It has been reported that there are two alternatively spliced variants of phospholipase C-δ4 (PLCδ4), termed ALT I and II, that contain an additional 32 and 14 amino acids in their respective sequences in the linker region between the catalytic X and Y domains (Lee, S. B., and Rhee, S. G. (1996) J. Biol. Chem. 271, 25–31). We report here the isolation and characterization of a novel alternative splicing isoform of PLCδ4, termed ALT III, as a negative regulator of PLC. In ALT III, alternative splicing occurred in the catalytic X domain, i.e. 63 amino acids (residues 424–486) containing the C-terminal of the X domain and linker region were substituted for 32 amino acids corresponding to the insert sequence of ALT I. Although the expression level of ALT III was found to be much lower in most tissues and cells compared with that of PLCδ4, it was significantly higher in some neural cells, such as NIE-115 cells and p19 cells differentiated to neural cells by retinoic acid. Interestingly, recombinant ALT III protein did not retain enzymatic activity, and the activity of PLCδ4 overexpressed in COS7 cells was markedly decreased by the co-expression of ALT III but not by ALT I or II. Moreover, N-terminal pleckstrin homology domain (PH domain) of ALT III alone could inhibit the increase of inositol-1,4,5-triphosphate levels in PLCδ4-overexpressing NIH3T3 cells, whereas a PH domain deletion mutant could not, indicating that the PH domain is necessary and sufficient for its inhibitory effect. The ALT III PH domain specifically bound to phosphatidylinositol (PtdIns)-4,5-P₂ and PtdIns-3,4,5-P₃ but not PtdIns, PtdIns-4-P, or inositol phosphates, and the mutant R36G, which retained only weak affinity for PtdIns-4,5-P₂, could not inhibit the activity of PLCδ4. These results indicate that PtdIns-4,5-P₂ binding to PH domain is essential for the inhibitory effect of ALT III. ALT III also inhibited PLCδ4 activity and partially suppressed PLCγ1 activity, but not PLCβ1 in vitro; it did inhibit all types of isozymes tested in vivo. Taken together, our results indicate that ALT III is a negative regulator of PLC that is most effective against the PLC-δ-type isoforms, and its PH domain is essential for its function.

Phospholipase C (PLC)1 plays a crucial role in the inositol phospholipid signaling by hydrolyzing phosphatidylinositol (PtdIns)-4,5-bisphosphate. This reaction produces two intracellular second messengers, inositol 1,4,5-trisphosphate (Ins-1,4,5-P₃) and diacylglycerol, which cause the increase of intracellular calcium concentration and the activation of protein kinase C (PKC), respectively (1). These cascades are thought to be terminated by the dephosphorylation of Ins-1,4,5-P₃ by inositol phosphate phosphatases (2) and the phosphorylation of diacylglycerol by diacylglycerol kinases (3). However, since two signals are generated simultaneously by one enzyme (an enzyme of PLC), there might be also a mechanism that turns off these cascades simultaneously, i.e. a negative regulation of PLC.

The PLC family is comprised of 10 subtypes found in mammalian species, and on the basis of their structure, they have been divided into three classes, β (β1–4), γ (γ1 and 2), and δ (δ1–4) types (4). Positive regulation mechanisms of PLC by association with membrane receptors are well characterized in β- and γ-type isoforms. β-Type isoforms are activated by the Gα or Gβγ subunit released from heterotrimeric G proteins after ligand stimulation (4–7). γ-Type isoforms are activated by the phosphorylation of specific tyrosine residues through the activation of receptor or nonreceptor tyrosine kinases (4, 8, 9). The mechanism by which δ-type isoforms are coupled to membrane receptors remains unclear.

Several recent studies indicate that the pleckstrin homology (PH) domains of PLC isoforms are also important for the activity of the isoforms. First, PLCδ1 was shown to be activated by the binding of PtdIns-4,5-P₂ to its PH domain and inhibited by the binding of Ins-1,4,5-P₃ to the same domain (10–12). On the basis of the information obtained from the three-dimensional structure of PLCδ1, a catalytic mechanism comprising two steps, tether and fix, was proposed (13); the PH domain tethers the enzyme to the membrane by specific binding to PtdIns-4,5-P₂, and the C2 domain fixes the catalytic domain in a productive orientation on the membrane. Therefore, the PH domain plays a important role for the first contact to the plasma membrane. Second, more recent studies indicate that the PtdIns-3,4,5-P₃ binding to the PH domain is involved in the protein tyrosine kinase-independent activation of PLCγ1. It has been reported that the overexpression of PtdIns-3 kinase, which produces PtdIns-3,4,5-P₃, results in the activation of PLCγ1 by the binding of PtdIns-3,4,5-P₃ to the N-terminal PH domain (14). Furthermore, SH2-containing inositol-5′-phosphatase, which removes 5′-phosphates from phosphoinositides, was shown to eliminate the PtdIns-3,4,5-P₃-dependent activation of PLCγ1 in B cells, resulting in a decrease of growth factor; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium.

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‡ The abbreviations used are: PLC, phospholipase C; PtdIns, phosphatidylinositol; Ins, inositol; PH, pleckstrin homology; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium.

A Novel Phospholipase C δ4 (PLCδ4) Splice Variant as a Negative Regulator of PLC*

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in Ins-1,4,5-P3 formation and decreased calcium mobilization (15, 16). Therefore, SH2-containing inositol-5'-phosphatase functions as a negative regulator of PLCδ1 by inhibiting PtdIns-3,4,5-P3-dependent activation, at least in B cells.

Negative regulations of PLC by protein kinase C (PKC) and CAM-dependent protein kinase are also known (4, 17–21). The proposed targets for phosphorylation by these kinases include cell-surface receptors, G proteins, and PLC itself. CAM-dependent protein kinase directly phosphorylates the serine residues of PLCβ2 and -β3 and inhibits the stimulation of their activity by Gβγ or Goα, respectively (17, 18). PLCβ1 is phosphorylated by PKCα, resulting in the inhibition of its activation by Gβγ (21). It was also reported that gelsolin and profilin, which bind to PtdIns-4,5-P2, compete with PLC for PtdIns-4,5-P2, resulting in the inhibition of the activity of PLCδ and -γ, respectively (22, 23). However, the negative regulation mechanism of PLCδ isoforms remains unknown.

Here, we report that a novel spliced variant of PLCδ4, termed ALT III, functions as a negative regulator of PLC through its PH domain. ALT III inhibited the activity of PLCδ most effectively among the PLC isoforms in vivo and in vitro. This is the first demonstration of a negative regulator of PLCδ-type isoforms.

**EXPERIMENTAL PROCEDURES**

**Materials**

[3H]PtdIns-4,5-P2, [3H]Ins-1,4,5-P3, and [3H]Ins-1,3,4,5-P4 were obtained from NEN Life Science Products. Phosphatidyl ethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidylinositol (PtdIns) were from Doosan Sedary Research Laboratories. Ins-1,4,5-P3 and bradykinin were from Sigma, and platelet-derived growth factor (PDGF) was from Boehringer Mannheim (Mannheim, Germany). PtdIns-4,5-P2 and PtdIns-4-P were purified from bovine spinal cords. PtdIns-3,4,5-P2 (both acyl groups are palmitoyl) was chemically synthesized. The transfected cell selection kit, the MACSelect4 Refineur-sulfate (RITC) and the MACSelect4-phosphate (FITC) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). The Ins-1,4,5-P2 assay kit was obtained from Amersham Pharmacia Biotech. The rabbit polyclonal anti-PLCδ1 antibody was raised against the C-terminal 157 amino acid tag of the vector pQE-31. PLCβ1,-γ1, and -δ1 were purified from bovine brain by using a three-step column chromatography technique (24).

**Methods**

Isolation of a Novel Spliced Variant of PLCδ4, Termed ALT III—Total RNA was isolated from rat tissues by the guanidinium isothiocyanate method and from culture cells by the Nonidet P-40 method. Total RNA was isolated from rat tissues by the guanidinium isothiocyanate method and from culture cells by the Nonidet P-40 method. A 1,491-bp cDNA encoding the PH domain (nucleotides 1–376), APH domain (nucleotides 202–375), and ALT III PH (nucleotides 1–376), were subcloned into the vector pFastbac (Life Technologies, Inc.). Recombinant, clonal baculoviruses were generated according to the protocol described by Life Technologies, Inc. SF9 cells were grown at 28 °C in SF900 II SFM (Life Technologies, Inc.) containing 10% FBS. Baculoviruses were used to infect a density of 1 × 10^6 cells. The proteins were purified according a method described previously (25).

Measurement of PLC Activity—PLC activity was assayed with [3H]PtdIns-4,5-P2, as the substrate. The PtdIns-4,5-P2-hydrolyzing activity was measured with mixed phospholipid micelles containing 40 μM phosphatidylethanolamine, 5 μM PtdIns-4,5-P2, and 1 μCi/ml [3H]PtdIns-4,5-P2. The lipids in chloroform were dried under a stream of nitrogen gas, suspended in assay buffer (20 mM HEPES, pH 7.0, 120 mM NaCl, 2 mM MgCl2, and 100 μM CaCl2), and subjected to sonication. All proteins added to the reaction mixture were dialyzed overnight against binding buffer (20 mM HEPES, pH 7.0, 120 mM NaCl, 2 mM MgCl2, 40 or 100 μM CaCl2, and 1 mg/ml bovine albumin serum (Bayer, Leverkusen, Germany)), and subjected to sonication. All enzymes added to the reaction mixture were dialyzed overnight against the assay buffer. Incubation was performed at 10 min at 37 °C in a 50-μl reaction mixture containing lipids micelles (5 μM [3H]PtdIns-4,5-P2, 20,000 dpm). The reaction was stopped by adding 2 ml of chloroform/methanol (2:1, v/v). Insoluble triphosphates were extracted with 0.5 ml of 1 N HCl, and the radioactivities in the upper aqueous phase were measured.

Assay of [3H]PtdIns-4,5-P2 Binding to the ALT III PH Domain—Recombinant glutathione S-transferase (GST) fusion proteins containing the ALT III PH domain (nucleotides 1–880) and R560 ALT III PH domain were expressed and purified. Site-directed mutagenesis by a two-stage PCR method was performed as described (28). These proteins were expressed in Escherichia coli, and the lysates were mixed with glutathione-Sepharose beads. After three washes, the proteins were eluted and dialyzed overnight against binding buffer (20 mM HEPES, 120 mM NaCl, 2 mM MgCl2, pH 7.4). Purified fusion proteins were mixed with micelles containing 2 μM [3H]PtdIns-4,5-P2, 20,000 dpm, 6 μM phosphatidylinositol trisphosphate (Ins-1,4,5-P3) as the substrate. The binding reaction was initiated by the addition of the purified fusion protein, and the reaction was stopped by adding 2 ml of chloroform/methanol (2:1, v/v). Insoluble triphosphates were extracted with 0.5 ml of 1 N HCl, and the radioactivities in the upper aqueous phase were measured.

Cell Culture, Transfection, and Selection of Transfected Cells Using the MACSelect 4 Kit—COS7, NIE-115, and BOSC3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NIH3T3 cells were maintained in DMEM with 10% calf serum. PC12 cells were maintained in DMEM with 10% horse serum and 5% FBS. C6 cells were maintained in Ham's F-10 nutrient medium (Life Technologies, Inc.) with 10% horse serum and 2.5% FBS. NG108-15 cells were maintained in DMEM supplemented with 10% FBS, 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM glycine. p19 cells were maintained in α-minimal essential medium with 10% FBS. The induction of the differentiation of p19 cells with retinoic acid was performed as described previously (26). NG108-15 and p19 cells were kindly provided by Dr. M. Ichikawa (University of Tokyo). PLCδ4, ALT I, II, and III were subcloned into the expression vector pcDNA3 (kindly provided from Dr. F. Shibasaki (Tokyo Metropolitan Institute of Medical Science)). The pMACS 4 vector carrying cDNA of CD4 was co-transfected with pcDNAs carrying cDNAs encoding PLCδ4, ALT I, II, and III in COS7 cells. Transfections were performed by electroporation (Bio-Rad). The cells were incubated at 37 °C for 2 days and harvested. The cells were then applied on the MACS high-gradient magnetic separation columns to collect the cells expressing CD4 protein, and after 3 washes, the cells bound to the columns were eluted according to the manufacturer's protocol. Lysates from the harvested cells were then used for the PLC assay.

Retrovirus Expression—The retrovirus vector, pMX, was kindly provided by Dr. T. Kitamura (University of Tokyo). cDNAs of PLCδ4, ALT III, and ALT III PH (nucleotides 1–376), PH domain (nucleotides 202–375), and PLCδ1 were subcloned into this vector. Recombinant retroviruses were generated and infected to NIH3T3 cells as described (27). In brief, cDNAs of interest were transfected in Bosc23 cells, in which the recombinant retrovirus is produced, and the cells were incubated for 2 days. The supernatant of the cells that contained the recombinant virus was then collected, and exponentially growing NIH3T3 cells were infected for 2 days. After that, the cells were harvested. Alternatively, cells were further incubated in starvation medium (DMEM supplemented with insulin/transferrin/selenium supplement (ITS) and bovine albumin serum (Boehringer Mannheim)) for 24 h, stimulated by 1 μM bradykinin or 50 ng/ml PDGF for the indicated time (see figure legends) and harvested. Cells infected with the recombinant viruses containing cDNA of green fluorescent protein were used as the control.

Purification of PLCδ4 and ALT III PH Protein Generated by an siRNA/ Bacteriophage Expression System—Recombinant PLCδ4 and ALT III PH proteins were prepared using a baculovirus expression system. cDNA of PLCδ4 and ALT III PH was subcloned into the vector pFastbac (Life Technologies, Inc.). Recombinant, clonal baculoviruses were generated according to the protocol described by Life Technologies, Inc. SF9 cells were grown at 28 °C in SF900 II SFM (Life Technologies, Inc.) containing 10% FBS. Baculoviruses were used to infect at a density of 1 × 10^6 cells. The proteins were purified according a method described previously (25).
serine, and 18 μM phosphatidylcholine in 50 μl of the binding buffer for 1 h at room temperature. Then 50 μl of glutathione-Sepharose beads were added, and the tubes were incubated for 15 min at room temperature with occasionally mixing. The tubes were centrifuged at 2,000 rpm for 2 min, and the radioactivity bound to beads was then measured. 

**RESULTS**

**Isolation of ALT III, a Novel Alternatively Spliced Variant of PLCδ4**—PLCδ4 is an enzyme that is inducible in response to mitogenic stimuli, and its expression is restricted in several tissues and cells (30). In addition to such characters, two alternatively spliced variants of PLCδ4, termed ALT I and ALT II, are also known to exist (25). To understand the specific function of PLCδ4 and the mechanisms of its regulation, we tried to obtain another isoform of PLCδ4. The entire coding region of PLCδ4 cDNA was amplified by RT-PCR as described by Lee and Rhee (25), subcloned into the vector pBluescript KS(−), and sequenced. The complete sequence of these products revealed that a novel splice variant termed ALT III could be obtained in addition to the reported isoforms, including PLCδ4, ALT I, and ALT II. In ALT III, 63 amino acids containing the C-terminal of the catalytic X domain and linker region between the catalytic X and Y domains were substituted for 32 amino acids corresponding to the insert sequence of ALT I (Fig. 1A). ALT III protein was highly expressed in some neural cells (Fig. 2B), indicating that it exists in living cells and is not an artifact obtained by PCR. An analysis of the genomic DNA sequence of PLCδ4 revealed that PLCδ4, ALT I, II, and III were generated from a single gene by alternative splicings. ALT I and II mRNA are comprised of exons IX, XI, and XI, and new exons X or X′ between exons X and XI of the gene, respectively. A splicing donor site for the exon X was not recognized in ALT III, and ALT III is comprised of exons IX, XI, and a new exon, X. Thus, ALT III contains amino acids VMKCPMSCLICVHVLAQAPNISPESILLPKR instead of the region from 424 to 486 (Fig. 1B).

**Distribution of PLCδ4 Isoforms in Tissues and Cells**—We next examined the distribution of PLCδ4 isoforms in rat tissues and culture cells. Lysates from rat tissues and culture cells were subjected to an immunoblot analysis with affinity purified polyclonal antibodies to PLCδ4 (Fig. 2A and C). The expressions of PLCδ4 isoforms mRNA were also examined by RT-PCR with a pair of PLCδ4-specific primers (Fig. 2B). Both analyses indicated that PLCδ4 was expressed in the brain and testis, and weak signals were observed in the heart and lung. The expressions of ALT I and II were abundant in the testis. A strong signal for ALT III was detected by immunoblotting in the heart. Some differences between the results of the RT-PCR and those of the immunoblotting were observed. For instance, although the expression of PLCδ4 mRNA was detected by RT-PCR in the spleen and kidney, the proteins were not detected. Also, ALT II and III proteins were detected by immunoblotting in the liver, and PLCδ4 protein was detected in the thymus, but the respective mRNAs were not detected. Although the ALT III expression was at quite a lower level compared with that of PLCδ4 in most tissues and cells, it was abundant in NIE-115 cells (a mouse neuroblastoma cell line) and p19 cells differentiated to neural cells by 5 × 10⁻⁵ M retinoic acid (Fig. 2C). In C6 cells, a rat glioblastoma cell line, PLCδ4, and ALT III were quite rich. We also detected PLCδ4 and ALT III mRNA in these cell lines by RT-PCR (data not shown). It was noteworthy that the expression level of ALT III was significantly higher in differentially neural p19 cells compared with that in undifferentiated embryonic p19 cells.

**Inhibition of PLCδ4 Activity by ALT III Caused by the PH Domain**—Since ALT III lacks a part of the X domain and almost the entire linker region, we expected that ALT III would not retain enzymatic activity. To investigate this, we subcloned the full-length of all PLCδ4 isoforms into the vector pcDNA3, and the encoded genes were co-expressed with the vector carrying CD4 gene in COS7 cells. Transfected cells were separated by magnetic beads to collect the cells expressing CD4 protein. At least 30% of the cells expressing CD4 protein were co-transfected with PLCδ4 (data not shown). Therefore, PLCδ4-overexpressing cells were concentrated enough to detect the activity of PLCδ4 in their lysates. Fig. 3A shows that PLCδ4 and ALT II retained their activity up to 2.7- and 2.8-fold, respectively, compared with the control, whereas the activity of ALT I only reached approximately 1.5-fold. ALT III does not have PLC activity at all. We also confirmed that recombinant ALT III protein generated by the Sf9/baculovirus system did not retain PLC activity (data not shown). Moreover, the activity of PLCδ4 was markedly decreased by the co-expression of ALT

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2 K. Fukami, K. Takenaka, A. Ito, K. Nagano, and T. Takenawa, submitted for publication.
III, to less than 40%, but not by ALT I (Fig. 3B). On the other hand, ALT II is catalytically active as PLCδ4, but PLCδ4 activity was rather suppressed by the co-expression of ALT II, slightly (Fig. 3B). We do not know why it happened. However, we confirmed that the activity of recombinant PLCδ4 protein was not inhibited by the addition of the recombinant ALT II protein generated by Sf9/baculovirus system (data not shown). Therefore, this partial suppression by ALT II may be caused by some factors that affect the function of ALT II in vivo.

In order to clarify how PLCδ4 is inhibited by ALT III in vivo, we next investigated whether the PH domain alone or the PH domain deletion mutant of ALT III could inhibit the activity of PLCδ4. PLCδ4, ALT III, the PH domain coding region, and PH domain deletion mutant cDNAs were expressed by a retrovirus system in NIH3T3 cells. The cells expressing various constructs were then harvested, and the Ins-1,4,5-P3 level was measured. As a result, ALT III did not retain enzymatic activity and inhibited the activity of PLCδ4 in vitro (Fig. 3C). Moreover, the ALT III PH domain alone could completely block the activity of PLCδ4, whereas the PH domain deletion mutant could not (Fig. 3C). These results indicate that the ALT III PH domain is necessary and sufficient for its inhibitory effect.

The ALT III PH domain specifically binds to PtdIns-4,5-P2 and PtdIns-3,4,5-P3, but not PtdIns, PtdIns-4-P, or inositol phosphates—we then analyzed the binding of inositol phospholipids on the binding of [3H]PtdIns-4,5-P2 to the PH domain. Unexpectedly, the R36G mutant could also bind to PtdIns-4,5-P2 with quite low affinity. Next, to determine the binding specificity of the ALT III PH domain to inositol phospholipid, we examined the competitive effect of various unlabeled inositol phospholipids on the binding of [3H]PtdIns-4,5-P2 to the PH domain. We found that the binding of [3H]PtdIns-4,5-P2 was inhibited to 60% by the addition of a 10-fold excess amount of unlabeled PtdIns-4,5-P2, and to 50% by PtdIns-3,4,5-P3, but not PtdIns or PtdIns-4-P, in a dose-dependent manner (Fig. 4B), showing that the ALT III PH domain has strong binding affinity for PtdIns-4,5-P2 and PtdIns-3,4,5-P3. We also examined the binding of the ALT III PH domain to inositol phosphates. Interestingly, the ALT III PH domain did not bind to Ins-1,4,5-P3 or Ins-1,3,4,5-P4 at all (Fig. 4C and D).

Inhibitory Effect of ALT III on PLC Activity in Vitro—To investigate the importance of the PH domain for the inhibitory effect of ALT III on PLC activity in vitro, the activities of recombinant PLCδ4 protein generated by the Sf9/baculovirus system were measured in the presence of recombinant ALT III protein generated by the bacteria system. As in the in vivo experiments (Fig. 3C), the activity of recombinant PLCδ4 protein was inhibited to 70% by the addition of equal amounts of ALT III and PH domain protein and to 40 and 55% by 2-fold excess amounts of ALT III and PH domain proteins, respectively (Fig. 5A). The activity of PLCδ4 was suppressed by a 2-fold excess amount of X-Y domain protein. The mutant R36G, of which the potency for PtdIns-4,5-P2 binding is markedly reduced, did not inhibit the activity at all, indicating that PtdIns-4,5-P2 binding to PH domain is essential for the inhibitory effect of ALT III. Next, we investigated the effect of ALT III protein on the other PLC activities, such as PLCδ1, -γ1, and -δ1 purified from bovine brain. As shown in Fig. 5B, ALT III inhibited the activity of PLCδ1 most effectively. The activity of PLCδ1 was partially suppressed, and PLCδ1 was not affected at all. It is difficult to compare the sensitivity of PLCδ1 and that of PLCδ4, because the sources of the proteins are different, i.e. PLCδ1 is purified from bovine brain whereas PLCδ4 is a recombinant protein. However, at least these data show that ALT III is more effective against PLCδ-type isoforms than PLCδ1 and -γ1 in vitro.

The Suppression of Ins-1,4,5-P3 Production by ALT III in Vivo—To determine which PLC isoforms were inhibited by ALT III in vivo, we next investigated whether the overexpression of ALT III in NIH3T3 cells affects the Ins-1,4,5-P3 production generated by the stimulation of bradykinin, PDGF, or the overexpression of PLCδ1. As shown in Fig. 6, A and B, in contrast to the results of the present in vitro experiments, the PLC activities stimulated by bradykinin and PDGF were inhibited by ALT III, although it was demonstrated that bradykinin and PDGF activate mainly PLCδ1 and PLCγ1, respectively. Moreover, the increase in the production of Ins-1,4,5-P3 by the overexpression of PLCδ1 in NIH3T3 cells reverted to the control level by the co-expression with ALT III (Fig. 6C), indicating that the activity of PLCδ1 is inhibited by ALT III in vitro and in vivo.

**DISCUSSION**

We demonstrated that a novel alternatively spliced variant of PLCδ4 acts as a negative regulator of PLC isozymes. Although some of the genes that encode PLC isozymes are also known to produce splice variants, such as normA (32) and plc21 (33), both of which encode phospholipase C in Drosophila, PLCδ1 (34) and -δ4 (35), all of their splicing differences occur outside of the catalytic X and Y domains. Therefore, ALT III is the first example of a splice variant in which the difference occurs inside the catalytic domain. Although the expression
were co-expressed with PLC
represent inhibition (%) against PLC
6 cells. All data are means
the PH domain deletion mutant did not (Figs. 3
stimulation, it may be not sensitive to ALT III. Besides, the
activity in the control cells was not so high without agonist
upstimulated control cells (Fig. 3,
did not result in the inhibition of endogenous PLC activity in
(Figs. 3, 5, and 6). Unexpectedly, the overexpression of ALT III
4,5-P2 binding to the ALT III PH domain, resulting in the
very low, did not inhibit the activity of PLC
domains that bind to PtdIns-3,4,5-P3 with high affinity rather
various inositol phospholipids and are divided into four groups
for the inhibition by ALT III.

Different PH domains have different binding specificities for
various inositol phospholipids and are divided into four groups
based on their selectivities (36, 37). The first group is the PH
domains that bind to PtdIns-3,4,5-P3, with high affinity rather
than to PtdIns-4,5-P2, such as the PH domains of Bruton’s
tyrosine kinase-1, general receptor for phosphoinositides-1, N-
ter nal T lymphoma invasion and metastasis protein-1, and
son of sevenless-1. The second group is the PH domains that
bind to PtdIns-4,5-P2 with high affinity and to PtdIns-3,4,5-P3
with relatively weak or the same affinity, such as those of
PLCδ1, β-adrenergic receptor kinase, spectrin, and oxysterol-
binding protein-1. The third group of PH domains bind specifically
to PtdIns-3,4-P2, including that of Akt/protein kinase B.
The fourth group of PH domains bind with low affinity and no
selectivity to inositol phospholipids. In regard to the PLC PH
domain, PLCδ1 belongs to the second group with high affinity
for PtdIns-4,5-P2 (11, 38–40), whereas the PLCγ1 PH domain
would be in the first group with high affinity for PtdIns-
3,4,5-P3 (14, 15). Since the PLCβ1 PH domain is known to bind
lipids nonspecifically (39), it might belong to the fourth group.

Our present results showed that the ALT III PH domain can
specifically bind to PtdIns-4,5-P2 and PtdIns-3,4,5-P3 but not
PtdIns or PtdIns-4-P (Fig. 4B), suggesting that the ALT III PH
domain belongs to the second group, to which the PLCδ1 PH
domain belongs. Interestingly, despite the sequence similarity
of ALT III PH domain with the PLCδ1 PH domain which binds
to PtdIns-4,5-P2 and Ins-1,4,5-P3 with high affinity, the ALT III
PH domain could not bind to Ins-1,4,5-P3 or Ins-1,3,4,5-P3 (Fig.
4, C and D). One possible reason is that the binding of the ALT
III PH domain to PtdIns-4,5-P2 and PtdIns-3,4,5-P3 is required
for additional contact with a hydrophobic region such as the
acyl chain of lipids. This explanation is not inconsistent with
our finding that the R36G mutant still bound to PtdIns-4,5-P2
with low affinity. In fact, some of the proteins, such as Bruton’s
tyrosine kinase, bind to inositol phospholipid with higher af-
finity than to inositol phosphate, and it is considered that the
natural ligand of such protein is the lipid rather than the
inositol phosphate (37).

Our results showed that ALT III inhibited the activity of PLC
δ-types the most efficiently and were less effective against
PLCδ1 and -γ1 (Fig. 5B) in vitro. The different effects on PLC

level of ALT III was quite lower than that of PLCδ4 in most of
the tissues and cells examined, it was significantly higher in
NI1-15 cells, a rat neuroblastoma cell line (Fig. 2). Remark-
ably, p19 cells, a rat embryonal carcinoma cell line, normally
expressed ALT III at a low level, but the expression was higher
compared with that of PLCδ4 when p19 cells were differenti-
tated to neural cells by retinoic acid (Fig. 2C). These results
suggest that ALT III is expressed and functions specifically in
some neural cells.

Since ALT III lacks the C-terminal of the X domain and
linker region, we first studied whether this molecule had PLC
activity; we found that it does not (Fig. 3A). We next examined
the biological functions of this molecule, since it is presumed
that ALT III inhibits PLC isoforms by competition for PtdIns-
3,4,5-P3 (14, 15). Since the PLC
activity; we found that it does not (Fig. 3
PLCδ4 -types the most efficiently and were less effective against
PLCδ1 and -γ1 (Fig. 5B) in vitro. The different effects on PLC

Negative Regulation of PLC

Fig. 3. Enzymatic activity of PLCδ4 was inhibited by ALT III, and the PH domain was necessary and sufficient for its inhibitory
effect. A, activity of PLCδ4 isoforms. PLCδ4 isoforms were co-expressed with a vector carrying the CD4 gene in COS7 cells. Transfected cells were
separated by magnetic beads that could harvest the cells expressing CD4 protein. cDNA of CD4 alone was also overexpressed, and transfected cells
were separated and used for the control. PLC assays were performed using their lysates, and the PLC activity is presented as the fold increase
against control lysates. B, enzymatic activity of PLCδ4 was inhibited by the co-expression of ALT III but not by ALT I or II. PLCδ4 splicing isoforms
were co-expressed with PLCδ4 and CD4, and lysates from transfected cells harvested by magnetic beads were used for the PLC assay. Data
represent inhibition (%) against PLCδ4-overexpressing cell lysates. C, the PH domain alone could inhibit the activity of PLCδ4, whereas the PH
domain deletion mutant could not. Various constructs (indicated in the figure) were overexpressed by a retrovirus expression system in NIH3T3
cells, and Ins-1,4,5-P3 levels were measured with an assay kit. Data represent inhibition (%) against Ins-1,4,5-P3 levels in PLCδ4-overexpressing
cells. All data are means ± S.E. (n = 4).
isozymes imply that the inhibition is not caused by the simple competition for PtdIns-4,5-P2 as a substrate with the PLC. It is known that the activity of PLC\(\delta1\) is activated by the binding of PtdIns-4,5-P2 to its PH domain (10), but PLC\(\beta1\) and -\(\gamma1\) are not known to be activated by PtdIns-4,5-P2 (39, 41). Therefore, PLC\(\delta1\) needs more PtdIns-4,5-P2 than the other isozymes for binding to the PH domain and for a substrate, whereas PLC\(\beta1\) and -\(\gamma1\) need PtdIns-4,5-P2 only as a substrate. The specificities of the inhibitory effect on PLC\(\delta1\) are reflected by the specific-ALT III PH domain and the mutant R36G. Recombinant GST fusion proteins were expressed in E. coli. Eluted proteins were mixed with various amounts of \([3H]\)PtdIns-4,5-P2, and then glutathione-Sepharose beads were added to the reaction mixture. After centrifugation, radioactivities bound to beads were measured. B, binding specificity of the ALT III PH domain to inositol phospholipids. Binding assays of the ALT III PH domain were performed with 100 pmol of \([3H]\)PtdIns-4,5-P2 in the presence of various amounts of competitive inhibitors. The concentrations of competitive unlabeled lipids are indicated as the relative molar ratio of \([3H]\)PtdIns-4,5-P2.

**Fig. 4.** Binding of the ALT III PH domain to inositol phospholipids and inositol phosphates. A, binding of PtdIns-4,5-P2 to the

**Fig. 5.** Inhibitory effect of ALT III in vitro. A, effect of various constructs of ALT III on the activity of PLC\(\delta4\). PLC assays were performed as described under “Experimental Procedures” in the presence of various amounts of ALT III generated by the Sf9/baculovirus system and GST fusion proteins containing the ALT III PH domain, ALT III X-Y domain (nucleotides 202–1844), and R36G mutant generated by bacteria system. The amounts of proteins are indicated as the molar ratio against PLC\(\delta4\) protein. B, effect of ALT III on the activity of PLC\(\beta1\), -\(\gamma1\), and -\(\delta1\) in vitro. The activity of purified PLC\(\beta1\), -\(\gamma1\), and -\(\delta1\) was assayed in the presence of various amounts of recombinant ALT III protein in vitro. The amounts of ALT III protein are indicated as the molar ratio against each PLC isozymes. Data represent duplicate determinations in one of two experiments; error bars give the range of duplicates.
FIG. 6. ALT III effectively inhibited the activity of PLCβ1 and suppressed the activity of PLCβ2 and -γ in vivo. A–C, effects of ALT III on the activities of PLCβ, -γ, and -δ1 in vivo. ALT III was overexpressed using a retrovirus expression system in NIH3T3 cells, and then the cells were serum-starved in starvation medium for 24 h, stimulated by bradykinin (1 μM) (A) or PDGF (50 ng/ml) (B) for the indicated times, and harvested. The Ins-1,4,5-P3 production stimulated by PDGF was suppressed (data not shown). Unfortunately, since the intracellular calcium concentration stimulated by bradykinin was also suppressed (data not shown), we do not know whether ALT III could suppress it or not. However, we cannot analyze the inhibitory effect of ALT III, we do not know whether ALT III could suppress it or not. Therefore, the competitive binding of ALT III to PtdIns-4,5-P2 may cause the inhibition of PLCβ1 activation which is induced through the binding of PtdIns-4,5-P2 to its PH domain, i.e. the feature of PLCβ1 that is activated through the binding of PtdIns-4,5-P2 to its PH domain may cause the sensitivity to ALT III. In contrast, the low sensitivity of PLC-γ and -δ1 to ALT III are reflected by their activation mechanism which is independent of the additional binding of PtdIns-4,5-P2 and may be caused by specificity of their PH domain binding to PtdIns-4,5-P2. Alternatively, PLCβ1 and -γ may hydrolyze PtdIns-4,5-P2 bound to ALT III, whereas PLCδ1 and PLCδ4 may not do so in vitro.

In contrast to the results of the present in vitro experiments, the overexpression of ALT III suppressed the activity of PLCβ and -γ in vivo (Fig. 6, A and B), and the increase in the intracellular calcium concentration stimulated by bradykinin was also suppressed (data not shown). Unfortunately, since the level of the transient calcium increase stimulated by PDGF was not high enough to analyze the inhibitory effect of ALT III, we do not know whether ALT III could suppress it or not. However, the Ins-1,4,5-P3 production stimulated by PDGF was suppressed more efficiently than was that stimulated by bradykinin (Fig. 6, A and B), indicating that ALT III is more effective against PLCγ than PLCβ in vivo. Since the activities of PLCβ1 and -γ1 were not suppressed by ALT III in vitro, we do not think that ALT III inhibits their activity directly. One possible explanation for the suppression in vivo is that PLCβ and -γ are suppressed by ALT III indirectly, i.e. competition for PtdIns-4,5-P2 with the other membrane targeting molecules that affect the PLCβ and -γ activity causes the suppression of them by ALT III in vivo. However, in the case of PLCγ, there still remains a possibility that competitive binding of ALT III to PtdIns-3,4,5-P3 causes the direct inhibition of PLCγ activation which is induced through binding of PtdIns-3,4,5-P3 to its PH domain. Thus, the suppressions of PLCβ and -γ by ALT III in vivo are meaningful events in the physiological condition and may be suppressed not directly but indirectly, and specific targets for the direct inhibition by ALT III are thought to be PLCδ-type isozymes.

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