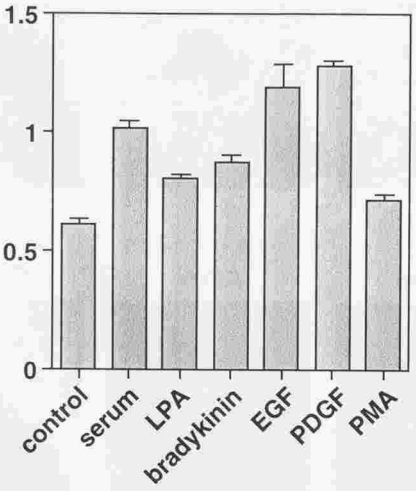


Fig.6

(C) Quantitative representation of (B). Each band corresponding to the phosphorylated or the unphosphorylated form was quantified with densitometry. The ratio of phosphorylated form against unphosphorylated form was calculated. Results are representative of three independent experiments.

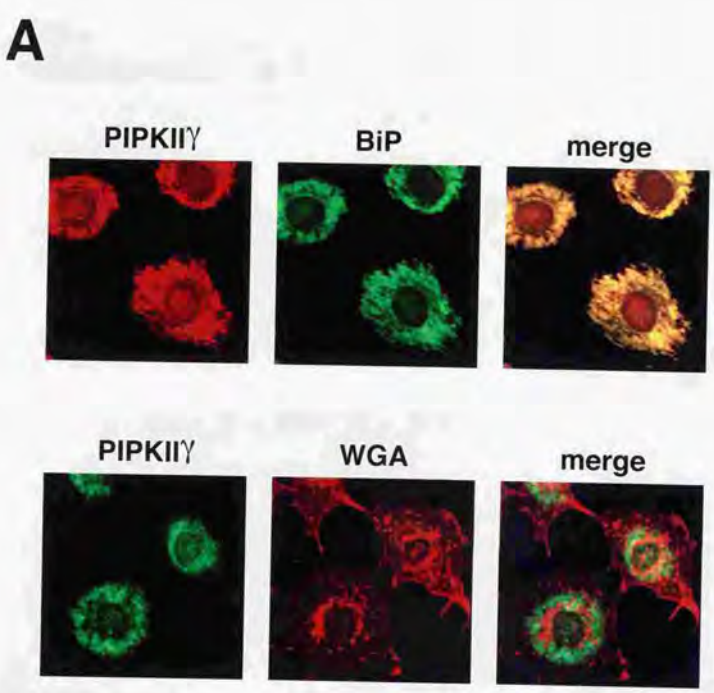


Fig.7
Intracellular localization of PIPKIIγ
(A) Immunofluorescence of PIPKIIγ. 3Y1 fibroblasts were fixed with 3.7% formaldehyde and permeabilized with 0.2%TritonX-100. Then, the cells were stained with anti-PIPKIIγ (PIPKIIγ), anti-BiP (BiP) antibody and rhodamine-conjugated wheat germ agglutinin (WGA). The overlapping image is also presented (merge).

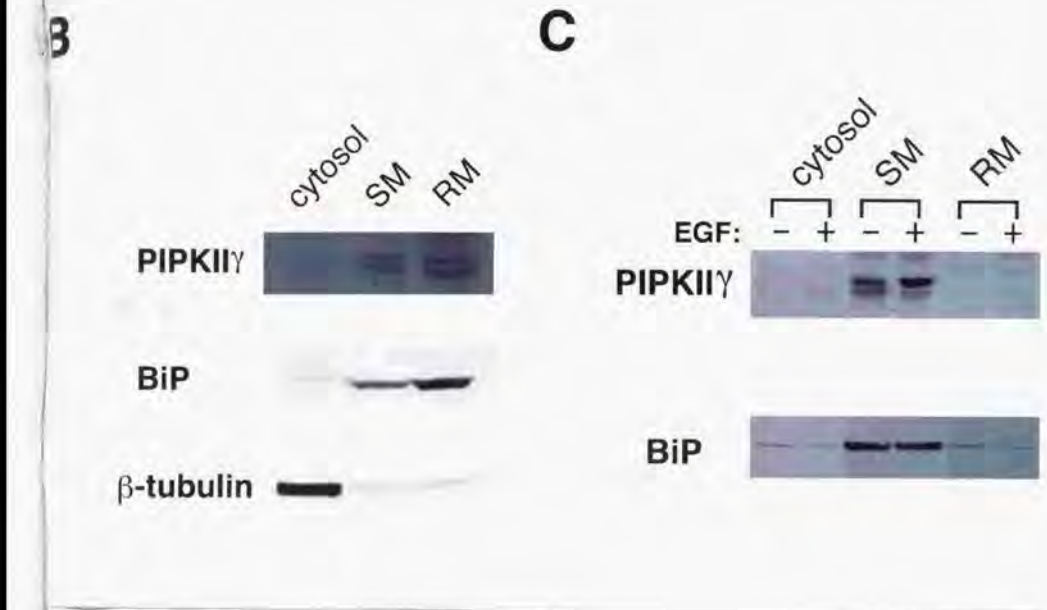
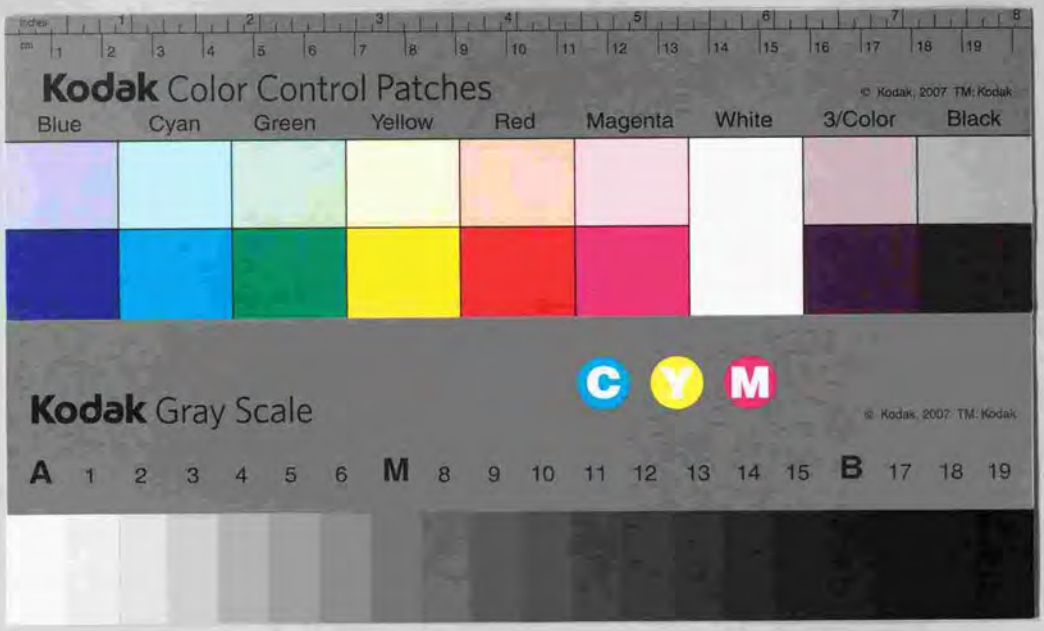


Fig.7
(B) Subcellular fractionation of PIPKIIγ in rat liver. Rat liver was subjected to subcellular fractionation as described in Materials and Methods. About 20μg of protein in each fraction (cytosol, SM(smooth microsome), RM (rough microsome)) was subjected to Western blotting by anti-PIPKIIγ, anti-BiP and anti-β-tubulin antibody.

(C) Serum-starved 3Y1 fibroblasts were stimulated with 100ng/ml EGF for 10 min, then subcellular fractionation was carried out. Detection of each protein was done as in (B).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C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19