

Inositol 5'-phosphatase, SHIP1 interacts with phospholipase C- γ 1 and modulates EGF-induced PLC activity

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Abbreviations: DAG, diacylglycerol; EGF, epidermal growth factor; GST, glutathione S-transferase; IP, inositol phosphates; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetrakisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PI-3-kinase, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLC, phospholipase C; RasGAP, GTPase-activation protein of Ras; SH domain, Src homology domain; SHIP1, SH2 domain-containing inositol 5'-phosphatase 1

Abstract

Phospholipase C- γ 1, containing two SH2 and one SH3 domains which participate in the interaction between signaling molecules, plays a significant role in the growth factor-induced signal transduction. However, the role of the SH domains in the growth factor-induced PLC- γ 1 regulation is unclear. By peptide-mass fingerprinting analysis, we have identified SHIP1 as the binding protein for the SH3 domain of PLC- γ 1. SHIP1 was co-immunoprecipitated with PLC- γ 1 and potentiated EGF-induced PLC- γ 1 activation. However, inositol 5'-phosphatase activity of SHIP1 was not required for the potentiation of EGF-induced PLC- γ 1 activation. Taken together, these results suggest that SHIP1 may function as an adaptor protein which can potentiate EGF-induced PLC- γ 1 activation without regards to its inositol 5'-phosphatase activity.

Keywords: epidermal growth factor; phospholipase C- γ 1; SH2 domain-containing inositol 5'-phosphatase; SH3 domain

Introduction

Phospholipase C (PLC) plays a pivotal role in transmembrane signaling. In response to various extracellular stimuli, such as numerous hormones, growth factors, and neurotransmitters, PLC hydrolyzes a minor membrane phospholipid, PIP₂, producing the two second messengers DAG and IP₃, which mediate the activation of PKC and intracellular Ca²⁺ release, respectively (Berridge and Irvine, 1989; Shin *et al.*, 2002). Until now, eleven mammalian isozymes of PLC have been identified and classified into β , γ , δ , ϵ and ζ subfamilies based on overall sequence similarities and the conservation of X and Y domains which are essential for the enzymatic activity (Rhee and Bae, 1997; Kim *et al.*, 2000; Song *et al.*, 2001).

Among various isozymes, PLC- γ uniquely contains two SH2 domains and an SH3 domain between the X and Y domains (Stahl *et al.*, 1988; Suh *et al.*, 1988). The SH2 domains of PLC- γ 1 are responsible for the association between PLC- γ 1 and phosphorylated tyrosine residues on the activated receptor tyrosine kinase. The SH3 domain of PLC- γ 1 is reported to be important for the mitogenic effect of PLC- γ 1. Microinjection of the GST-fused SH3 domain of PLC- γ 1 into G₀/G₁ phase-arrested NIH 3T3 cells has been reported to induce a mitogenic response (Smith *et al.*, 1996). In addition, we have reported that overexpression of the SH2-SH2-SH3 domain of PLC- γ 1 could induce cellular transformation in 3Y1 rat fibroblast and could induce tumor when transplanted into nude mice (Chang *et al.*, 1997). To elucidate the physiological role of SH2-SH2-SH3 domains in the function of PLC- γ 1, it is important to identify SH2-SH2-SH3 domain-binding signaling molecules.

In this study, we found SHIP1 as a binding protein for the SH3 domain of PLC- γ 1 by pull-down assay with GST-fusion protein. SHIP1 was initially identified as a tyrosine-phosphorylated protein after stimulation of blood cells by any of a broad number of cytokines and growth factors (Damen *et al.*, 1993; Kavanaugh *et al.*, 1994; Lioubin *et al.*, 1994). SHIP1 contains several protein-protein interaction domains, such as a N-terminal SH2 domain, a potential phosphotyrosine binding domain site, and a C-terminal proline-rich sequences allowing binding of the SH3 domain (Pesesse *et al.*, 1997). SHIP1 selectively removes the 5'-phosphate from IP₄ and PIP₃, suggesting that it may act as a downstream of or counter to PI 3-kinase (Damen *et al.*, 1996; Lioubin *et al.*, 1996). As a inositol 5'-phosphatase, SHIP1 is thought to be a negative regulator of PIP3 downstream signal transduction. Indeed, overexpression of SHIP1 can antagonize PI-3-kinase function in *Xenopus* oocytes (Deuter-Reinhard *et al.*, 1997). Also, SHIP1 is reported to have a role in the negative signaling mediated by the inhibitory receptor Fc γ R1b

(Ono *et al.*, 1997). However, it is still unclear whether SHIP1 is involved in growth factor-mediated PLC- γ 1 activation mechanism.

In this study, we showed the physical association between SHIP1 and the SH3 domain of PLC- γ 1. Also, EGF-induced PLC- γ 1 activity was increased by SHIP1 expression without regard to its inositol 5'-phosphatase activity. Our findings suggest the possibility that the domain function of SHIP1 is involved in growth factor-mediated PLC- γ 1 activation.

Materials and Methods

General reagents

Mammalian cell culture media were purchased from GIBCO-BRL (Gaithersburg, MD). LipofectAMINE was purchased from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-SHIP1 antibody (#06-632) was purchased from UBI (Lake Placid, NY). pFLAG-CMV-2 vector, mouse monoclonal anti-FLAG M5 antibody, and anti-FLAG M2 agarose were purchased from Eastman Kodak (Rochester, NY). Mouse monoclonal anti-PLC- γ 1 antibody (F7) was prepared as previously described (Suh *et al.*, 1988). A horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA+IgM+IgG were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Myo-[2-³H] inositol (18.3 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). The enhanced Chemiluminescence (ECL) reagent, glutathione-agarose and protein A-agarose were purchased from Amersham Pharmacia International (Buckinghamshire, UK). PMSF, leupeptin, pepstatin A, and aprotinin were obtained from Boehringer Mannheim (Mannheim, Germany). Fetal calf serum and bovine calf serum were purchased from HyClone (Logan, UT). Silver staining kit and Dowex AG 1-X8 formate column were purchased from Bio-rad (Hercules, CA).

Plasmid constructs and mutagenesis

To generate GST-fusion proteins containing the src homology (SH) domains of PLC- γ 1, cDNAs encoding the SH domains were amplified by polymerase chain reaction using rat PLC- γ 1 cDNA as the template. The mammalian expression vector for FLAG-epitope-tagged PLC- γ 1 (pFLAG-WT) was made by PCR. The amplified products were inserted in-frame with the FLAG-epitope tag of pFLAG-CMV-2. The mammalian expression vector for mouse SHIP1 (pEBB-HA tagged-wtSHIP1) was a gift from Dr. Kodimangalam S. Ravichandran. And catalytically inactive mutant of SHIP1 was constructed using site-directed mutagenesis.

Expression and purification of GST-fusion proteins

Recombinant proteins were purified from *Escherichia coli* strain DH5 containing the appropriate constructs. Expression was induced by IPTG (0.1 mM) for 3-5 h at 25°C. Cells were pelleted and resuspended in buffer containing phosphate-buffered saline, 1% Triton X-100,

1 mM PMSF, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 2 μ M pepstatin A, and then lysed by sonication and clarified by centrifugation at 15,000 *g* for 20 min. The soluble fraction was incubated with glutathione-coated Sepharose beads for 3 h at 4°C and then washed 4 times with ice-cold washing buffer (1% Triton X-100, 200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 20 mM NaF, 1 mM PMSF, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 2 μ M pepstatin A).

in vitro binding experiments

Clarified lysates (500 μ g) of SP2 cells were incubated with 5 μ g of GST-fusion proteins immobilized on glutathione-agarose beads in a final volume of 1 ml of lysis buffer for 2 h at 4°C. Protein complexes were collected by centrifugation and washed 4 times with NP40 containing lysis buffer (1% NP40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM NaF, 500 μ M sodium orthovanadate, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 2 μ M pepstatin A). Associated protein complexes were dissociated by heating in SDS sample buffer. The protein complexes were resolved by 8% SDS-PAGE and transferred to a nitrocellulose membrane. SHIP1 was immunoblotted using rabbit anti-SHIP1 antibody. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and ECL according to the manufacturer's instructions.

Identification of p145 by protein peptide mass fingerprinting

The fraction containing p145 after pull-down from SP2 cell was separated on 8% SDS-PAGE and stained by silver staining kit. The band corresponding to 145 kDa protein were excised, rinsed several times with 3% methanol and digested with trypsin for 6 h at 37°C. Following the extraction of digested peptides from the gel, the extract was lyophilized, resuspended in 100 μ l of 100 mM ammonium bicarbonate and analyzed by MALDI-TOF (Matrix Associated Laser Desorption Ionization-Time of Flight) mass spectrometry (PerSeptive Biosystems, Framingham, MA). Identification of p145 as achieved by database searching program Profound.

Cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (FCS), 50 U/ml penicillin, 50 μ g/ml gentamycin sulfate, and 50 μ g/ml streptomycin. Cells were plated on cultureware dish and incubated in a humidified 5% CO₂ atmosphere at 37°C. Cells were seeded at 2 \times 10⁵ cells/35 mm well 24 h before transfection using liposome-mediated transfection. LipofectAMINE was used for the transfection. We followed the manufacturer's instructions using 1 μ g of plasmid DNA and 6 μ l of LipofectAMINE reagent/well, incubated the cells with this mixture for 6 h, and then replaced the medium. Forty-eight hours later, cells were harvested. In the case of serum starvation, transfected cells were serum-

starved for 18 h at 30 h post-transfection.

Co-immunoprecipitation and immunoblotting

Confluent COS-7 cells in 100 mm plates were serum-starved in DMEM medium supplemented with only 0.1% bovine calf serum for 18 h at 37°C. Cells were lysed in 1 ml of cold NP40 lysis buffer (1% NP40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM NaF, 500 μ M sodium orthovanadate, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 2 μ M pepstatin A). The cell lysates were clarified by centrifugation at 14,000 g for 15 min at 4°C. The clarified lysates were precleared with 30 μ l of 50% protein A-Sepharose for 30 min at 4°C. The clarified cell lysates were mixed with 5 μ g of anti-FLAG antibody immobilized to protein A-Sepharose for 3 h at 4°C. The immune complexes were collected by centrifugation (4 min at 3,000 g), washed 4 times with cold NP40 lysis buffer, and

dissociated by heating in SDS sample buffer. Proteins were separated by 8% SDS-PAGE. The separated proteins were then transferred to nitrocellulose filters. Blocking was performed with TTBS buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk powder. The filters were then incubated with anti-HA antibody for 4 h at room temperature. Immunoblots were subsequently washed and incubated with horseradish peroxidase-linked secondary antibody for 1 h at room temperature, washed 4 times in TTBS buffer, and developed with horseradish peroxidase-dependent chemiluminescence (ECL).

Measurement of inositol phosphates accumulation

COS-7 cells were transfected with the cDNA of appropriate constructs. 24 h after transfection, cells were labeled with 1 μ ci/ml myo-[2- 3 H]inositol in 2.0 ml of inositol free DMEM for 18 h. After washing with medium

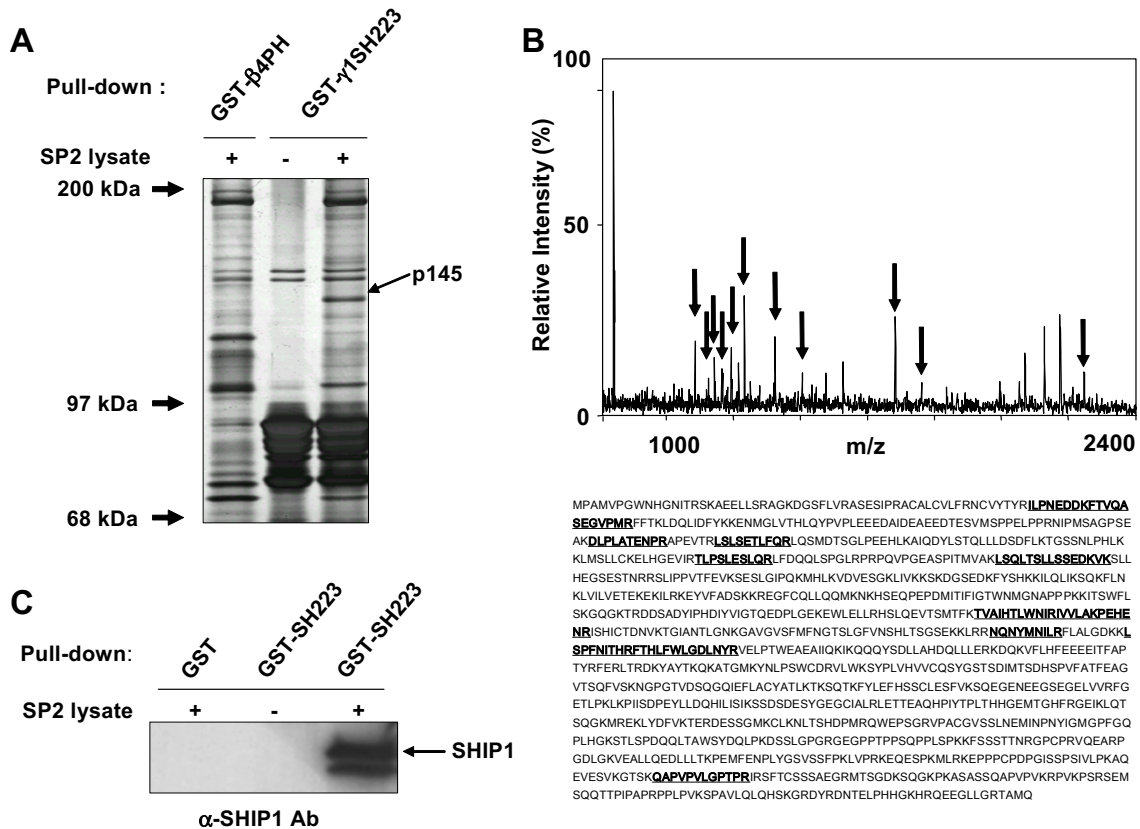


Figure 1. SHIP1 binds to the SH2-SH2-SH3 domain of PLC- γ 1 *in vitro*. (A) 500 μ g of solubilized SP2 cell lysate were subjected to pull-down assay using 5 μ g of each GST fusion proteins. Precipitates were subjected to SDS-PAGE and resulting polyacrylamide gel were stained with silver reagent. Several specific SH2-SH2-SH3 domain binding proteins were selected and excised. Excised bands were subjected to peptide mass finger printing using MALDI-TOF mass spectrometry. (B) The map was produced by MALDI-TOF mass spectrometry using the peptide supernatant obtained after 'in gel' digestion of the excised band with trypsin as described under "Experimental procedure". A database search for the measured tryptic peptide masses uniquely identified p145 as SHIP1. Coverage map for SHIP1 were shown and the matching peptides are underlined. (C) The identification of p145 as SHIP1 using MALDI-TOF mass-spectrometry was also confirmed by immunoblotting using anti-SHIP1 antibody. 500 μ g of SP2 cell lysate were precipitated with 5 μ g of each GST-fusion proteins immobilized on glutathione-agarose beads. Collected protein complexes were washed with lysis buffer and then subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane. Immunoblot analysis was performed with anti-SHIP1 antibody.

containing 2 mM LiCl for 10 min, cells were incubated for 10 min at room temperature with 10 mM EGF in the same medium containing LiCl. The reactions were quenched with 5% perchloric acid. IP and total inositol fractions were resolved on a Dowex AG 1-X8 formate column.

Results

SHIP1 binds to the SH2-SH2-SH3 domain of PLC- γ 1 *in vitro*

To elucidate the possible role of SH2-SH2-SH3 domain-binding proteins in the regulation of PLC- γ 1, we identified SH2-SH2-SH3 domain-binding proteins. SH2-SH2-SH3 domain of PLC- γ 1 was expressed as GST fusion proteins in *E. coli* and immobilized on GSH agarose. The immobilized GST fusion proteins were incubated with extracts of SP2 cell. Several proteins seemed to specifically interact with SH2-SH2-SH3 domain of PLC- γ 1 *in vitro* and a protein of apparent molecular mass of 145 kDa (p145) showed the strongest intensity among SH2-SH2-SH3 domain-binding proteins (Figure 1A).

To identify p145, target band was excised from silver-stained gel and digested with trypsin. Mass spectrometric fingerprinting analysis was performed as described under "Materials and Methods" resulting in the identification of p145 as SH2 domain-containing inositol 5'-phosphatase, SHIP1. A search for these masses in a comprehensive sequence database showed that 11 peptide masses matched to calculated tryptic peptide masses of SH2 domain-containing 5'-inositol phosphatase, SHIP1 with an accuracy of less than 50 ppm. These peptides covered 12% of the whole sequence of SHIP1 (Figure 1B). To further verify the identity of p145, immunoblot analysis was performed using anti-SHIP1 antibody (Figure 1C). Taken together, these data demonstrate that SHIP1 interacts with SH2-SH2-SH3 domain of PLC- γ 1 *in vitro*.

PLC- γ 1 interacts with SHIP1 in cells

In order to further investigate the physical association between SHIP1 and whole PLC- γ 1 molecule, HA-tagged SHIP1 and FLAG-tagged PLC- γ 1 were transiently expressed in COS-7 cells. SHIP1 was co-immunoprecipitated with PLC- γ 1 in resting cells (Figure 2). Both PLC- γ 1 and SHIP1 are known to be activated by EGF stimulation. However, the physical association between PLC- γ 1 and SHIP1 was not affected by EGF treatment (data not shown). These results indicate that SHIP1 is associated with whole PLC- γ 1 molecule in cell.

SHIP1 interacts with SH3 domain of PLC- γ 1

To map the region in PLC- γ 1 which is responsible for the interaction with SHIP1, GST-fused SH domains of PLC- γ 1 were used in pull-down assay. Purified fusion proteins of various SH domains were incubated with SP2 cell lysates. SHIP1 was co-precipitated with GST-

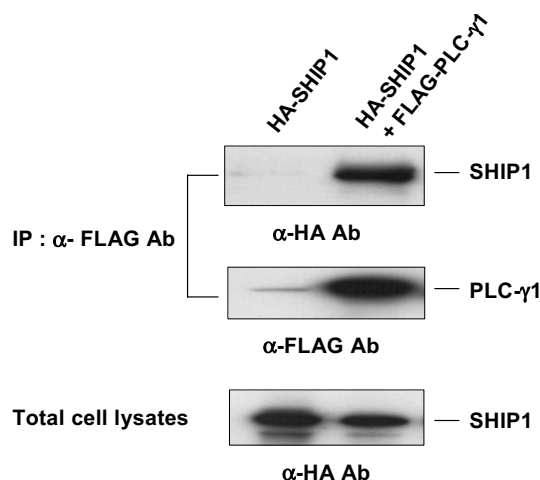


Figure 2. SHIP1 was co-immunoprecipitated with PLC- γ 1 in COS-7 cell. PLC- γ 1 and SHIP1 were transiently expressed in COS-7 cells. COS-7 cells were solubilized with lysis buffer and precleared with protein A-Sepharose. Then 500 μ g of each cell lysate was precipitated with 5 μ g of anti-FLAG antibody immobilized to protein A-Sepharose. Resulting precipitates were subjected to SDS-PAGE and immunoblot analysis. Coprecipitation of SHIP1 was checked with anti-HA antibody (upper panel). Precipitated PLC- γ 1 and expressed SHIP1 were shown using each antibody (middle and lower panel).

SH3 and GST-SH2-SH2-SH3 fusion proteins *in vitro*, whereas the SHIP1 did not interact with two SH2 domains of PLC- γ 1 (Figure 3A).

To further demonstrate the involvement of the SH3 domain of PLC- γ 1 in the interaction with SHIP1 in intact cell, we transiently expressed FLAG-tagged wild-type PLC- γ 1 or mutants PLC- γ 1 lacking SH domains with HA-tagged SHIP1 into COS-7 cells. After Serum-starvation, transfected cells were lysed and immunoreacted with anti-FLAG antibody, and resulting precipitates were analyzed with immunoblot analysis using anti-FLAG antibody and anti-HA antibody. SHIP1 was co-immunoprecipitated with wild type and SH2 domain deletion mutants of PLC- γ 1 (Figure 3C). The deletion of the SH3 domain from PLC- γ 1 abolished the interaction with SHIP1. This result shows that SH3 domain of PLC- γ 1 is necessary for the interaction with SHIP1.

SHIP1 potentiates EGF-induced PLC activation

SHIP1 was originally identified as an inositol polyphosphate-5-phosphatase in cytokine-stimulated hematopoietic cells (Damen *et al.*, 1996). Also, SHIP1 has an important role in growth factor mediated signaling such as HGF-induced vasculogenesis in epithelial cells (Stefan *et al.*, 2001).

The effect of SHIP1 on EGF-induced PLC- γ 1 activation was tested to investigate the physiological meaning of the interaction between PLC- γ 1 and SHIP1. EGF is a well-known agonist to activate PLC- γ 1 in COS-7 cell. COS-7 cells were transfected with HA-tagged SHIP1 or vector plasmid. After 24 h serum depletion, EGF-induced inositol phosphates generation was mea-

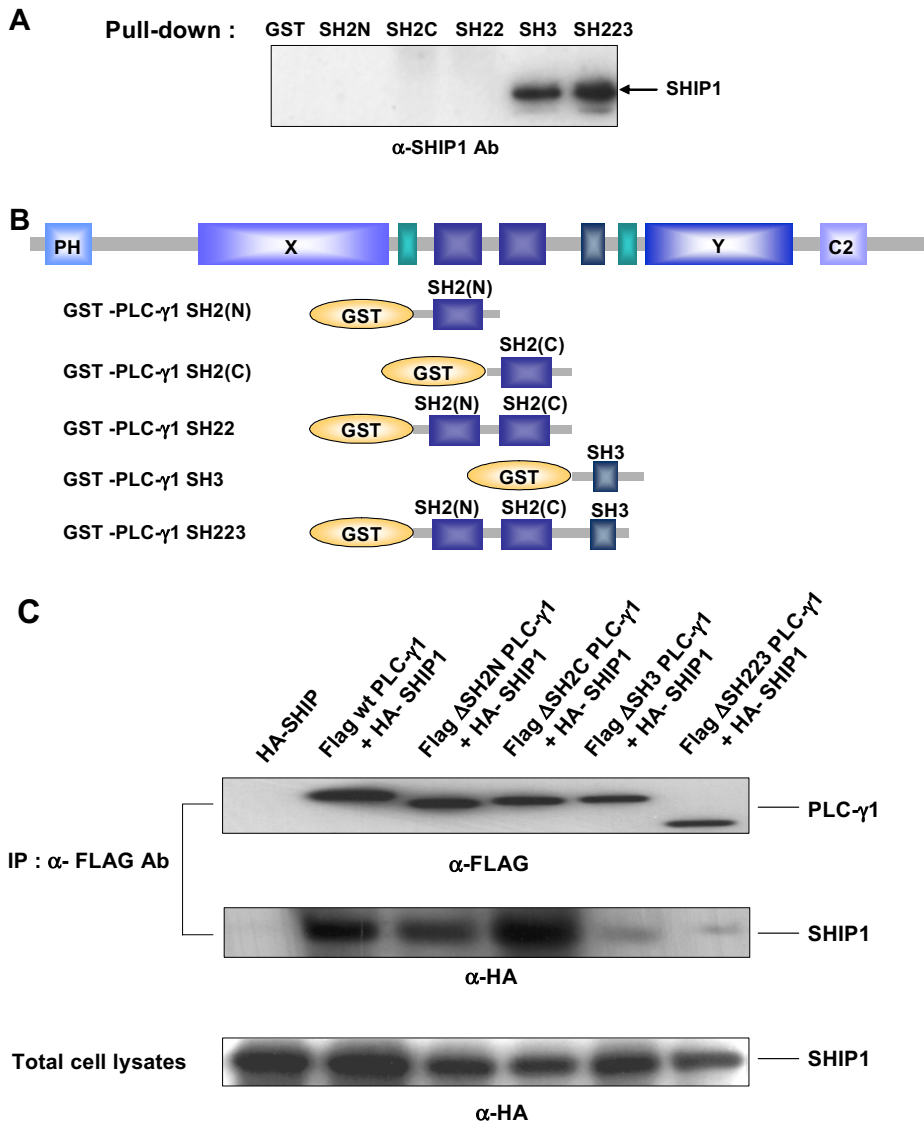


Figure 3. SHIP1 interacts with the SH3 domain of PLC- γ 1. (A) Combinations of SH2N, SH2C, SH3 domain containing GST fusion proteins were constructed and expressed. Pull-down assay was performed using these GST fusion proteins and SP2 cell lysates, and the resulting precipitates were subjected to immunoblot analysis using anti-SHIP1 antibody. As shown above, SHIP1 was precipitated with only GST fusion protein of SH3 and SH2-SH2-SH3 domain of PLC- γ 1. (B) Schematic diagram of various SH2-SH2-SH3 domain fusion protein of PLC- γ 1. (C) COS-7 cells were transfected with HA-tagged SHIP1/FLAG-tagged wild type or various SH domain deletion mutant of PLC- γ 1. After solubilization, resulting lysates were immunoprecipitated with anti-FLAG antibody and analyzed with anti-HA antibody. Total cell lysates (40 μ g) were also immunoblotted with anti-HA antibody.

sured. Basal inositol phosphates level was increased by SHIP1 expression. Also, EGF-induced inositol phosphates generation was increased by 50 percent in cells expressing SHIP1 compared with vector control (Figure 4A) To check whether the 5'-inositol phosphatase activity of SHIP1 is involved in the increase of inositol phosphates generation, catalytically inactive mutant of SHIP1 was used (Deuter-Reinhard *et al.*, 1997). Catalytically inactive mutant of SHIP1 also increase the basal and EGF-induced inositol phosphates generation as those of wild type SHIP1 (Figure 5A). These results indicate that SHIP1 potentiate EGF-induce PLC- γ 1 activation without regard to its inositol 5'-phosphatase activity. Therefore, these data suggests that the physical interaction between PLC- γ 1 and SHIP1 might be responsible for the increase of EGF-induced inositol phosphates generation.

Discussion

Among several molecules involved in growth factor-mediated signal transduction, PLC- γ 1 plays a pivotal role for cellular proliferation by growth factor. Not only its lipase activity but also central SH2-SH2-SH3 domain of PLC- γ 1 is thought to be important for the PLC- γ 1-mediated cellular proliferation. Thus, it has become an important issue to identify the proteins that could specifically bind to the SH2-SH2-SH3 domain of PLC- γ 1. Therefore, we set out to find the binding proteins to the SH2-SH2-SH3 domain of PLC- γ 1 which might participate in PLC- γ 1-mediated physiological function. In this study, we demonstrated that SHIP1 associates with the SH3 domain of PLC- γ 1 and potentiates EGF-induced PLC- γ 1 activation.

SHIP1 has several SH3 domain consensus (PXXP) binding motif in carboxy-terminal which is known to

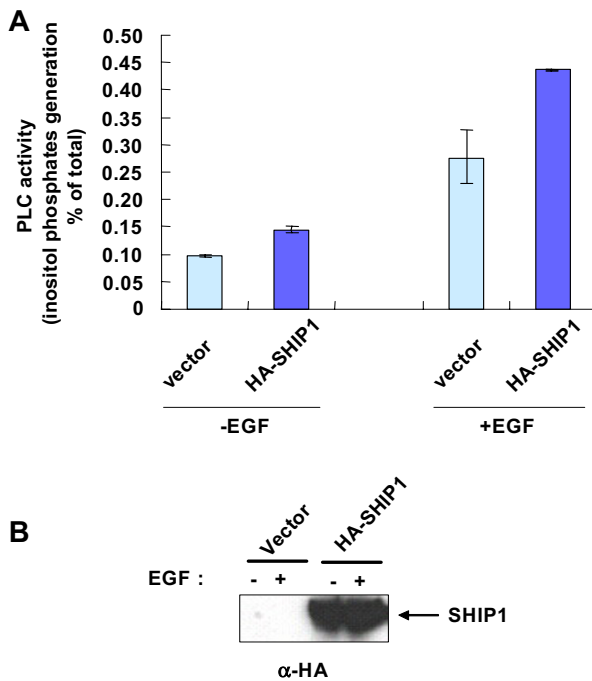


Figure 4. EGF-induced PLC activity is potentiated by SHIP1 expression. (A) COS-7 cells were transfected with vector, wild type of SHIP1 expression vector. 24 h after transfection, cells were labeled with 1 μ Ci/ml myo-[2-³H]inositol in 2 ml of inositol free DMEM for 18 h. Total inositol phosphate accumulation was determined after cells were treated for 10 min with 100 ng/ml EGF. Total inositol fractions were resolved on a Dowex AG1-X8 formate column. Data was presented as mean \pm S.E. of one of the three experiments performed in duplicate. (B) Expression level of wild type of SHIP1 was shown with anti-HA antibody.

mediate association with SH3 domains of Grb2 and Src (Lucas *et al.*, 1999). Also, we showed that SH3 domain of PLC- γ 1 was responsible for the association with SHIP1 (Figure 3A, C) and this physical interaction was not changed by EGF stimulation (data not shown). The SH3 domain, which is not essential for tyrosine phosphorylation, has a role in anchoring PLC- γ 1 to the membrane. Although SH2(N) domain is essential for the membrane localization of PLC- γ 1, SH3 domain may contribute to the membrane attachment of PLC- γ 1. The SH3 domain of PLC- γ 1 binds to cytoskeletal components, and translocation of activated PLC- γ 1 molecules to the detergent-insoluble cell cytoskeleton has been reported (Bar-Sagi *et al.*, 1993; Weng *et al.*, 1993; Pei *et al.*, 1996). Thus, SH3 domain of PLC- γ 1 may be important for stabilizing the association of PLC- γ 1 with the membrane through the interaction with cytoskeletal components. As SHIP1 bind to SH3 domain of PLC- γ 1 in resting state and has to be recruited to EGF receptor for its activation, SHIP1 may function in the membrane translocation process of PLC- γ 1 through the interaction with SH3 domain of PLC- γ 1 like other cytoskeletal components.

SHIP1 is known as a negative regulator of calcium signalling (Choquet *et al.*, 1993; Sarmay *et al.*, 1996;

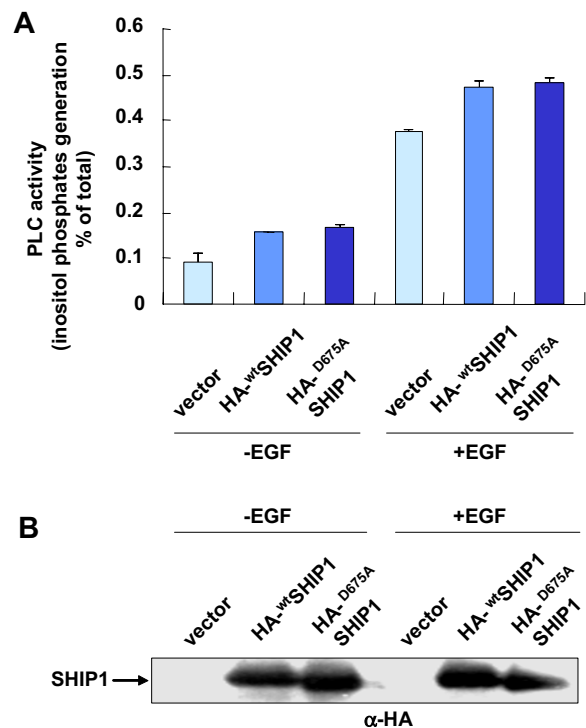


Figure 5. Inositol 5'-phosphatase activity of SHIP1 is not required for the potentiation of EGF-induced PLC activation. (A) COS-7 cells were transfected with vector, wild type or catalytically inactive mutant of SHIP1 expression vector. 24 h after transfection, cells were labeled with 1 μ Ci/ml myo-[2-³H]inositol in 2 ml of inositol free DMEM for 18 h. Total inositol phosphate accumulation was determined after cells were treated for 10 min with 100 ng/ml EGF. Total inositol fractions were resolved on a Dowex AG1-X8 formate column. Data was presented as mean \pm S.E. of one of the three experiments performed in duplicate. (B) Expression level of wild type and catalytically inactive mutant of SHIP1 was probed with anti-HA antibody.

Tridandapani *et al.*, 1997). When this gene was knocked out, calcium and IP₃ response was not efficiently terminated. And the inositol 5'-phosphatase activity of SHIP1 is believed to be essential in the negative regulation of IP₃ generation and calcium release. As PLC- γ 1 was reported to be activated by PIP₃ and EGF activate PI3K in COS-7 cells, we postulated that expression of inositol 5'-phosphatase, SHIP1 will reduce EGF-induced PLC- γ 1 activation. Not in accordance with our expectation, EGF-induced inositol phosphates generation was potentiated when SHIP1 was transiently expressed in COS-7 cell (Figure 4A). Moreover the inositol 5'-phosphatase activity of SHIP1 was not required for the elavation of EGF-induced inositol phosphate generation (Figure 5A). Potentiation of EGF-induced PLC- γ 1 activation by SHIP1 expression and dispensibility of inositol 5'-phosphatase activity of SHIP1 for such potentiation implies another role of SHIP1 on growth factor-mediated PLC- γ 1 activation process. Inositol 5'-phosphatase activity which cleaves 5'-phosphate from PIP₃ contribute termination of growth factor-mediated PLC- γ 1 activation, but SHIP1 may assist PLC- γ 1

activation through the association with SH3 domain.

Independently of its 5'-phosphatase activity, SHIP1 may function as an adaptor under some circumstances. That suggestion is based on the report that SHIP1 reduces Ras activity by recruiting the RasGAP (GTPase-activation protein of Ras)-binding protein, p62Dok (Tamir *et al.*, 2000). As an adaptor, SHIP1 may support the membrane targeting and phosphorylation of PLC- γ 1. PLC- γ 1 is recruited by growth factor receptor, phosphorylated and activated. Also, SHIP1 has to be recruited by growth factor receptor for its function. SHIP1 was associated with PLC- γ 1 basally, and this association was maintained during EGF-induced activation process. Moreover, expression of catalytically inactive SHIP1 potentiated EGF-induced inositol phosphate generation. Taken together, SHIP1 may function as an adaptor in the activation process of PLC- γ 1 irrespective of its 5'-inositol phosphatase activity.

In conclusion, this study demonstrates a novel association of PLC- γ 1 and the inositol phosphatase SHIP1. By this interaction, PLC- γ 1 activity potentiated without regards to inositol 5'-phosphatase activity of SHIP1. Also, we suggest another function of SHIP1 as an adaptor protein in the growth factor mediated activation process of PLC- γ 1.

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