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The SH3 Domain of Phospholipase C-γ1 Associates with Shc

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The SH3 domain of PLC-γ1 has been known to induce DNA synthesis. However, little is known about the putative effector proteins that associate with the domain. In this report, we provide evidence that the SH3 domain of PLC-γ1 associates with Shc, which has been implicated in the activation of p21Ras in response to many growth factors. The association between Shc and PLC-γ1 is enhanced either by *v-Src*-induced transformation or EGF-stimulation *in vivo* and *in vitro*. Furthermore, from transient expression studies with COS-7 cells, we show that the SH3 domain of PLC-γ1 is required for association with Shc *in vivo*, whereas tyrosyl phosphorylation of PLC-γ1 is not. Taken together, we suggest that Shc might be involved in the PLC-γ1-mediated signaling pathway.

Keywords: GST, PLC-γ1, Protein–Protein Interaction, SH3 domain, Shc.

Introduction

Phosphoinositide-specific phospholipase C (PLC) plays a role in transmembrane signaling. In response to various extracellular stimuli, such as numerous hormones, growth factors, and neurotransmitters, PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Rhee *et al.*, 1989; Rhee and Choi, 1992; Noh *et al.*, 1995).

There are multiple PLC isozymes, and, in spite of low overall homology between the predicted amino acid sequences, significant sequence similarity is obvious in two domains which are designated the X- and the Y-domain. On the basis of the relative locations of the X- and Y-domains in the primary structure, PLC isozymes are classified into three types,viz., β, γ, and δ. Two γ-type PLCs, PLC-γ1 and -γ2, but not β- and δ-type isozymes, are activated through phosphorylation by growth factor-receptor tyrosine kinase or nonreceptor tyrosine kinase (Suh *et al.*, 1990; Kamat and Carpenter, 1997). PLC-γ1 is involved in cellular proliferation. It is known that the expression level for PLC-γ1 is increased in human colorectal cancer, human breast carcinomas, familial adenomatous polyposis, and human skin in hyperproliferative conditions (Arteaga *et al.*, 1991; Nanney *et al.*, 1992; Noh *et al.*, 1994; Park *et al.*, 1994). Smith *et al.* (1989; 1990) reported that microinjection of purified PLC-γ1 into quiescent NIH 3T3 cells results in the induction of DNA synthesis and that the injection of antibodies against PLC-γ1 into the same cells blocks the serum-induced DNA synthesis. Recent studies showed that a PLC-γ1 mutant lacking lipase activity also induces DNA synthesis, implying that regions other than the catalytic domain may be responsible for the mitogenic effect (Smith *et al.*, 1994; Huang *et al.*, 1995). Among multiple PLC-isozymes, only PLC-γ isozymes contain a Src-homology (SH) domain (two SH2 domains and one SH3 domain) (Kamat and Carpenter, 1997). These domains have been found in a number of proteins involved in the regulation of cell proliferation and differentiation (Pawson, 1995). The SH2 domains of PLC-γ1 has been known to mediate the association between PLC-γ1 and phosphorylated tyrosines on the activated receptor tyrosine kinase or src tyrosine kinase (Kim *et al.*, 1993; Kamat and Carpenter, 1997). The SH3 domain of PLC-γ1 has been known to be responsible for the mitogenic effect of PLC-γ1, e.g., microinjection of the GST-fused SH3 domain of PLC-γ1 into G_0 growth-arrested NIH 3T3 cells has been reported to induce DNA synthesis (Huang *et al.*, 1995; Smith *et al.*, 1996). However, little is known about the putative effector proteins that associate with the PLC-γ1 SH3 domain.

We tried to identify proteins that associate with the SH3 domain of PLC-γ1 and possibly mediate the mitogenic effect of PLC-γ1. For this purpose, we prepared various glutathione S-transferase (GST)-fused SH domains of PLC-γ1 using these fusion proteins in *in vitro* binding

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experiments. In this paper, we provide evidence that the SH3 domain of PLC-γ1 associates with Shc which has been implicated in the activation of p21Ras in response to many growth factors.

Materials and Methods

Cells, antibodies, and reagents COS-7, 3Y1, and *v-Src* transformed 3Y1 cells (SR-3Y1 cells) were cultured in DMEM supplemented with 10% bovine calf serum. Rabbit anti-Shc polyclonal antibody and anti-phosphotyrosine (4G10) antibody were purchased from UBI (Lake Placid, USA); Mouse anti-Shc monoclonal antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, USA); pFLAG-CMV-2 vector, anti-FLAG M5 monoclonal antibody, and anti-FLAG M2 agarose were purchased from EASTMAN KODAK (Rochester, USA). Mouse anti-PLC-γ1 monoclonal antibody (F7) was prepared as previously described (Suh *et al.*, 1988a). Glutathione-agarose and Protein A-agarose were purchased from Pharmacia Biotech (Piscataway, USA)

Plasmid constructs For the generation of glutathione S-transferase (GST) fusion proteins containing the src homology (SH) domains of PLC-γ1, DNAs encoding the SH domains were amplified by PCR using rat PLC-γ1 cDNA (Suh *et al.*, 1988b) as template. The following primer pairs were used:

GST-γ1SH223
Forward primer: 5′-ACTGGATCCCCGAAGGAGGCCAGTGGCAGC3′
Reverse primer: 5′-AGTCCCGGGATGCTCCCTCTCCGGCTCTAG3′

GST-γ1SH22
Forward primer: 5′-ACTGGATCCCCGAAGGAGGCCAGTGGCAGC3′
Reverse primer: 5′-AGTCCCGGGGTTGCGGCCCTCATACAGTGC3′

GST-γ1SH2N
Forward primer: 5′-ACTGGATCCCCGAAGGAGGCCAGTGGCAGC3′
Reverse primer: 5′-AGTCCCGGGCTCTTTGCTCTCATGGGCATT3′

GST-γ1SH2C
Forward primer: 5′-ACTGGATCCATGCGCCTTTCAGAGCCTGTT3′
Reverse primer: 5′-AGTCCCGGGGTTGCGGCCCTCATACAGTGC3′

GST-γ1SH3
Forward primer: 5′-ACTGGATCCAAGATTGGGACAGCTGAACCC3′
Reverse primer: 5′-AGTCCCGGGATGCTCCCTCTCCGGCTCTAG3′

Underlined nucleotides indicate the *Bam*HI and *Sma*I sites in the forward and reverse primers, respectively. Amplified products were digested with *Bam*HI and *Sma*I and subcloned into pGEX-4T2 (Pharmacia Biotech). The mammalian expression vectors for FLAG-epitope tagged PLC-γ1 (pFLAG-WT, pFLAG-Y771, pFLAG-Y783, or pFLAG-Y771/783) were made by PCR using point mutant PLC-γ1 cDNAs [Tyrosine residues Y771 and/or Y783 were replaced with phenylalanine residue(s)] provided by Dr. Rhee, S. G. (National Institute of Health, USA) as templates (Kim *et al.*, 1991). The amplified products were inserted in-frame with the FLAG-epitope tag of the pFLAG-CMV-2 (Eastman Kodak). The mammalian expression vector for mutant PLC-γ1 lacking the SH3 domain (pFLAG-ΔSH3, lacking the region corresponding to amino acid residues 796 ~ 902) was constructed by polymerase chain reaction.

Expression and purification of GST-fusion proteins Recombinant proteins were purified from *E. coli* strain DH5α containing the appropriate constructs. Expression was induced by IPTG (0.1 mM) for 2–4 h at 25°C. Cells were pelleted and resuspended in lysis buffer containing phosphate-buffered saline, 1% Triton X-100, 0.1 mM PMSF, and then lysed by sonication and clarified. The soluble fraction was incubated with glutathione-coated Sepharose beads (Pharmacia Biotech Inc.) for 3 h at 4°C and then washed four times with lysis buffer.

Immunoprecipitation and immunoblotting Confluent 100-mm plates of 3Y1 and SR-3Y1 cells were serum-starved in DMEM supplemented with only 0.1% bovine calf serum for 18 h at 37°C. Cells were then treated with 100 ng/ml of EGF for 2 min and lysed with 1 ml of cold Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris·HCl, pH 8.0, 20 mM NaF, 1 mM $MgCl_2$, 200 μM sodium orthovanadate, 1 mM PMSF, 10 μM leupeptin, and 2 μM pepstatin). The lysates were clarified by centrifugation at 14,000 × *g* for 15 min at 4°C. The clarified lysates (500 μg) were pre-cleared with 30 μl of 50% protein A-sepharose for 30 min at 4°C. The clarified lysates were mixed with appropriate antibody pre-coupled to protein A-sepharose for 3 h at 4°C. The immune complexes were collected by centrifugation (4 min at 3000 × *g*), washed four times with cold lysis buffer, and eluted by heating in SDS sample buffer. Proteins were separated by 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. The filters were then incubated with primary antibody at the concentration recommended by the manufacturer for 2 h at room temperature. Immunoblots were subsequently washed and incubated with horseradish peroxidase-linked secondary antibody for 1 h at room temperature, washed five times in TTBS buffer, and developed with horseradish peroxidase-dependent chemiluminescence (ECL) (Amersham Co, Arlington Heights, USA).

Transient expression Transient transfection of the COS-7 cells was done by the DEAE-dextran method (Cullen *et al.*, 1984). Cells were seeded at 1×10^6 cells/100-mm dish and transfected 24 h later by incubation with 2 ml of transfection cocktail [2 μg of plasmid DNA (mammalian expression vectors for FLAG-epitope tagged PLC-γ1 mutants) and 500 μg of DEAE-dextran in PBS] for 1 h. Then, 7 ml of serum-free DMEM containing 100 μM chloroquine was added. After 2.5 h, the medium was aspirated and the cells were treated with 10% DMSO in DMEM for 2.5 min, washed with PBS, and incubated in a 5% CO_2 incubator. The cells were serum-starved for 18 h at 30 h post-transfection and then immunoprecipitated with anti-FLAG M2 agarose (EASTMAN KODAK).

***In vitro* binding experiment** Clarified lysates (200 μg) of 3Y1 and SR-3Y1 cells were incubated with the GST-fused SH domains of PLC-γ1 (5 μg) immobilized on glutathione-agarose beads in a final volume of 1 ml of lysis buffer for 1.5 h at 4°C. Protein complexes were collected by centrifugation and washed four times with lysis buffer. Associated proteins were eluted by heating in SDS sample buffer.

Results

Shc is co-precipitated with the GST-fused SH3 domain of PLC-γ1 in SR-3Y1 fibroblast cells To identify proteins that associate specifically with the SH3 domain of PLC-γ1, we prepared five constructs of GST-fused SH domains of PLC-γ1 (GST-γ1SH3, GST-γ1SH2N, GST-γ1SH2C, GST-γ1SH22, and GST-γ1SH223) (Fig. 1A). We used these constructs in *in vitro* binding experiments with the lysate of *v-Src*-transformed 3Y1 (SR-3Y1) fibroblast cells. Previously, Sillman *et al.* (1995) identified Syk as a PLC-γ1 binding protein in B lymphocytes, and recently Crowley *et al.* (1996) have suggested that Syk-associated p145 is likely to be the same protein as the previously identified Shc-associated p145. Because Shc has been known to be involved in the activation of p21Ras in response to many proliferative signals (Bonfini *et al.*, 1996), we chose Shc as a possible candidate for the associating protein of PLC-γ1. We tested whether Shc, one of the p21Ras regulators, could associate with PLC-γ1. As shown in Fig. 1B, we detected three Shc proteins, p66, p52, and p46, in SR-3Y1 cells. Regardless of EGF-stimulation, all three forms of Shc proteins in SR-3Y1 cells associated with the GST-γ1SH3 domain of PLC-γ1. Among the three isoforms of Shc, the p52 Shc protein showed the strongest association with the SH3 domain of PLC-γ1. The Shc-binding to GST-γ1SH3 was comparable to that of GST-γ1SH223. In addition, small amounts of p52 Shc protein also associated with the GST-γ1SH2N fusion protein, but not with GST-γ1SH2C (Fig. 1B).

***v-Src* is involved in the association between Shc and the SH3 domain of PLC-γ1** Since the *in vitro* association between Shc and the GST-fused SH domains of PLC-γ1 was not changed upon EGF-stimulation in SR-3Y1 cells, we postulated that this EGF-independent association may be an effect of the persistently activated *v-Src* tyrosine kinase. To confirm this hypothesis, we compared *in vitro* binding activities of Shc to the GST-γ1SH3 domain fusion protein using lysates of serum-starved SR-3Y1 cells and serum-starved normal 3Y1 cells. As shown in Fig. 2A, the complex formation of Shc with GST-γ1SH3 was much higher in SR-3Y1 cells than in normal 3Y1 cells, although the level of Shc expression in the SR-3Y1 cells was comparable to that in the 3Y1 cells. In search of an explanation for the observed difference in *in vitro* binding activity, we compared the extent of tyrosyl-phosphorylation of the Shc proteins in SR-3Y1 cells and 3Y1 cells. As shown in Fig. 2B, Shc protein in SR-3Y1 cells was highly tyrosyl-phosphorylated, whereas Shc protein in normal 3Y1 cells did not have detectable tyrosyl-phosphorylation. This result is consistent with a previous report that Shc is phosphorylated on tyrosine residues by *v-Src* (McGlade *et al.*, 1992). Therefore, it is likely that tyrosyl phosphorylation of Shc by *v-Src* enhances the association of Shc with the SH3 domain of PLC-γ1.

Increased *in vivo* association of Shc with PLC-γ1 in *v-Src*-transformed 3Y1 cells To demonstrate the association of Shc with PLC-γ1 in intact cells, we

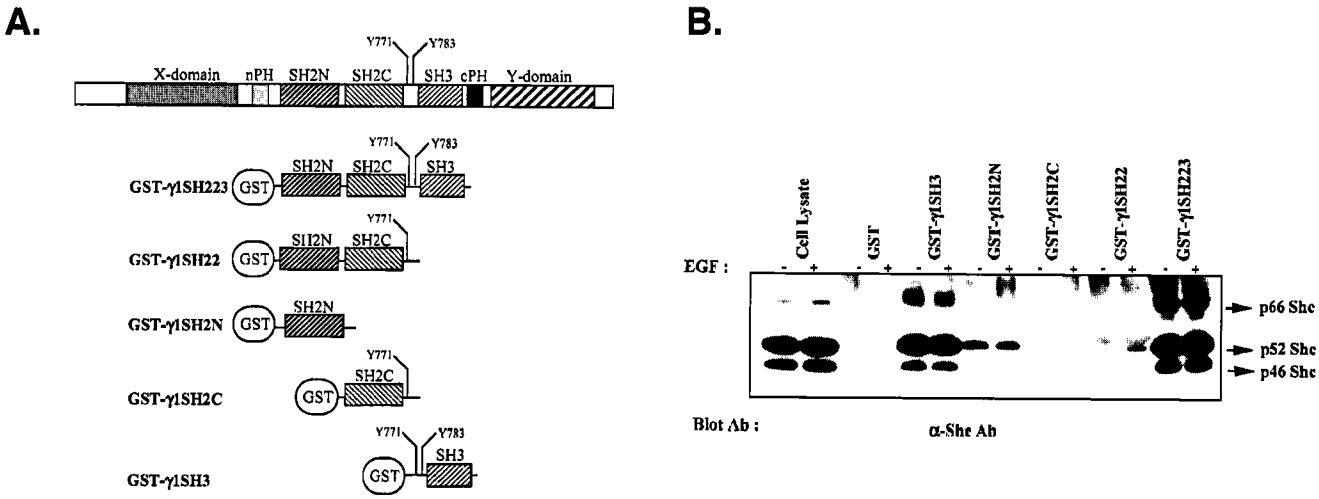

Fig. 1. A. Constructs of PLC-γ1 GST-fusion proteins. The locations of tyrosine residues neighboring the SH3 domain of PLC-γ1 and which are known to be phosphorylated by activated growth factor PTK are indicated in the top diagram. Lower diagrams show the different portions of PLC-γ1 that were included in each of the GST fusion protein constructs. B. SH3 domain of PLC-γ1 in association with Shc in *v-Src* transformed 3Y1 cells. Triton X-100 lysates (200 μg) of SR-3Y1 cells treated or not treated with EGF (100 ng/ml) for 2 min were precipitated with 5 μg of GST, GST-γ1SH223, GST-γ1SH22, GST-γ1SH2N, GST-γ1SH2C, or GST-γ1SH3. Bound proteins were analyzed on Western blots probed with anti-Shc polyclonal antibody.

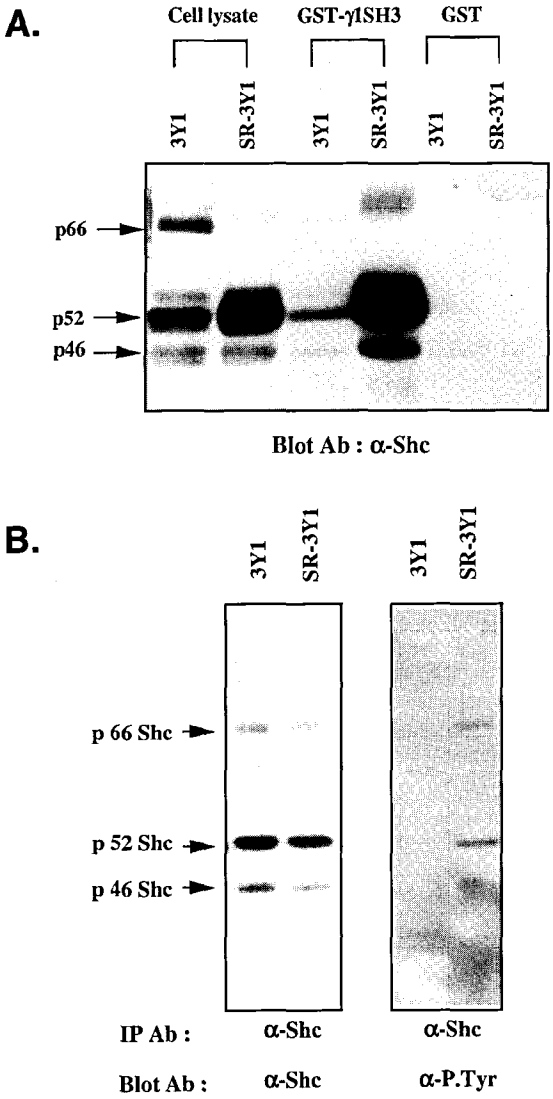

Fig. 2. A. *v-Src*'s involvement in the association between Shc and the SH3 domain of PLC-γ1. Serum-starved SR-3Y1 cells and serum-starved normal 3Y1 cells were lysed. Cell lysates (500 μg) were reacted with 5 μg of GST or GST-γ1SH3. Bound proteins were analyzed on Western blots probed with anti-Shc polyclonal antibody. B. Comparison of tyrosyl phosphorylation of Shc in *v-Src* transformed 3Y1 and normal 3Y1 cells. SR-3Y1 and 3Y1 cells were serum-starved for 18 h in serum-free medium. Cell lysates (500 μg) were immunoprecipitated with anti-Shc monoclonal antibody and Western blotted. The blot was probed with anti-Shc monoclonal antibody. It was then stripped and re-probed with anti-PY antibody (4G10).

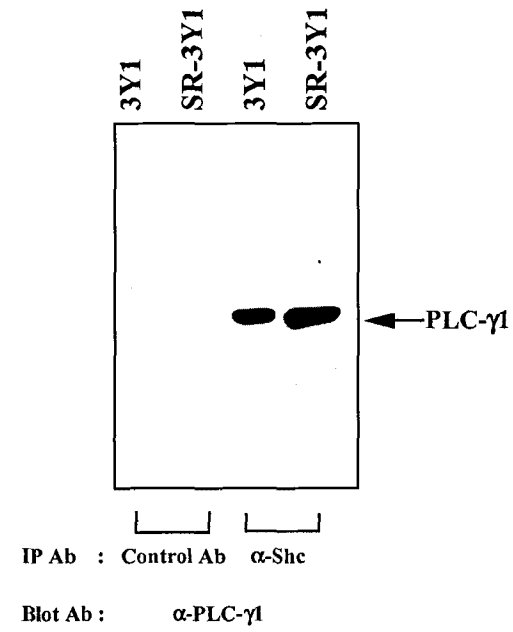

Fig. 3. Increased association between PLC-γ1 and Shc *in vivo* in *v-Src* transformed cells. 3Y1 cells and SR-3Y1 cells cultured in complete medium were lysed and equal amounts of cellular protein (500 μg) were immunoreacted with anti-Shc monoclonal antibody or mouse pre-immune serum. Immunoprecipitates were resolved by SDS-PAGE, blotted, and analyzed with anti-PLC-γ1 monoclonal antibody (F7). The position of PLC-γ1 is indicated.

performed co-immunoprecipitation experiments using lysates of SR-3Y1 cells and 3Y1 cells cultured in complete media. As shown in Fig. 3, PLC-γ1 co-immunoprecipitated with Shc in lysates from both SR-3Y1 cells and 3Y1 cells. Although the level of expression of PLC-γ1 in SR-3Y1 was comparable to that in 3Y1 cells and the amount of Shc immunoprecipitated by anti-Shc antibody was not different between the two cell lines as shown in Fig. 2B, more PLC-γ1 co-precipitated with Shc in lysates of SR-3Y1 cells. These results, therefore, demonstrate that Shc does associate with PLC-γ1 *in vivo*, and that the *in vivo* association between Shc and PLC-γ1 is enhanced by *v-Src*-induced transformation.

EGF treatment also enhances association between Shc and the SH3 domain of PLC-γ1 *in vitro* Since Shc is being phosphorylated on tyrosine residues by growth factor receptor kinase as well as Src tyrosine kinase (McGlade *et al.*, 1992; Okada *et al.*, 1995), we tested whether activation of growth factor receptor kinases such as the EGF receptor could enhance the association of Shc with PLC-γ1. As shown in Fig. 4, in *in vitro* binding experiments using lysates of normal 3Y1 cells, EGF-stimulation enhanced the association between Shc and GST-γ1SH3 (Fig. 4, lower panel), while no activated EGF receptor was observed in this GST-γ1SH3 protein complex (Fig. 4, upper panel). Although three isoforms of Shc proteins (p66, p52, and p46) existed in the 3Y1 cells, the p52 Shc isoform showed the strongest association with the GST-γ1SH3 protein. The other two Shc isoforms (p66 and p46) exhibited only weak binding to the GST-γ1SH3 protein (Fig. 4, lower panel). Upon EGF-treatment, the three isoforms of the Shc proteins translocated to the particulate fraction. Therefore,

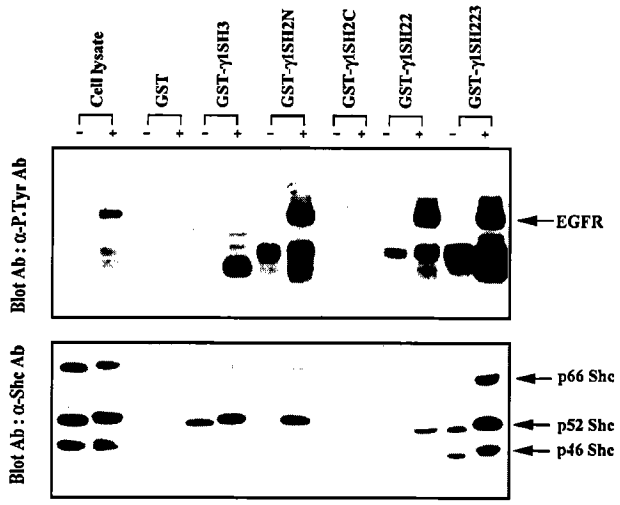

Fig. 4. EGF-stimulation enhances the association between Shc and the SH3 domain of PLC-γ1 in 3Y1 cells. 3Y1 cells were serum-starved for 18 h. Cells were then either left untreated or stimulated with 100 ng/ml EGF at 37°C for 2 min. Cells were solubilized in Triton X-100 lysis buffer. Cleared cell lysates (200 μg) were allowed to react with 5 μg of GST, GST-γ1SH223, GST-γ1SH22, GST-γ1SH2N, GST-γ1SH2C, or GST-γ1SH3. The bound proteins were then separated by SDS-PAGE (8%), and transferred to a nitrocellulose membrane. The high molecular weight portion of the nitrocellulose was probed with anti-PY monoclonal antibody (4G10) to detect tyrosyl phosphorylated EGF-receptors, while the lower molecular weights were probed with anti-Shc polyclonal antibody.

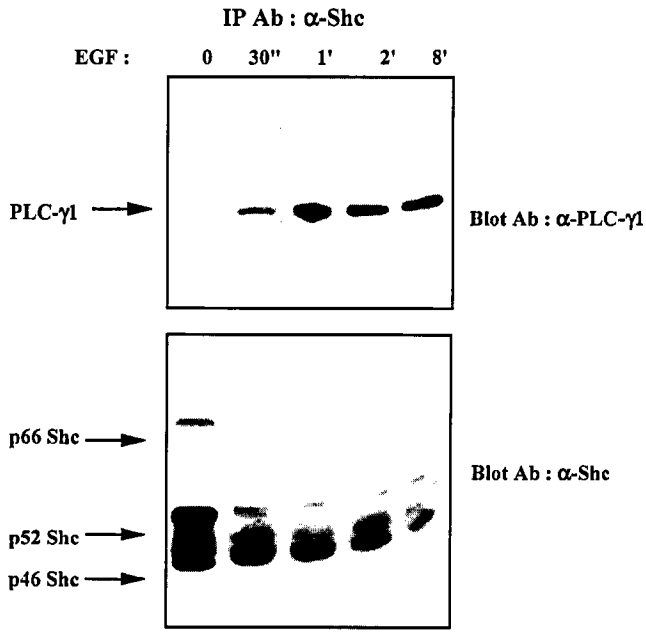

Fig. 5. EGF-dependent Shc association with PLC-γ1 *in vivo*. Serum-starved COS-7 cells were stimulated with EGF (100 ng/ml) for the indicated times. Cells were lysed, and equal amounts of cellular protein (500 μg) were immunoreacted with mouse anti-Shc monoclonal antibody. The immune complexes were then separated by SDS-PAGE (8%) and transferred to a nitrocellulose membrane. The high molecular weight portion of the blot was then probed with anti-PLC-γ1 antibody (F7) to detect PLC-γ1 proteins co-immunoprecipitated with Shc, while the lower molecular weights were probed with anti-Shc polyclonal antibody. The three isoforms of Shc proteins and PLC-γ1 are indicated with arrows.

taken together with the data shown in Fig. 2, these results suggest that some modification of Shc, most possibly tyrosyl-phosphorylation of Shc, induced by EGF or *v-Src* tyrosine kinase, enhances the association between the SH3 domain of PLC-γ1 and Shc. Our data are also consistent with a previous report that only the N-terminal SH2 domains of PLC-γ1 mediate binding to activated EGF receptor (Gergel *et al.*, 1994) (Fig. 4, upper panel). The amount of Shc protein bound to GST-γ1SH2N, therefore, may represent the amount of Shc protein that originated from the EGF receptor-complex precipitated with this GST-fusion protein (Fig. 4, lower panel). On the other hand, GST-γ1SH2N may be bound with the phosphotyrosine residue of Shc. This phenomenon requires further study through the mutation of Shc.

Shc associates with PLC-γ1 in an EGF-dependent manner *in vivo* To investigate whether EGF-stimulation could affect the association of Shc with PLC-γ1 *in vivo*, we performed immunoprecipitation experiments with anti-Shc antibody using COS-7 cell lysates. As shown in Fig. 5, PLC-γ1 did co-immunoprecipitate with Shc. The amount of PLC-γ1 co-precipitated with Shc increased with EGF-stimulation.

The SH3 domain of PLC-γ1 is required for the association with Shc *in vivo* In order to demonstrate the involvement of the SH3 domain of PLC-γ1 in the association with Shc *in vivo*, we transiently expressed FLAG-tagged wild-type PLC-γ1 (pFLAG-WT transfection) or a mutant PLC-γ1 lacking the SH3 domain (pFLAG-ΔSH3 transfection) in COS-7 cells. Transfected cells were serum-starved and treated with EGF. Cells were lysed and immunoprecipitated with FLAG-M2 agarose. Among the three isoforms of Shc, the p52 Shc isoform showed the strongest association with wild-type PLC-γ1 upon EGF-stimulation. In response to the EGF-stimulation, most of the Shc proteins co-precipitated with FLAG-tagged wild-type PLC-γ1 showed an electrophoretic mobility-shift. Around 50% of p52 Shc in the cell lysate was co-immunoprecipitated with FLAG-tagged wild-type PLC-γ1. On the other hand, about 5% of p52 Shc in the cell lysate was precipitated with PLC-γ1 lacking the SH3 domain. The deletion of the SH3 domain in PLC-γ1 did not affect the ability to associate with activated EGF receptor (Fig. 6). These results demonstrate that the SH3 domain of PLC-γ1 is required for the association with Shc in intact cells.

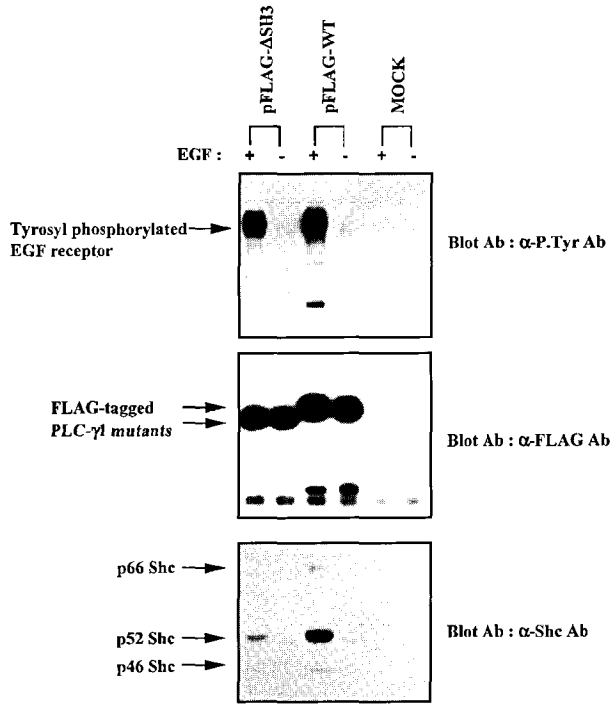

Fig. 6. The SH3 domain of PLC-γ1 is required for the association with Shc *in vivo*. COS-7 cells were transfected with either the vector (Mock), pFLAG-WT, or pFLAG-ΔSH3. Transfected cells were either stimulated with EGF (100 ng/ml) for 2 min or not treated. FLAG-tagged PLC-γ1 mutants were immunoprecipitated with anti-FLAG M2 agarose. The washed immunoprecipitates were then separated by SDS-PAGE (8%) and transferred to a nitrocellulose membrane. The high molecular weight portion of the nitrocellulose blot was probed with anti-PY antibody (4G10) to detect tyrosyl phosphorylated EGF receptor. Then this blot was stripped and re-probed with anti-FLAG (M5) antibody. The lower molecular weights were probed with anti-Shc polyclonal antibody.

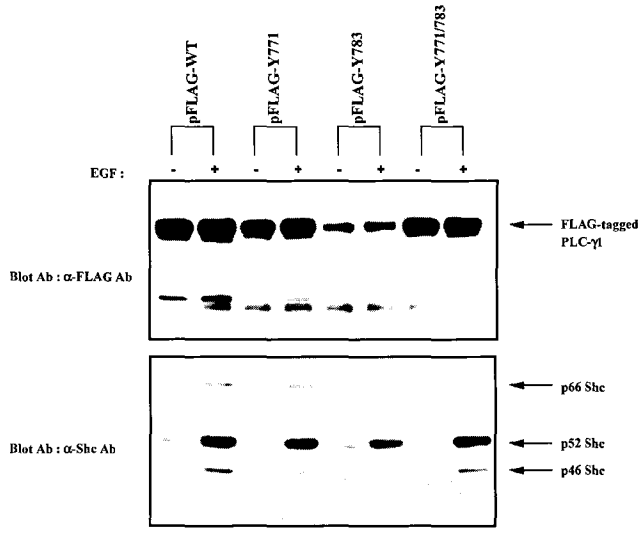

Fig. 7. Tyrosyl phosphorylation of PLC-γ1 is not needed for the association with Shc. COS-7 cells were transfected either with pFLAG-WT, pFLAG-Y771, pFLAG-Y783, or pFLAG-Y771/783. Transfected cells were either stimulated with EGF (100 ng/ml) for 2 min or not stimulated. FLAG-tagged PLC-γ1 mutants were immunoprecipitated with anti-FLAG M2 agarose. The washed immunoprecipitates were then separated by SDS-PAGE (8%) and transferred to a nitrocellulose membrane. The high molecular weight portion of the nitrocellulose blot was then probed with anti-FLAG antibody (M5) to detect FLAG-tagged PLC-γ1 proteins, while the lower molecular weights were probed with anti-Shc polyclonal antibody.

Tyrosyl-phosphorylation of PLC-γ1 is not needed for the association with Shc *in vivo* Previously, it had been known that residues Y771 and Y783 in PLC-γ1 can be phosphorylated by an activated EGF receptor (Kim *et al.*, 1991). It is believed that the tyrosyl phosphorylation of these residues may up-regulate the enzymatic activity of PLC-γ1 (Kamat and Carpenter, 1997). The Y771 and Y783 residues are located in the immediate upstream region of the SH3 domain of PLC-γ1. To investigate the involvement of tyrosyl phosphorylation of PLC-γ1 in the association with Shc, we made an additional three FLAG epitope-tagged mammalian expression vectors of PLC-γ1 mutants (pFLAG-Y771, pFLAG-Y783, and pFLAG-Y771/Y783), which contain phenylalanine(s) instead of tyrosine 771 and/or tyrosine 783. We transfected these mutants into COS-7 cells. After serum-starvation, transfected cells were stimulated with EGF and subsequently immunoprecipitated with anti-FLAG antibody. As shown in Fig. 7, all three FLAG-tagged PLC-γ1 mutants could bind to Shc upon EGF-stimulation. They exhibited similar binding abilities to Shc as wild-type PLC-γ1. Therefore, we suggest that tyrosyl phosphorylation of PLC-γ1 is not needed for association with Shc *in vivo*.

Discussion

In this report, we showed that the SH3 domain of PLC-γ1 associates with Shc, which has been known to be involved in the activation of p21Ras in response to many growth factors such as epidermal growth factor, nerve growth factor, and platelet-derived growth factor (Okada *et al.*, 1995; Bonfini *et al.*, 1996).

PLC-γ1 has been studied for the last decade in the view of its enzymatic role in that it hydrolyzes PIP_2 to generate two second messengers, IP_3 and DAG, which are responsible for the activation of protein kinase C and the increase of the level of intracellular calcium, respectively (Rhee *et al.*, 1989; Rhee and Choi, 1992; Noh *et al.*, 1995). However, a recent report that lipase-deficient PLC-γ1 can induce DNA synthesis raised the possibility that PLC-γ1 can also play a role as an adaptor which relays a signal from activated receptor tyrosine kinase to downstream

signaling molecules by protein–protein interaction mediated by its Src homology domain (Smith *et al.*, 1989). Recently, the SH3 of PLC-γ1 has been identified as a region responsible for the induction of DNA synthesis (Huang *et al.*, 1995; Smith *et al.*, 1996).

Nonetheless, little is known about possible effector proteins that can bind to the SH3 domain of PLC-γ1. Therefore, we tried to find proteins that can specifically associate with the SH3 domain of PLC-γ1 and that can possibly mediate the mitogenic effect of PLC-γ1. First, we examined whether the proteins that previously have been reported to be involved in the mediation of growth factor-stimulated cellular proliferation can associate with the PLC-γ1 SH3 domain. Among many signaling molecules, Shc was candidated as a possible associating protein of the PLC-γ1 SH3 domain, since Shc is known as a key mediator that can link many kinds of activated receptor tyrosine kinase to p21Ras (Bonfini *et al.*, 1996). Indeed, Shc was proven to associate with the SH3 domain of PLC-γ1. The association between Shc and PLC-γ1 was potentiated either by the activation of EGFR or *v-Src*-induced transformation *in vivo* and *in vitro*.

Recently, Roche *et al.* (1996) showed that PDGF-induced c-Fos expression, a p21Ras-dependent event important for S-phase entry, can be inhibited by 80% by introducing dominant negative forms of PLC-γ1 (GST-fused SH2 domain of PLC-γ1 or anti-PLC-γ1 antibody raised against SH2 domains) into NIH 3T3 fibroblast cells. Furthermore, 3Y1 rat fibroblast cells overexpressing either wild-type PLC-γ1 or the SH2-SH2-SH3 domain of PLC-γ1 showed comparatively elevated p21Ras activity compared to vector transfected 3Y1 cells (Chang *et al.*, unpublished data). Therefore, it will be of great importance to study the possibility that the PLC-γ1 SH3-induced mitogenic effect could be mediated by the p21Ras signaling pathway .

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