Direct Interaction of SOS1 Ras Exchange Protein with the SH3 Domain of Phospholipase C-γ1[†]

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Received November 5, 1999; Revised Manuscript Received March 20, 2000

ABSTRACT: A recent report that microinjection of the SH3 domain of PLC-γ1 could induce DNA synthesis raised the functional importance of the SH3 domain of PLC-γ1 in mitogenic signaling. In this report, we provide evidence that SOS1, a p21Ras-specific guanine nucleotide exchange factor, directly binds to the SH3 domain of PLC-γ1, and that the SH3 domain of PLC-γ1 is involved in SOS1-mediated p21Ras activation. SOS1 was coprecipitated with the GST-fused SH3 domain of PLC-γ1 in vitro. The interaction between SOS1 and the PLC-γ1 SH3 domain is mediated by direct physical interaction. The carboxyl-terminal proline-rich domain of SOS1 is involved in the interaction with the PLC-γ1 SH3 domain. Moreover, PLC-γ1 could be co-immunoprecipitated with SOS1 antibody in cell lysates. From transient expression studies, we could demonstrate that the SH3 domain of PLC-γ1 is necessary for the association with SOS1 in vivo. Intriguingly, overexpression of the SH3 domain of PLC-γ1, lipase-inactive PLC-γ1, or wild-type PLC-γ1 elevated p21Ras activity and ERK activity when compared with vector transfected cells. The PLC-γ1 mutant lacking the SH3 domain could not activate p21Ras. p21Ras activities in cell lines overexpressing either PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1 were elevated about 2-fold compared to vector transfected cells. This study is the first to demonstrate that the PLC-γ1 SH3 domain enhances p21Ras activity, and that the SH3 domain of PLC-γ1 may be involved in the SOS1-mediated signaling pathway.

Phosphoinositide-specific phospholipase C (PLC)[1] plays a pivotal role in transmembrane signaling. In response to various extracellular stimuli, such as numerous hormones, growth factors, and neurotransmitters, this enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), producing the two second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (*1*−*3*). There are multiple PLC isozymes, and despite low overall homology between the predicted amino acid sequences, significant sequence similarity is apparent in two domains which are designated the X- and Y-domain. On the basis of the relative locations of the X- and Y-domains in the primary structure of PLC, PLC isozymes are classified into three types: β, γ, and δ (*4*−*6*). Two types of PLC-γ, PLC-γ1 and -γ2, but not the β- and δ-type isozymes, are activated through phosphorylation of the growth factor receptor tyrosine kinases or nonreceptor tyrosine kinases (*6*).

PLC-γ1 is involved in cellular proliferation. It has been reported that the level of expression of PLC-γ1 is increased in human colorectal cancer (*7*), human breast carcinoma (*8*), familial adenomatous polyposis (*9*), and human skin in hyperproliferative conditions (*10*). Smith et al. (*11*, *12*) reported that microinjection of purified PLC-γ1 into quiescent NIH 3T3 cells results in the induction of DNA synthesis, and that the injection of anti-PLC-γ1 antibodies into the same cells blocks serum-induced DNA synthesis. Recent studies have shown that PLC-γ1 mutant lacking the lipase activity also induce DNA synthesis, implying that regions other than the catalytic domain may be responsible for the mitogenic effect (*13*, *14*). Among multiple PLC isozymes, only PLC-γ isozymes contain a Src-homology domain (SH domain): two SH2 domains and one SH3 domain (*4*−*6*, *15*). These domains have been found in a number of proteins involved

[†] This work was supported in part by the Korean Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling Research at Ewha Woman's University (KOSEF Grant 98-0403-08-01-5) and by the Brain Science & Engineering Research Program (Grant 98-J04-02-01-A-03) of the Ministry of Science and Technology in Korea. M.J.K. was supported by a Creative Research Initiative program grant from the Ministry of Science and Technology in Korea.

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[1] Abbreviations: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; BCS, bovine calf serum; SH, Src homology; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GST, glutathione *S*-transferase; kDa, kilodalton(s); aa, amino acid(s); PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH2, src homology 2; SH3, src homology 3; (h)SOS, (human) son of sevenless; SR-3Y1, *v-Src* transformed 3Y1.

10.1021/bi992558t CCC: $19.00
Published on Web 06/27/2000

in the regulation of cell proliferation and differentiation (*16*). The SH2 domains of PLC-γ1 are known to mediate the association between PLC-γ1 and phosphorylated tyrosine residues on the activated receptor tyrosine kinase or src tyrosine kinase (*17*). The SH3 domain of PLC-γ1 is known to be responsible for the mitogenic effect of PLC-γ1 (*14, 18*); namely, microinjection of the GST-fused SH3 domain of PLC-γ1 into G_0 growth-arrested NIH 3T3 cells has been reported to induce a mitogenic response (*14, 18*). Recently, we also observed that overexpression of the SH2−SH2−SH3 domain of PLC-γ1 in 3Y1 rat fibroblast can induce cellular transformation and that 3Y1 cells overexpressing the SH2−SH2−SH3 domain of PLC-γ1 are tumorigenic when transplanted into nude mice (*19*). Until now, however, little was known about the putative effector proteins that bind to the PLC-γ1 SH3 domain in vivo, and those that can mediate the mitogenic activity of the PLC-γ1 SH3 domain.

To identify proteins that interact with the SH3 domain of PLC-γ1, we prepared various glutathione *S*-transferase (GST)-fused SH domains of PLC-γ1 and used these fusion proteins in in vitro binding experiments. Here we demonstrate that SOS1, a p21Ras-specific guanine nucleotide exchange factor (*20, 24*−*27*), directly binds to the SH3 domain of PLC-γ1, and that SOS1-mediated signaling is enhanced by the SH3 domain of PLC-γ1.

EXPERIMENTAL PROCEDURES

Materials. Rabbit polyclonal anti-ERK-1/2 antibody (#06-182) was purchased from UBI (Lake Placid, NY); mouse monoclonal anti-GST antibody (catalog #sc-138) and rabbit polyclonal anti-SOS antibody (catalog #sc-259) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); 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-phospho-ERK antibody was purchased from NEB. Mouse monoclonal anti-PLC-γ1 antibody (F7) was prepared as previously described (*21*). Glutathione−agarose and protein A−agarose were purchased from Pharmacia Biotech (Piscataway, NY). PMSF, leupeptin, pepstatin A, and aprotinin were obtained from Boehringer Mannheim (Mannhein, Germany). Fetal calf serum and bovine calf serum were purchased from HyClone (Logan, UT).

Cell Culture. Rat embryonic fibroblast 3Y1 cells and *v-Src* transformed 3Y1 cells (SR-3Y1 cells) were cultured at 37 °C in a humidified 5% CO_2 atmosphere in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. COS-7 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. Vector transfected 3Y1 cell and 3Y1 cells expressing either wild-type PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1 were previously described (*19*).

Plasmid Construction. To generate glutathione *S*-transferase (GST) fusion proteins containing the src homology (SH) domains of PLC-γ1, cDNAs encoding the SH domains were amplified by PCR using rat PLC-γ1 cDNA (*15*) as the template. The following primer pairs were used: GST-γ1SH223:

forward primer: 5′-ACTGGATCCCCGAAGGAGG-CCAGTGGCAGC-3′

reverse primer: 5′-AGTCCCGGGATGCTCCCTCTC-CGGCTCTAG-3′

GST-γ1SH22:

forward primer: 5′-ACTGGATCCCCGAAGGAGGC-CAGTGGCAGC-3′

reverse primer: 5′-AGTCCCGGGGTTGCGGCCCTC-ATACAGTGC-3′

GST-γ1SH2N:

forward primer: 5′-ACTGGATCCCCGAAGGAGGC-CAGTGGCAGC-3′

reverse primer: 5 ′-AGTCCCGGGCTCTTTGCTCTC-ATGGGCATT-3′

GST-γ1SH2C:

forward primer: 5′-ACTGGATCCATGCGCCTTTCA-GAGCCTGTT-3′

reverse primer: 5′-AGTCCCGGGGTTGCGGCCCTC-ATACAGTGC-3′

GST-γ1SH3:

forward primer: 5′-ACTGGATCCAAGATTGGGAC-AGCTGAACCC-3′

reverse primer: 5′-AGTCCCGGGATGCTCCCTCTC-CGGCTCTAG-3′

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 vector (Pharmacia Biotech, Sweden). The mammalian expression vector for FLAG-epitope-tagged PLC-γ1 (pFLAG-WT) was made by a polymerase chain reaction. The amplified products were inserted in-frame with the FLAG-epitope tag of pFLAG-CMV-2 (Eastman Kodak). The mammalian expression vector for PLC-γ1 lacking the SH3 domain (pFLAG-ΔSH3, lacking the region corresponding to amino acid residues 796−902) was constructed by polymerase chain reaction. The DNA fragment encoding the SH3 domain of rat PLC-γ1 was isolated by digesting GST-γ1SH3 with *Bam*HI and *Sma*I. Isolated insert was subcloned into the *Bam*HI/*Sma*I site of pFLAG-CMV2 vector and named pFLAG-γ1SH3. For the construction of lipase-inactive mutant of PLC-γ1 (H335Q), two-step PCR amplification of mutant DNA oligos containing a transversion from T to A was used. Primers used were as follows:

5′-CTTCCTCGCAAAATACGTATC-3′

5′-GATACGTATTTTGCGAGGAAG-3′

An amplified PCR product was digested with *Mlu*I and *Bst*EII. A FLAG-epitope-tagged mammalian expression vector for lipase-inactive PLC-γ1 was made by replacing the digested PCR fragment with the corresponding region in pFLAG-WT, and named pFLAG-lipase-inactive. To confirm that this mutant PLC-γ1 is lipase-inactive, we overexpressed this mutant PLC-γ1 into COS-7 cells, and we isolated mutant PLC-γ1 by immunoprecipitation using anti-

FLAG antibody. By performing an in vitro PLC assay (*31*), we could confirm that this mutant PLC-γ1 has no PIP_2-hydrolyzing activity (data not shown). The mammalian expression vector for human SOS1 (pEXV3-EEhSOS1) was a gift from E. Porfiri (ONYX Pharmaceuticals) (*22*). GST-fused SOS1 constructs were made by PCR amplification using pEXV3-EEhSOS1 as the template.

The following primer pairs were used: GST-SOS-N (amino acid residues in hSOS1: 1−309):

forward primer: 5′-GCTGCCCGGGATGCAGGAGC-AGCAGCTG-3′

reverse primer: 5′-GCCAGTCGACCGATCATGAAA-ACCAGGTCG-3′

GST-SOS-Dbl-PH (amino acid residues in hSOS1: 285−616):

forward primer: 5′-GCTGCCCGGGGACTTAGCAG-AGGAACTGGC-3′

reverse primer: 5′-GCCAGTCGACATATGGTACGT-AAGCCTCTC-3′

GST-SOS-GRF (amino acid residues in hSOS1: 576−1027):

forward primer: 5′-GCTGCCCGGGAGATTTGCAG-AGCCTGACTC-3′

reverse primer: 5′-GCCAGTCGACGAAATCTTGG-GAGAGGCTTA-3′

GST-SOS-C (amino acid residues in hSOS1: 1013−1333):

forward primer: 5′-GCTGCCCGGGTCCCTAGAAA-TAGAACCACG-3′

reverse primer: 5′-GCCAGTCGACTCAGGAAGAA-TGGGCATTCT-3′

Underlined nucleotides indicate the *Sma*I and *Sal*I sites in the forward and reverse primers, respectively. Amplified products were digested with *Sma*I and *Sal*I and then subcloned into pGEX-4T2 (Pharmacia Biotech).

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 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 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 μM pepstatin A, and then lysed by sonication and clarified by centrifugation at 15000*g* for 20 min. The soluble fraction was incubated with glutathione-coated Sepharose beads (Pharmacia Biotech Inc.) 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 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 μM pepstatin A).

In Vitro Binding Experiments. Clarified lysates (500 μg) of 3Y1, SR-3Y1, or COS-7 cells were incubated with 5 μg of GST-fusion proteins 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 4 times with Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 20 mM NaF, 200 μM sodium orthovanadate, 1 mM PMSF, 1 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 μM 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. SOS1 was immunoblotted using rabbit anti-SOS antibody. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and ECL according to the manufacturer's instructions.

Far-Western Analysis. Confluent COS-7 cells in complete medium (10% BCS−DMEM) were washed with PBS and then lysed in Triton X-100 lysis buffer. Cleared cell lysates were allowed to immunoreact with rabbit polyclonal anti-SOS antibody. The resulting immunoprecipitates were separated by SDS−PAGE on an 8% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in blocking buffer [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20, 5% skimmed milk powder] for 2 h and then incubated with 2 μg/mL GST-fusion protein in blocking buffer for 1 h. After the membrane was rinsed with blocking buffer, bound GST-fusion proteins were detected by immunoprobing the membrane with anti-GST monoclonal antibody followed by peroxidase-conjugated goat anti-mouse immunoglobulin antibody and ECL.

Coimmunoprecipitation 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 then treated with 100 ng/mL EGF and lysed in 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, 200 μM sodium orthovanadate, 1 mM PMSF, 1 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 μM pepstatin A). The cell lysates were clarified by centrifugation at 14000*g* for 15 min at 4 °C. The clarified lysates were precleared with 30 μL of 50% protein A−Sepharose for 30 min at 4 °C. The clarified cell lysates were mixed with 5 μg of rabbit anti-SOS polyclonal antibody precoupled to protein A−Sepharose for 3 h at 4 °C. The immune complexes were collected by centrifugation (4 min at 3000*g*), washed 4 times with cold Triton X-100 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-PLC-γ1 monoclonal antibody (F7) 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) (Amersham Corp.).

Transfections in COS-7 Cells. COS-7 cells (ATCC, Fairfax, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum at 37 °C in 95% air, 5% CO_2. Cells were seeded at 2×10^5 cells/35 mm well 24 h before transfection using liposome-mediated transfection. LipofectAMINE (Life Technologies, Inc.) was used for the transfection. We followed the manufacturer's instructions using 1 μg of plasmid DNA and 6 μL 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 (Figures 5 and 6), transfected cells were serum-starved for 18 h at 30 h post-transfection.

Ras Activity Assay (RBD Binding Assay) and ERK Activity Assay. COS-7 cells were transfected with vector, pFLAG-WT, pFLAG-γ1SH3, or pFLAG-lipase-inactive, in a 100 mm dish by using LipofectAMINE (Life Technologies, Inc.). After 48 h, transfected COS-7 cells were stimulated with 10% fetal calf serum for 5 min, and lysed in MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/mL leupeptin, 10 μg/mL aprotinin). Resulting cell lysates were clarified by centrifugation at 14000*g* for 15 min. Then 500 μg of clarified cell lysates was incubated with 5 μg of purified GST-Raf-1-RBD proteins (RBD, residues 1−149 of human Raf-1) for 30 min at 4 °C (*23*). Bound protein complexes were subjected to 15% SDS−PAGE and transferred onto nitrocellulose paper. Coprecipitated p21ras was analyzed with immunoblot analysis using rat monoclonal anti-p21ras antibody Y13-Z5P from Calbiochem. Expression of transfected wild-type PLC-γ1 and lipase-inactive PLC-γ1 was analyzed with immunoblotting using anti-PLC-γ1 antibody (F7), and the expression of the FLAG-epitope-tagged SH3 domain of PLC-γ1 was detected with anti-FLAG antibody (M5). To check the effect on ERK activity, clarified cell lysates (20 μg) were loaded onto 10% SDS−PAGE, and then transferred onto nitrocellulose. The blot was immunoprobed with mouse monoclonal anti-phospho ERK-1, -2 antibody from NEB. Then this blot was striped and reprobed with rabbit polyclonal anti-ERK-1/2 antibody from UBI to detect ERK-1 and ERK-2.

In Vivo GTP/GDP Binding Assays (Ras Activity). Vector transfected 3Y1 cell and 3Y1 cells expressing either wild-type PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1 were metabolically labeled with [^{32}P]orthophosphate (1 mCi/mL) in phosphate-free, serum-free medium for 8 h and lysed. Ras proteins were immunoprecipitated with the anti-v-H-ras(Ab-1) antibody (Calbiochem) mAb Y13-259, and guanine nucleotides bound to Ras proteins were eluted and fractionated by thin-layer chromatography. The percentage of total Ras protein bound to GTP was calculated according to the following formula: % GTP = cpm in GTP/(cpm in GTP + cpm in GDP), using cpm normalized for moles of phosphate.

RESULTS

SOS1 Is Coprecipitated with the GST-Fused SH3 Domain of PLC-γ1 in Vitro. To identify proteins that bind specifically to 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) (Figure 1). We used these GST-fusion proteins in in vitro binding experiments with lysates of 3Y1 and *v-Src* transformed 3Y1 (SR-3Y1) fibroblast cells. Because SOS1 has an SH3 domain binding motif in its carboxyl-terminal region and was known to be involved in the mitogenic signaling by activating p21Ras in response to many proliferative signals (*20, 24*−*27*), we considered SOS1 as a possible candidate for a binding protein to the PLC-γ1 SH3 domain. We thus tested whether SOS1, a p21Ras-specific guanine nucleotide exchange factor, could bind to the SH3 domain of PLC-γ1. As shown in Figure 2, regardless of EGF stimulation, most of the SOS1 proteins in 3Y1 cells (see upper panel of Figure 2A) or *v-Src* transformed 3Y1 (SR-3Y1) cells (see lower panel of Figure 2A) did bind to the GST-γ1SH3 fusion protein in vitro, whereas the SOS1 proteins did not interact with the two SH2 domains of PLC-γ1. Although SOS1 in SR-3Y1 cells exhibited lower electrophoretic mobility as compared to that in normal 3Y1 cells, *v-Src*-induced cellular transformation did not affect the in vitro binding activity between SOS1 and GST-γ1SH3 (Figure 2B). The binding affinity of PLC-γ1 SH3 and SOS1 was comparable to that of Grb2-N-SH3 and SOS1. SOS1 did not interact with the Abl SH3 domain (Figure 2C).

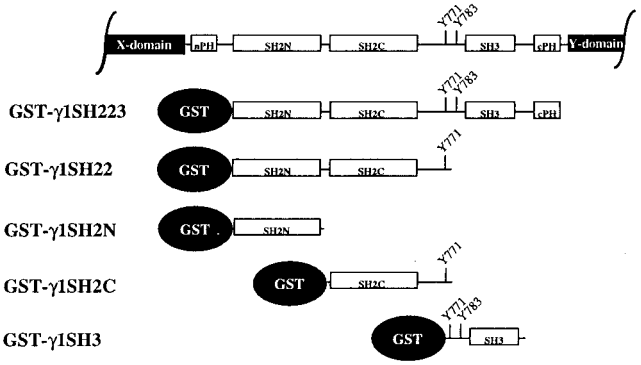

FIGURE 1: Constructs of various PLC-γ1 GST-fusion proteins. The locations of tyrosine residues neighboring the SH3 domain of PLC-γ1 which are known to be phosphorylated by activated growth factor PTK are indicated in the top diagram. The lower diagrams show the different portions of PLC-γ1 that were included in each of the GST-fusion protein constructs.

SOS1 Binds Directly to the SH3 Domain of PLC-γ1. To test whether SOS1 bound directly or indirectly to the SH3 domain of PLC-γ1, we examined the interaction in far-western blots as described under Experimental Procedures. SOS1 protein was specifically recognized by the GST-γ1SH3 fusion protein, but not by GST and the GST-γ1SH22 protein (data not shown). As shown in Figure 3, GST and the Abl SH3 domain did not interact with SOS1, and the interaction between the PLC-γ1 SH3 domain and SOS1 was comparable to that of the Grb2-N-SH3 domains and SOS1. Therefore, these results suggest that SOS1 binds directly to the SH3 domain of PLC-γ1.

The Carboxyl-Terminal Proline-Rich Domain of SOS1 Is Involved in the Interaction with PLC-γ1. To map the region on the SOS1 protein that is responsible for the interaction with the PLC-γ1 SH3 domain, we prepared four GST-fused human SOS1 proteins [GST-SOS-N (aa residues 1−309), GST-SOS-Dbl-PH (aa residues 285−616), GST-SOS-GRF (aa residues 576−1027), and GST-SOS-C (aa residues 1013−1333)], and used them in in vitro binding experiments. As shown in Figure 4, only the GST-SOS-C fusion protein containing the proline-rich tract specifically precipitated PLC-γ1. Thus, these data suggest that the carboxyl-terminal proline-rich domain of SOS1 might be involved in the interaction with PLC-γ1.

SOS1 Interacts with PLC-γ1 in Vivo. To investigate whether endogenous SOS1 proteins interact with PLC-γ1 in intact cells, we performed coimmunoprecipitation experiments using anti-SOS antibody. As shown in Figure 5, PLC-

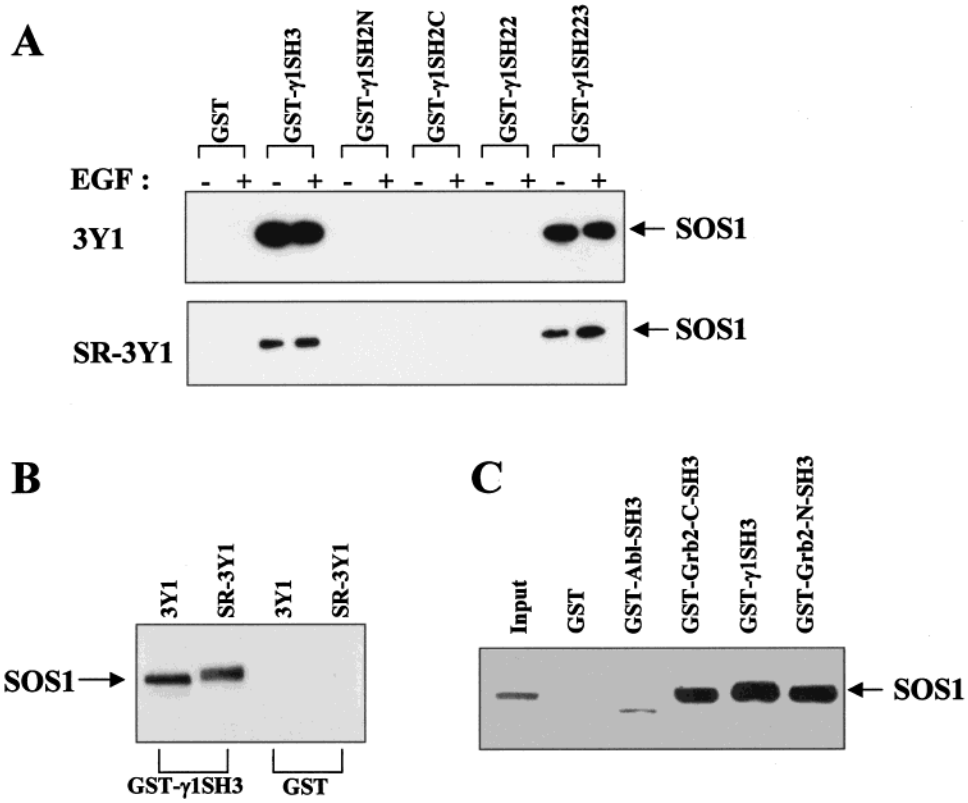

FIGURE 2: (Upper panel in A) The SH3 domain of PLC-γ1 in association with SOS1 in 3Y1. Triton X-100 lysates of 3Y1 cells treated or not treated with EGF (100 ng/mL) for 2 min were reacted with 5 μg of GST, GST-γ1SH223, GST-γ1SH22, GST-γ1SH2N, GST-γ1SH2C, or GST-γ1SH3. Bound proteins were analyzed on an immunoblot probed with polyclonal anti-SOS antibody. (Lower panel in A) The SH3 domain of PLC-γ1 in association with SOS1 in SR-3Y1 (*v-src* transformed 3Y1 cell). Triton X-100 lysates of SR-3Y1 cells treated or not treated with EGF (100 ng/mL) for 2 min were reacted 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 polyclonal anti-SOS antibody. (B) *v-Src*'s involvement in the association between SOS1 and the SH3 domain of PLC-γ1. SR-3Y1 cells and 3Y1 cells were grown until confluence and then lysed. Cell lysates (500 μg) were reacted with 5 μg of GST or GST-γ1SH3. Bound proteins were analyzed by immunoblot analysis using polyclonal anti-SOS antibody. (C) Interaction between SH3 domains and SOS1. 3Y1 cell lysates were incubated with GST, GST-Abl-SH3, GST-Grb2-C-SH3 (C-terminal SH3 domain of Grb2), GST-γ1SH3, or GST-Grb2-N-SH3 (N-terminal SH3 domain of Grb2). Bound proteins were analyzed by immunoblot analysis using polyclonal anti-SOS antibody. Input lane represents 5% of cell lysate used in in vitro binding experiment.

γ1 was coimmunoprecipitated with anti-SOS antibody in resting and EGF-stimulated cells. The amount of PLC-γ1 coprecipitated with SOS1 was slightly increased upon EGF-stimulation. About 5% of cellular PLC-γ1 was coprecipitated with SOS1 (data not shown). This result indicates that PLC-γ1 interacts with SOS1 in vivo.

The SH3 Domain of PLC-γ1 Is Required for the Association with SOS1 in Vivo. To further demonstrate the involvement of the SH3 domain of PLC-γ1 in the direct interaction with SOS1 in intact cells, we transiently expressed FLAG-tagged wild-type PLC-γ1 (pFLAG-WT transfection) or a mutant PLC-γ1 lacking the SH3 domain (pFLAG-ΔSH3 transfection) into COS-7 cells. After serum-starvation, transfected cells were either left untreated or stimulated with EGF. The cells were lysed and immunoreacted with FLAG-antibody, and then immune complexes were analyzed with immunoblot analysis using FLAG-antibody and SOS1 antibody. As shown in Figure 6, the SOS1 protein was coprecipitated with FLAG-tagged wild-type PLC-γ1. About 10% of cellular SOS1 was coprecipitated with FLAG-tagged wild-type PLC-γ1 (data not shown). The deletion of the SH3 domain from PLC-γ1 abolished the interaction with SOS1. This result shows that the SH3 domain of PLC-γ1 is necessary for the interaction with SOS1 in vivo.

The SH3 Domains of PLC-γ1 and Lipase-Inactive PLC-γ1 Mutant Elevate p21Ras Activity. Since SOS1 is a well-known activator of p21Ras and micro-injected SH3 domain of PLC-γ1 can induce DNA synthesis, we checked whether the SH3 domain of PLC-γ1 can affect p21Ras activity. Previously, many researchers have reported that the mitogenic effect of PLC-γ1 originates from its SH3 domain and is independent of its PIP_2-hydrolyzing activity. In addition, SOS1 was identified as a binding protein of the SH3 domain of PLC-γ1 in this study. Therefore, we tested the possibility that the SH3 domain of PLC-γ1 may induce p21Ras activiation. We transiently overexpressed the SH3 domain of PLC-γ1, lipase-inactive PLC-γ1, or wild-type PLC-γ1 in COS-7 cells. The p21Ras activities in transfected COS-7 cell were analyzed by using GST-Raf-1-RBD protein (*23*). As shown in Figure 7A, transient overexpression of the SH3 domain of PLC-γ1, lipase-inactive PLC-γ1, or wild-type PLC-γ1 elevated p21Ras activity when compared with vector transfected cell. To further confirm that PLC-γ1 SH3 is responsible for the activation of p21Ras, we used PLC-γ1 mutant lacking the SH3 domain. As shown in Figure 7C, PLC-γ1 mutant lacking the SH3 domain could not activate p21Ras activity. Transient overexpression of SOS1, wild-type PLC-γ1, or lipase-inactive PLC-γ1 could activate

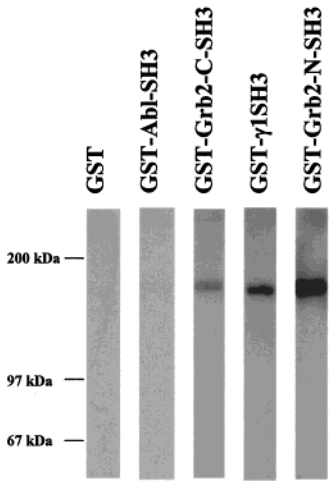

FIGURE 3: Direct interaction between SOS1 and the PLC-γ1 SH3 domain. COS-7 cells cultured in complete medium were lysed in RIPA buffer. Cell lysates were immunoprecipitated with anti-SOS polyclonal antibody. The immune complexes were then resolved by 8% SDS−PAGE and transferred onto a nitrocellulose filter. The resulting blot was overlaid with GST, GST-Abl-SH3, GST-Grb2-C-SH3, GST-γ1SH3, or GST-Grb2-N-SH3. Bound GST-fusion proteins were detected by immunoprobing the membrane with monoclonal anti-GST antibody followed by peroxidase-conjugated goat anti-mouse immunoglobulin antibody and ECL. Data are representative of two independent experiments.

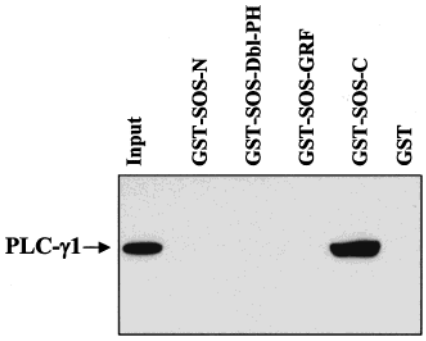

FIGURE 4: Carboxyl-terminal proline-rich tract of SOS1 binds to PLC-γ1. Triton X-100 lysates of COS-7 cells were incubated with GSH−agarose coupled with GST, GST-SOS-N, GST-SOS-Dbl-PH, GST-SOS-GRF, or GST-SOS-C. The agarose bead was washed extensively. Bound proteins were analyzed on immunoblots probed with monoclonal anti-PLC-γ1 antibody (F7). Input lane represents 20% of total cell lysate used in binding experiment. Data are representative of two independent experiments.

p21Ras. Therefore, these data suggest that PLC-γ1 may elevate p21Ras activity in a lipase-independent manner, and that the SH3 domain of PLC-γ1 may be involved in p21Ras activation by the interaction with SOS1.

Effects of the PLC-γ1SH3 Domain and Lipase-Inactive Mutant on ERK Activation. To further confirm that the PLC-γ1 SH3 domain and lipase-inactive PLC-γ1 can modulate p21Ras activity, we checked ERK activity, one of the key targets downstream of p21Ras signaling. As shown in Figure 7B, ERK activities were enhanced by either PLC-γ1 SH3 or the lipase-inactive mutant of PLC-γ1, as well as by wild-type PLC-γ1, suggesting that the SH3 domain of PLC-γ1 is involved in SOS1-mediated p21Ras activation and ERK activation. Although PLC-γ1 is known to be a key enzyme that hydrolyzes PIP_2 to generate two second messengers, IP_3 and DAG, in response to various growth factor stimuli, the data shown in Figure 7 suggest that the PLC-γ1 SH3 domain

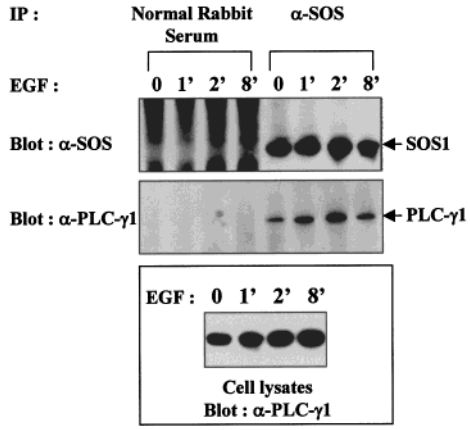

FIGURE 5: SOS1 interacts with PLC-γ1 in vivo. COS-7 cells were stimulated with EGF (100 ng/mL) for the indicated times, and then lysed. The resulting cell lysates were immunoprecipitated with normal rabbit serum or polyclonal anti-SOS antibody. The washed immunoprecipitates were then separated by SDS−PAGE (8%) and transferred to a nitrocellulose membrane. The nitrocellulose blot was then probed with anti-PLC-γ1 antibody (F7) to detect the PLC-γ1 that coprecipitated with SOS1. Afterward this blot was stripped and reprobed with anti-SOS antibody to detect immunoprecipitated SOS1. To show that equal amounts of cell lysates were used in immunoprecipitation, whole cell lysates were immunoblotted with anti-PLC-γ1 antibody (shown in inset). Similar results were obtained in three other experiments.

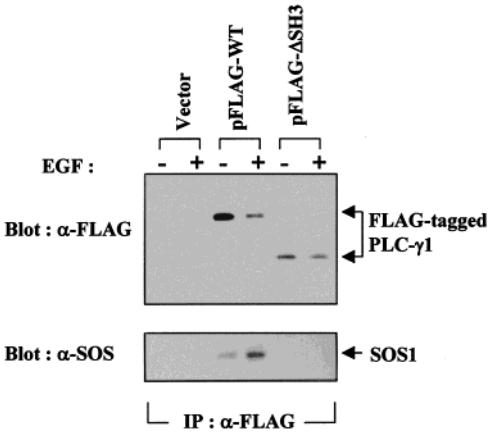

FIGURE 6: SH3 domain of PLC-γ1 is required for the association with SOS1 in vivo. COS-7 cells were transfected with vector (mock), pFLAG-WT, or pFLAG-ΔSH3. Transfected cells were either stimulated with EGF (100 ng/mL) for 2 min or not treated and then lysed. FLAG-tagged PLC-γ1 mutants were immunoprecipitated with anti-FLAG M2 agarose. The washed immunoprecipitates were then separated by SDS−PAGE (8%) and transferred to a nitrocellulose membrane. The blot was probed with anti-SOS antibody to detect the SOS1 coprecipitated with FLAG-tagged PLC-γ1. Later this blot was stripped and reprobed with anti-FLAG (M5) antibody. The data are representative of three independent experiments.

and the PLC-γ1 protein which has no catalytic activity play roles in p21Ras activation as an adapter molecule such as Grb2.

Effects of PLC-γ1 and the SH2−SH2−SH3 Domain on p21Ras Activity. To further confirm the data obtained from the transient expression study, we have measured p21Ras activities by using in vivo GTP/GDP binding assays in 3Y1 cell lines overexpressing either PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1. As shown in Figure 8, p21Ras activities in cell lines overexpressing either PLC-γ1 or the

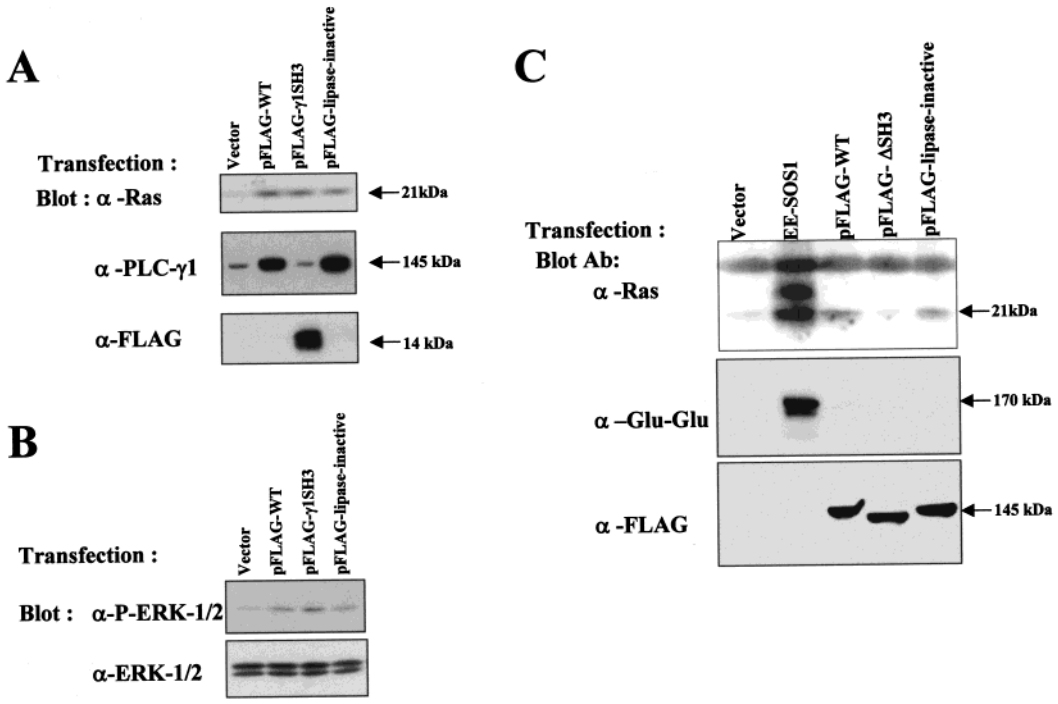

FIGURE 7: (A) Effects of the PLC-γ1SH3 domain and the lipase-inactive PLC-γ1 mutant on p21Ras activity. COS-7 cells were transfected with vector, pFLAG-WT, pFLAG-γ1SH3, or pFLAG-lipase-inactive. After 48 h, COS-7 cells were stimulated with 10% fetal calf serum and lysed with MLB buffer (see Experimental Procedures). 500 μg of clarified cell lysates was incubated with GST-Raf-1-RBD proteins. Coprecipitated p21Ras with GST-Raf-1-RBD protein was detected by immunoblot analysis using monoclonal anti-Ras antibody. Expression of transfected wild-type PLC-γ1 and lipase-inactive PLC-γ1 was analyzed with immunoblotting using anti-PLC-γ1 antibody (F7), and expression of the FLAG-epitope-tagged SH3 domain of PLC-γ1 was detected with anti-FLAG antibody (M5). (B) Effects of the PLC-γ1SH3 domain and the lipase-inactive mutant on ERK activity. Transfected cell lysates (20 μg) were subjected to immunoblot analysis with anti-phospho-ERK antibody (α-P-ERK-1/2 Ab). After stripping anti-phospho-ERK antibody, the membrane was reprobed with rabbit polyclonal anti-ERK-1/2 antibody (α-ERK-1/2 Ab). These data are representative of three independent experiments. (C) Effects of PLC-γ1 mutant lacking the SH3 domain on p21Ras activity. COS-7 cells were transfected with vector, EE-tagged SOS1, wild-type PLC-γ1, PLC-γ1 mutant lacking the SH3 domain, or lipase-inactive PLC-γ1. Transfected cells were lysed, and 1 mg of transfected cell lysates was subjected to the RBD binding assay (upper panel). To confirm expression of EE-tagged SOS1 and FLAG-tagged PLC-γ1 construct (wild-type PLC-γ1, PLC-γ1 lacking the SH3 domain, and lipase-inactive PLC-γ1), transfected cell lysates (20 μg) were subjected to immunoblot analysis using anti-Glu-Glu antibody (middle panel) or anti-FLAG antibody (bottom panel). These data are representative of three independent experiments.

SH2−SH2−SH3 domain of PLC-γ1 were elevated about 2-fold compared to vector transfected cells. Therefore, these data together with data in Figure 7 demonstrate that PLC-γ1 can activate p21Ras.

DISCUSSION

Although the SH3 domain of PLC-γ1 is known to induce a mitogenic response (*14, 18*), little is known about possible effector proteins. Thus, it has become an important issue to identify the mechanism by which the PLC-γ1 SH3 domain induces a mitogenic effect. Therefore, we set out to find a protein that could specifically bind to the SH3 domain of PLC-γ1 and that might possibly mediate PLC-γ1 SH3 domain-mediated mitogenic signaling. We first examined whether proteins involved in the activation of p21Ras could associate with the PLC-γ1 SH3 domain in vitro. Among many signaling molecules, SOS1 was a good candidate for a possible binding protein to the PLC-γ1 SH3 domain, since SOS1 was known to have the SH3 domain binding motif (*20, 24*−*27*). Indeed, SOS1 did associate with the SH3 domain of PLC-γ1 in vitro and in vivo.

Grb2, a major partner of SOS1, is known to mediate growth factor-induced p21Ras activation. Grb2 can dock through its SH2 domain with specific phosphotyrosine residues of the activated EGFR which subsequently allows relocalization of SOS1 to the plasma membrane (*20, 26, 32*). Here, we suggest that PLC-γ1 also participates in p21Ras activation through interaction with SOS1. SOS1, a p21Ras-specific guanine nucleotide exchange factor, was coprecipitated with the GST-fused SH3 domain of PLC-γ1 in vitro (Figure 2). The interaction between SOS1 and PLC-γ1 was mediated by a physical, direct interaction (Figure 3). Moreover, the carboxyl-terminal proline-rich domain of SOS1 was involved in the interaction with the PLC-γ1 SH3 domain (Figure 4), although a recent paper by Park et al. (*33*) showed that the proline-rich domain of SOS1 did not interact with the PLC-γ1 SH3 domain by using the yeast two-hybrid system. In our study, the binding between the PLC-γ1SH3 domain and SOS1 was comparable to that of Grb2 and SOS1 in a far-western assay and in vitro binding experiment. PLC-γ1 could be coimmunoprecipitated with anti-SOS antibody (Figures 5 and 6). The association between SOS1 and PLC-γ1 was influenced neither by the activation of EGF receptor nor by *v-Src*-induced transformation in vitro (Figure 2). These binding characteristics between SOS1 and PLC-γ1 are very similar to those between Grb2 and SOS1 (*20, 25*−*27*). Moreover, transient overexpressions of PLC-γ1, the PLC-γ1SH3 domain, or the lipase-inactive PLC-γ1 mutant resulted in elevation of the p21Ras activity and ERK activity (Figure 7A,B). PLC-γ1 mutant lacking the SH3

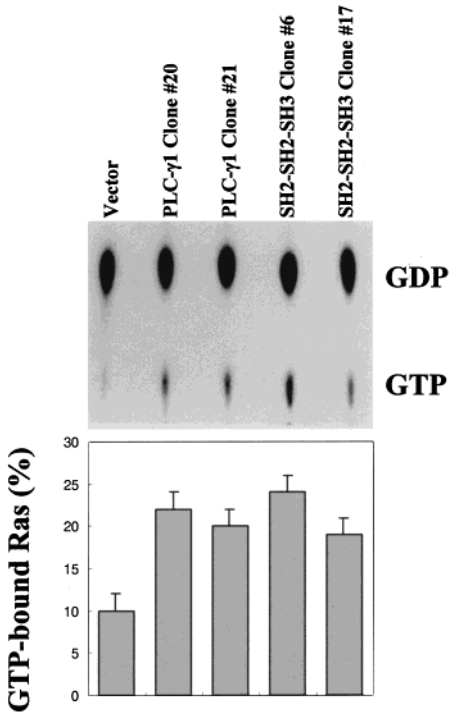

FIGURE 8: Effects of PLC-γ1 on p21Ras activity. Vector transfected 3Y1 cell and 3Y1 cells expressing either wild-type PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1 were metabolically labeled with [^{32}P]orthophosphate (1 mCi/mL) in phosphate-free, serum-free medium for 8 h and lysed. Ras proteins were immunoprecipitated with the anti-v-H-ras(Ab-1) antibody (Calbiochem) mAb Y13-259, and guanine nucleotides bound to Ras proteins were eluted and fractionated by thin-layer chromatography. The percentage of total Ras protein bound to GTP was calculated according to the following formula: % GTP = cpm in GTP/(cpm in GTP + cpm in GDP), using cpm normalized for moles of phosphate. These data are representative of four independent experiments.

domain could not activate p21Ras (Figure 7C). 3Y1 rat fibroblast cells that overexpress either wild-type PLC-γ1 or the SH2−SH2−SH3 domain of PLC-γ1 showed transformed phenotypes (*19*), and these cells showed considerably elevated p21Ras compared to vector transfected 3Y1 cells (Figure 8).

There are two possible hypotheses that could explain the mechanism by which the overexpression of the PLC-γ1 SH3 domain or lipase-inactive PLC-γ1 can enhance p21Ras activity and ERK activity. One is that PLC-γ1 SH3 domain overexpression breaks the equilibrium of the intracellular distribution of SOS1. It has been argued that the membrane localization of SOS1 proteins is critical for its exchange activity toward p21Ras (*28, 29*). However, overexpression of the PLC-γ1 SH3 domain or the lipase-inactive PLC-γ1 mutant did not change the amount of SOS1 which is located in the membrane fraction (data not shown). Therefore, it is very unlikely that the enhanced p21Ras activity during overexpression of the PLC-γ1 SH3 domain or the lipase-inactive PLC-γ1 mutant may be due to an altered intracellular localization of SOS1.

Another hypothesis is that overexpression of the PLC-γ1 SH3 domain or the lipase-inactive PLC-γ1 mutant may modulate the guanine nucleotide exchange activity of SOS1 on p21Ras. Recently, Corbalan-Garcia et al. (*30*) reported that the carboxyl-terminal region of SOS1 which is involved in the direct interaction with the Grb2 SH3 domain plays a role in the negative regulation of SOS1 activity by intramolecular interaction in vitro and in vivo. They found that truncation of the carboxyl-terminal region of SOS1 could increase the guanine nucleotide exchange activity of SOS1 on p21Ras in vitro and in vivo, resulting in enhanced p21Ras-signaling and -transforming activity of SOS1. They also observed that stable association of SOS1 with membrane is not essential for its activity and that transient interaction between SOS1 and Ras might be sufficient for its catalytic activity in vivo. Thus, the overexpression of the PLC-γ1 SH3 domain or lipase-inactive PLC-γ1 mutant may release the self-inhibitory effect of the carboxyl-terminal domain of SOS1, leading to enhancement of p21Ras activity and ERK activity.

In conclusion, we have identified that SOS1, one of the direct activators of p21Ras, is a binding protein of the SH3 domain of PLC-γ1, and that activation of p21Ras and ERK is enhanced when PLC-γ1SH3, the lipase-inactive PLC-γ1 mutant, or wild-type PLC-γ1 are overexpressed. We, therefore, suggest that the PLC-γ1 SH3 domain may be involved in the SOS1-mediated signal transduction.

ACKNOWLEDGMENT

We thank E. Porfiri (ONYX Pharmaceuticals) for the hSOS1expression vector, J. L. Bos (Utrecht University, The Netherlands) for the GST-Raf-1-RBD construct, Dr. B. K. Kay (University of North Carolina) for the GST-fused SH3 domain of Abl, and Grb2, and Ms. Gisela Hoscheck for proofreading the manuscript.

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BI992558T