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Siah Proteins Induce the Epidermal Growth Factor-dependent Degradation of Phospholipase Cε*

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Phospholipase Cε (PLCε) is activated by various growth factors or G-protein-coupled receptor ligands via different activation mechanisms. The Ras association (RA) domain of PLCε is known to be important for its ability to bind with Ras-family GTPase upon growth factor stimulation. In the present study, we identified Siah1 and Siah2 as novel binding partners of the PLCε RA domain. Both Siah1 and Siah2 interacted with the RA2 domain of PLCε, and the mutation of Lys-2186 of the PLCε RA2 domain abolished this association. Moreover, Siah induced the ubiquitination and degradation of PLCε upon epidermal growth factor (EGF) stimulation, and Siah proteins were phosphorylated on multiple tyrosine residues via an Src-dependent pathway upon EGF treatment. The Src inhibitor abolished the EGF-dependent ubiquitination of PLCε, and the Siah1 phosphorylation-deficient mutant could not increase the EGF-dependent ubiquitination and degradation of PLCε. The EGF-dependent degradation of PLCε was blocked in mouse embryonic fibroblast (MEF) cells derived from Siah1a/Siah2 double knockout mice, and the extrinsic expression of wild-type Siah1 restored the degradation of PLCε, whereas the phosphorylation-deficient mutant did not. Siah1 expression abolished PLCε-dependent potentiation of EGF-dependent cell growth. In addition, the expression of wild-type Siah1 in Siah1a/Siah2-double knockout MEF cells inhibited EGF-dependent cell growth, and this inhibition was abolished by PLCε knockdown. Our results suggest that the Siah-dependent degradation of PLCε plays a role in the regulation of growth factor-dependent cell growth.

Under the control of cell surface receptors, phosphoinositide-specific phospholipase C (PLC)[2] isozymes hydrolyze phosphatidylinositol 4,5-bisphosphate to generate two intracellular products, inositol 1,4,5-trisphosphate and diacylglycerol, which are implicated in calcium mobilization and protein kinase C activation, respectively. So far, 14 PLC isoforms have been cloned in mammals. Based on their functional and structural characteristics, they have been grouped into five classes: PLCβ (β1–β4), PLCδ (δ1–δ4), PLCγ (γ1 and γ2), PLCε, PLCζ, and PLCη (η1 and η2) (1–3).

PLCε plays a role in the interplay between PLC and small GTPases. Various G-proteins directly activate PLCε. For example, RhoA was found to stimulate PLCε activity by interacting with a 65-amino acid insert within the catalytic core of PLCε (4). Lysophosphatidic acid and thrombin stimulates PLCε by activating $G\alpha_{12}$ and/or $G\alpha_{13}$ and downstream RhoA (5). Moreover, Ras-family GTPases activate PLCε by binding with the RA domain of PLCε. Adrenaline and prostaglandin E_1 have been reported to activate PLCε by triggering adenylyl cyclase-coupled receptors, and Rap2B, which is regulated by Epac (a guanine-nucleotide-exchange factor regulated by cAMP), has been found to associate with the RA domain of PLCε during PLCε activation (6, 7). In addition, EGF treatment was found to induce an association between the PLCε RA domain and activated Ras, and this resulted in the recruitment of PLCε into the plasma membrane for activation (8).

Several physiological studies have indicated that PLCε is involved in development and cell growth. PLCε knockout mice had cardiac dysfunction resulting from defective heart development or were susceptible to hypertrophy in response to chronic cardiac stress (9, 10). Recently, mutations of PLCε in individuals with severe nephrotic syndrome were identified, and PLCε knockdown in zebra fish led to a loss of the filtration barrier maintained by glomerular podocytes, which in combination demonstrate the importance of PLCε in kidney development and function (11). Moreover, ablation of PLCε activity in mice led to reduced carcinogen-induced skin tumor formation, providing evidence that PLCε plays a positive role in tumor cell growth (12). Furthermore, the overexpression of PLCε in BaF3 cells induced platelet-derived growth factor-dependent cell growth (13).

Siah proteins are homologues of *Drosophila* SINA, which is a ring-finger protein involved in R7 cell development in the eye

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[2] The abbreviations used are: PLC, phospholipase C; EGF, epidermal growth factor; FBS, fetal bovine serum; RA, Ras association; E3, ubiquitin-protein isopeptide ligase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

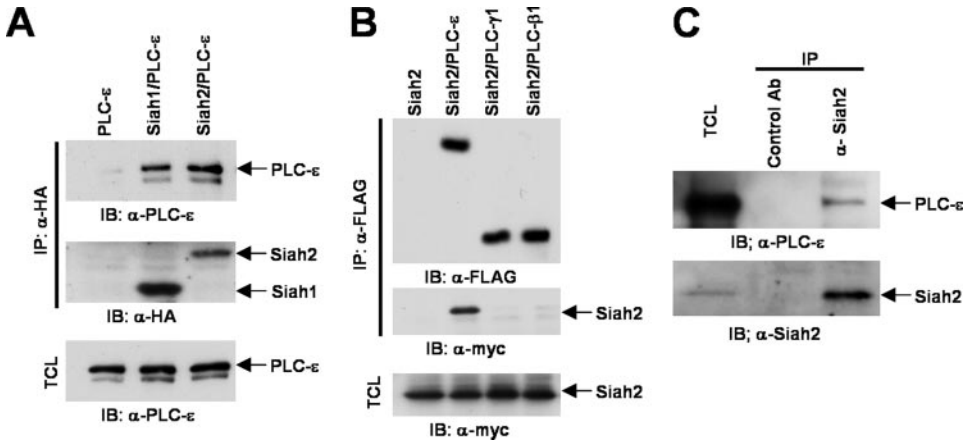

FIGURE 1. **Interaction between PLCε and the Siah proteins.** *A*, COS-7 cells were transfected with HA-Siah1/FLAG-PLCε or HA-Siah2/FLAG-PLCε. The proteasomal inhibitor MG132 was added to the media 12 h before cell lysis to inhibit the self-degradation of the Siah proteins. Siah proteins were immunoprecipitated with α-HA antibody, and immunocomplexes were subjected to immunoblotting with the indicated antibodies (*TCL*, total cell lysates). *B*, COS-7 cells were transfected with Myc-Siah2 and the indicated PLC isozymes. PLC isozymes were immunoprecipitated with α-FLAG antibody and the immunocomplexes were subjected to immunoblotting with α-Myc antibody to detect co-immunoprecipitated Siah2. *C*, MEF cells were incubated with MG132 (10 μM) for 12 h. Cell lysates were prepared, and Siah2 was immunoprecipitated with α-Siah2 antibody. The immunocomplexes were subjected to immunoblotting with the indicated antibodies. Goat α-AH receptor antibody was used for the control antibody.

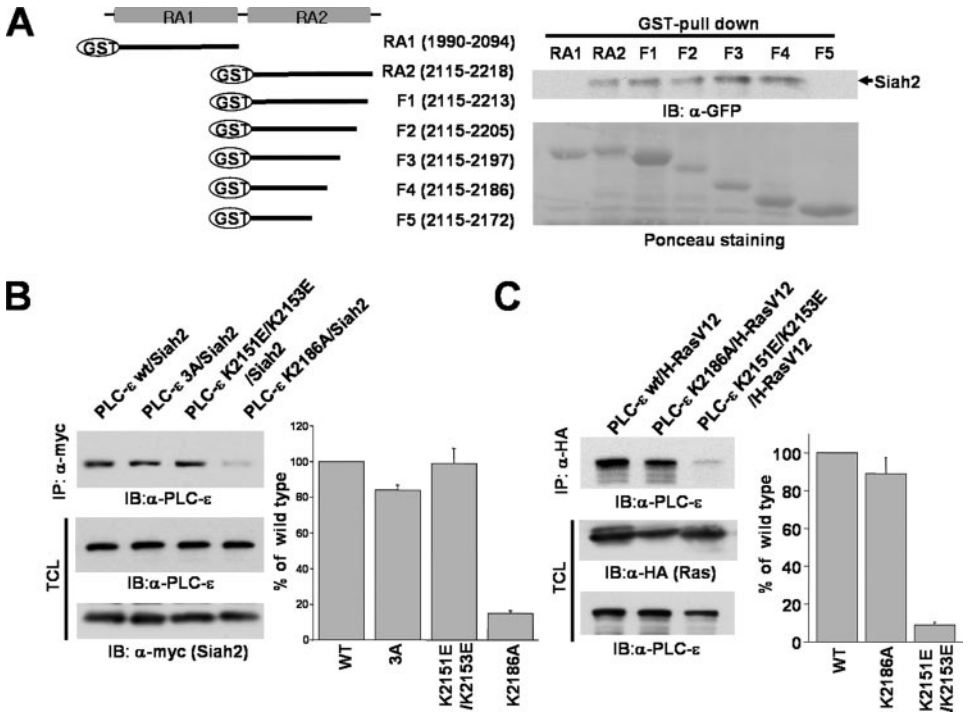

FIGURE 2. **Mapping of the Siah binding region in the RA domain of PLCε.** *A*, glutathione *S*-transferase fusion proteins containing the indicated region of the RA domain were incubated with GFP-Siah2 expressed in COS-7 cells. Bound Siah2 was detected with α-GFP antibody. *B*, COS-7 cells were transfected with Myc-Siah2 and FLAG-PLCε wild-type or PLCε RA domain mutants (PLCε 3A, mutant with VLK-(2173–2175) replaced by AAA; PLCε K2151E/K2153E, Ras binding-deficient mutant). Siah2 was immunoprecipitated with α-Myc antibody, and the immunocomplexes were subjected to immunoblotting with α-PLCε antibody. The relative binding of PLCε mutants with Siah2 was quantified with densitometry. The results are shown as the means ± S.D. ($n = 3$). *C*, COS-7 cells were transfected with the constitutively active form of HA-Ras (RasV12) and FLAG-PLCε constructs. RasV12 mutant was immunoprecipitated with α-HA antibody, and the immunocomplexes were subjected to immunoblotting with α-PLCε antibody. The relative binding of PLCε mutants with RasV12 was quantified with densitometry. The results are shown as the means ± S.D. ($n = 3$).

(14). Three murine (Siah1a, Siah1b, and Siah2) and two human (SIAH1 and SIAH2) homologues have been identified. The mammalian Siah proteins are highly homologous; Siah1a and Siah1b are 98% identical, whereas Siah1 proteins and Siah2 protein diverge significantly only at their N termini (15, 16). Siah proteins are RING finger proteins with E3 ligase activity and have been implicated in the ubiquitination and proteasome-dependent degradation of various substrate molecules. Substrates of Siah proteins are quite diverse and include transcriptional regulators (17–19), membrane receptors (20, 21), a microtubule-associated motor protein (22), and other proteins. In particular, the involvement of Siah proteins in cell growth regulation has been suggested in many reports. Siah1 expression is induced by tumor suppressor p53 in mammals and the overexpression of Siah1 inhibits cell proliferation and promotes apoptosis (23–25). Moreover, Siah-induced β-catenin degradation is important for the negative regulation of cell proliferation (26, 27), and the Siah-induced degradation of Kid is important for mitosis and contributes to cell growth arrest (28). Furthermore, mutations of Siah proteins in several cancers have been reported (29). These reports imply that Siah has tumor suppressor functions in some experimental settings.

To identify novel PLCε regulatory proteins, we performed yeast two-hybrid analysis using the RA domain of PLCε, and Siah1 and Siah2 were identified as PLCε-binding proteins. Here, we demonstrate that Siah proteins induce the proteasomal degradation of PLCε after EGF stimulation. Src-dependent phosphorylation was found to be required for the EGF-dependent degradation of PLCε. Moreover, the Siah-dependent degradation of PLCε was found to act as an important negative regulator of PLCε-dependent cell growth.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal antibody of PLCε was obtained from Dr. Tohru Kataoka (Kobe University, Japan). Mouse monoclonal antibody of Siah1 was described previously (30). Other antibodies used were: goat polyclonal anti-Siah2 antibody and goat polyclonal anti-AH

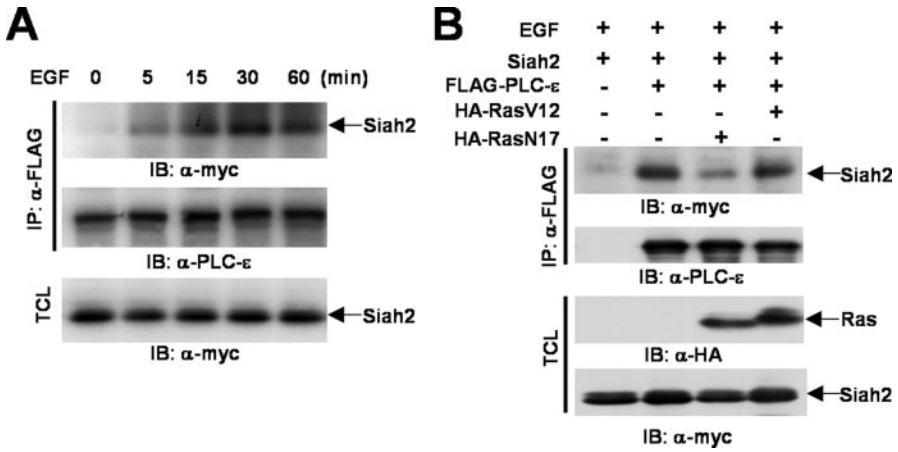

FIGURE 3. **EGF-dependent interaction between PLCε and Siah2.** *A*, COS-7 cells were transfected with Myc-Siah2 and FLAG-PLCε. After serum starvation for 12 h, cells were incubated with the proteasomal inhibitor MG132 (10 μM) for another 12 h. Cells were treated with EGF (100 ng/ml) for the indicated times. Cell lysates were prepared, and PLCε was immunoprecipitated with α-FLAG antibody. The immunocomplexes were subjected to immunoblotting with α-Myc antibody to detect co-immunoprecipitated Siah2. *B*, COS-7 cells were transfected with Myc-Siah2 and FLAG-PLCε in the presence or absence of HA-Ras mutants (RasV12, constitutively active mutant; RasN17, dominant negative mutant). After serum starvation and incubation with MG132 (10 μM), the cells were stimulated with EGF (100 ng/ml) for 30 min. Cell lysates were prepared, and PLCε was immunoprecipitated with α-FLAG antibody.

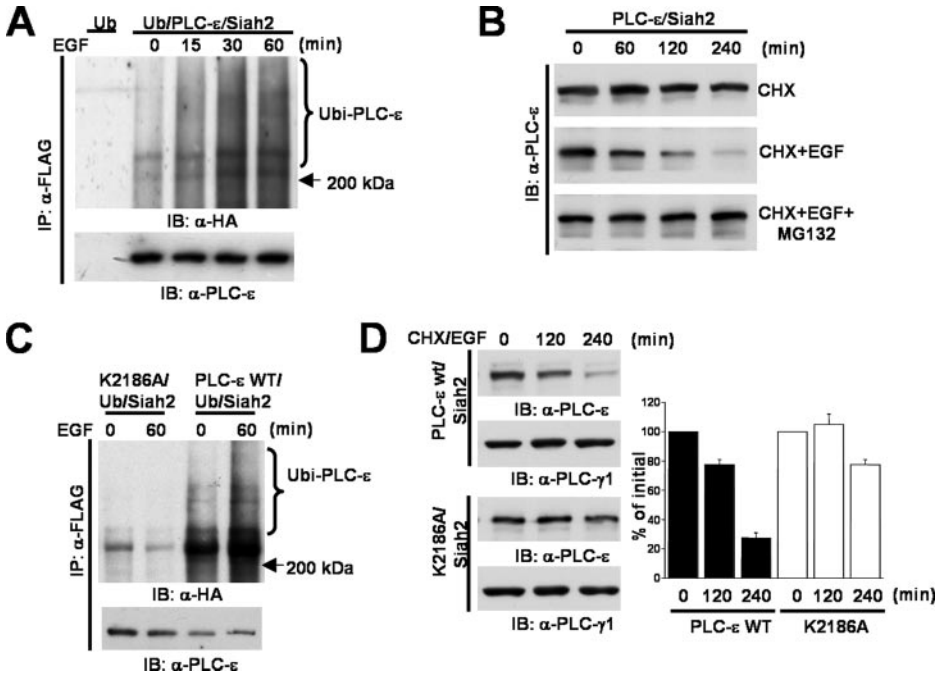

FIGURE 4. **EGF-induced ubiquitination and degradation of PLCε.** *A*, COS-7 cells were transfected with HA-ubiquitin, FLAG-PLCε, and Myc-Siah2. After serum starvation for 12 h, the cells were incubated with MG132 (10 μM) for an additional 12 h. The cells were stimulated with EGF (100 ng/ml) for the indicated times. PLCε was immunoprecipitated with α-FLAG antibody, and PLCε ubiquitination was detected with α-HA antibody. *B*, COS-7 cells were transfected with Myc-Siah2 and FLAG-PLCε. After serum starvation for 24 h, the cells were pretreated with cycloheximide (10 μg/ml) for 1 h in the presence or absence of MG132 (10 μM), and then EGF (100 ng/ml) was added to media for the indicated times (*CHX*, cycloheximide). Cell lysates were prepared, and the level of PLCε was monitored by immunoblotting with α-PLCε antibody. *C*, COS-7 cells were transfected with wild-type FLAG-PLCε or the K2186A mutant in the presence of HA-ubiquitin and Myc-Siah2. PLCε ubiquitination was detected after stimulation with EGF (100 ng/ml) for 60 min. *D*, COS-7 cells were transfected with FLAG-PLCε wild type or the K2186A mutant in the presence of Myc-Siah2. After serum starvation for 24 h, cells were pretreated with cycloheximide (10 μg/ml) for 1 h and then treated with EGF (100 ng/ml) for the indicated times. Cell lysates were prepared and subjected to SDS-PAGE and immunoblotting with α-PLCε antibody. The remaining PLCε level after EGF stimulation for the indicated time was quantified and expressed as a percentage of the PLCε level of unstimulated control cells. The results are shown as the means ± S.D. ($n = 3$).

receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-FLAG antibody (Sigma), mouse monoclonal anti-Myc antibody (Invitrogen), mouse monoclonal anti-HA antibody (Sigma, St. Louis, MO), Rhodamine-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-conjugated goat anti-mouse IgG, were purchased from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were from Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Cell Culture—COS-7 cells were grown in DMEM containing 10% bovine calf serum, antibiotics, and glutamine. Wild-type and Siah-deficient MEF cells were grown in DMEM containing 10% fetal bovine serum, 0.02 mM β-mercaptoethanol, antibiotics, and glutamine as previously described (30). Cells were grown to ~90% confluence for immunoprecipitation and Western blot experiments.

Yeast Two-hybrid Screening—PLCε RA domains (amino acids 1990–2218) were cloned into the pLexA (BD Clontech) in-frame with the LexA DNA-binding domain (referred to as pLexA-PLCε). The yeast strain, EGY48, carrying a reporter gene was cotransformed with the bait plasmid, pLexA-PLCε, and a human HeLa cDNA library fused to the VP16 activation domain. Transformation was carried out using the lithium acetate method (31). Leucine-positive colonies were identified by a filter-lifting assay for β-galactosidase activity. Library-derived DNA was prepared from candidate clones and analyzed by DNA sequencing.

Immunoprecipitation—Cells were lysed with TGH buffer (1% Triton X-100, 10% glycerol, 50 mM NaCl, 50 mM HEPES, pH 7.3, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethysulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml aprotinin). Lysates were then centrifuged at 14,000 × *g* for 10 min at 4 °C. Supernatants were incubated with anti-HA, anti-FLAG for 3 h and then washed with TGH buffer three times. Immunoprecipitates were subjected to SDS-PAGE and Western blotting.

PLCε Phosphorylation Analysis—COS-7 cell-transfected Siah proteins were serum-starved for 20 h and then incubated

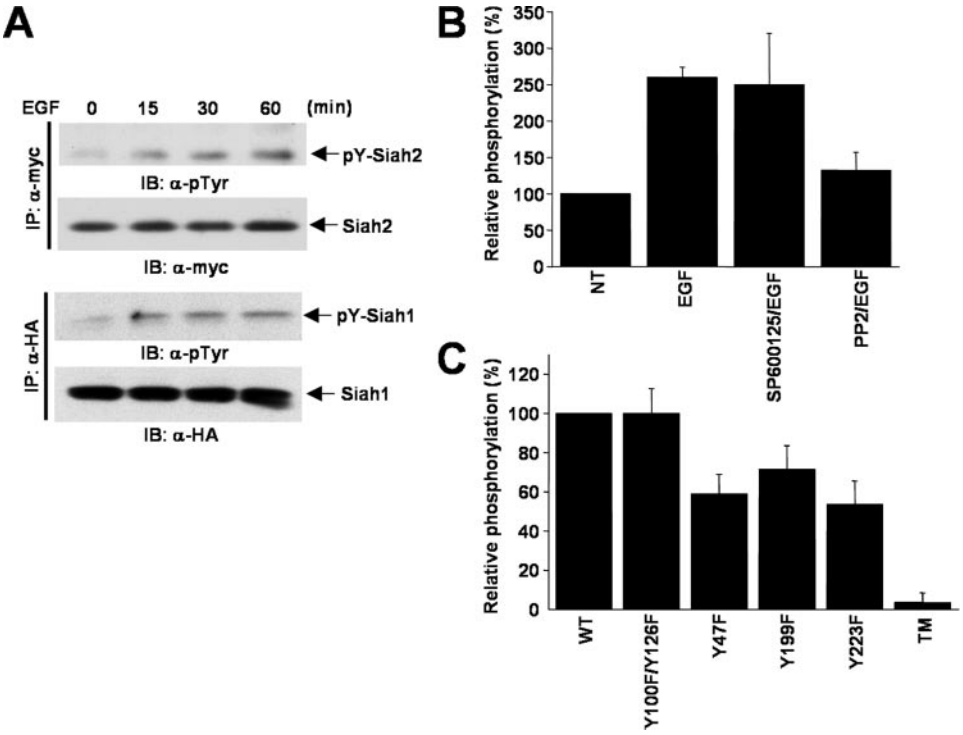

FIGURE 5. **EGF-induced tyrosine phosphorylation of Siah proteins.** *A*, COS-7 cells were transfected with HA-Siah1 or Myc-Siah2. After serum starvation for 12 h, the cells were incubated with MG132 (10 μM) for an additional 12 h. Cells were then treated with EGF (100 ng/ml) for the indicated times. Siah proteins were immunoprecipitated with α-HA antibody or α-Myc antibody, and the immunocomplexes were subjected to immunoblotting with α-phosphotyrosine antibody. *B*, COS-7 cells were transfected with Myc-Siah2. After serum starvation for 20 h, cells were labeled with [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 4 h and then pretreated with the indicated pharmacological inhibitors before EGF (100 ng/ml) stimulation for 30 min. Cells were co-incubated with MG132 (10 μM) for 12 h before EGF stimulation. Cell lysates were prepared and Siah2 was immunoprecipitated with α-Myc antibody. The immunocomplexes were subjected to autoradiography and immunoblotting with α-Myc antibody. The relative phosphorylation of Siah2 was quantified. The results are shown as the means ± S.D. (n = 3). *C*, COS-7 cells were transfected with HA-Siah1 wild-type or Siah1 phosphorylation site mutants (*TM*, phosphorylation-deficient mutant of Siah1). After serum starvation for 20 h, cells were labeled with [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 4 h and then treated with EGF (100 ng/ml) for 30 min. Cells were co-incubated with MG132 (10 μM) for 12 h before EGF stimulation. Siah1 was immunoprecipitated with α-HA antibody. The immunocomplexes were subjected to autoradiography and immunoblotting with α-HA antibody. The relative phosphorylation of Siah1 wild-type and mutants was quantified. Results are shown as the means ± S.D. (n = 3).

with 1 mCi of [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 4 h at 37 °C. Cells were treated with 100 ng/ml EGF for the indicated times. Cell lysates were prepared, and Siah proteins were immunoprecipitated. The immunocomplexes were subjected to SDS-PAGE and autoradiography. The amounts of immunoprecipitated Siah proteins were measured by immunoblotting and used for the quantitation of relative Siah phosphorylation.

Cell Growth Assay—MEF cells were seeded in triplicate into 6-well plates at a density of 2×10^5 cells per well and were transfected with PLCε siRNA or control scrambled siRNA. After 24 h, cells were incubated with serum-free DMEM for 24 h to reach quiescence. The cells were incubated in serum-free medium supplemented with 100 ng/ml EGF for 18 h prior to addition of ^{3}H-labeled thymidine for additional 6 h. Thymidine incorporation was measured as previously reported (32).

Plasmid Construction and Mutagenesis—FLAG-tagged mouse PLCε DNA is a generous gift from Dr. Tohru Kataoka (Kobe University, Japan). For the construction of Siah binding-deficient mutant, evolutionally conserved VLK (2173–2175) sequence was changed into AAA (called PLCε 3A) or Lys-2186 residue was changed into alanine (called PLCε K2186A) by site-directed mutagenesis. PLCε K2151E/K2153E is a Ras binding-deficient mutant. Siah1 wild-type and Y100F/Y126F mutant DNA was kindly provided by Dr. Zhiheng Xu (Columbia University) and introduced into pcDNA-HA vector by PCR amplification. To construct phosphorylation-deficient mutant of Siah1, Tyr-47, Tyr-199, and Tyr-223 were changed into phenylalanines (called Siah1 TM). For expression of Siah1 in knockout MEF cells, wild-type Siah1 or Siah1 TM were PCR-amplified and cloned into a lentivirus-derived C-FUW vector (33).

Production of Lentivirus-harboring Siah1—Lentivirus infection was performed as previously described (34).

PLCε Knockdown in MEF Cells—Synthetic siRNA against PLCε and control scrambled siRNA was introduced into MEF cells using LipofectAMINE (Invitrogen). PLCε siRNA (GCCAAATATTCCTACAGCA) and control scrambled siRNA (ACTGTCACAAGTACCTACA) have been previously described (35).

RESULTS

Interaction between PLCε and Siah Proteins—We sought to identify binding partners of the PLCε RA domain and performed a yeast two-hybrid analysis using bait containing the serial RA1 and RA2 domain of PLCε. Our yeast two-hybrid analysis revealed that the RA domain of PLCε interacts with various proteins other than small GTPases. Siah1 and Siah2 were identified as novel PLCε RA domain-binding proteins. The positive clones obtained from a HeLa cell cDNA library contained the substrate binding domain of the Siah proteins (Siah1, 177–282; Siah2, 217–324). We examined whether PLCε interacts with Siah proteins in cells by using co-immunoprecipitation analysis. Both Siah1 and Siah2 were found to associate with PLCε in COS-7 cells (Fig. 1*A*), but Siah2 did not interact with PLC-γ1 or PLC-β1 (Fig. 1*B*). These results indicate that Siah proteins interact with PLCε by specifically recognizing the RA domain, which is present only in the PLCε isozyme.

We then investigated whether endogenous PLCε and Siah2 can form a complex in MEF cells. As shown in Fig. 1*C*, Siah2 was immunoprecipitated with α-Siah2 antibody and the immunocomplex contained PLCε, which indicates that PLCε-Siah2 complex exists under physiological conditions.

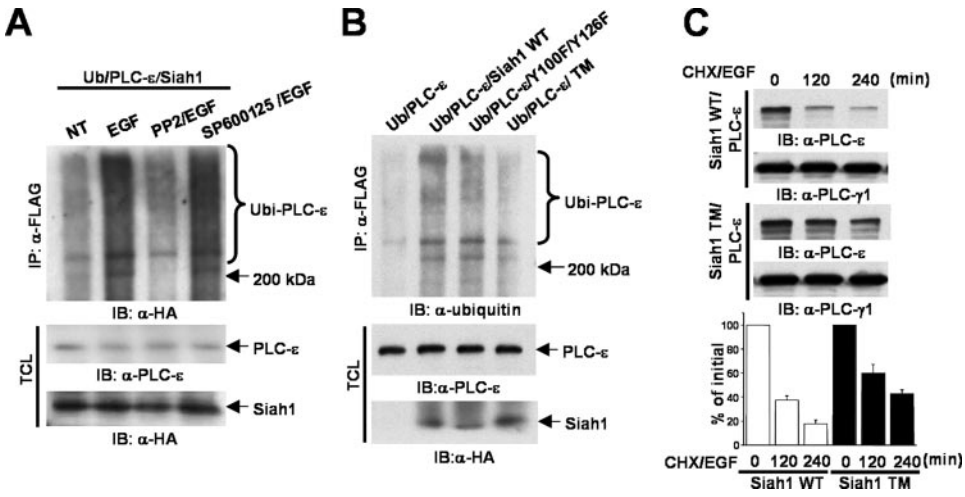

FIGURE 6. **Tyrosine phosphorylation of the Siah proteins is important for the EGF-induced degradation of PLCε.** *A*, COS-7 cells were transfected with FLAG-PLCε, HA-Siah1, and HA-ubiquitin. After serum starvation and incubation with MG132 (10 μM), the cells were pretreated with the indicated inhibitors and then treated with EGF (100 ng/ml) for 60 min. PLCε was immunoprecipitated and the immunocomplexes were probed with α-HA antibody to detect ubiquitinated PLCε. *B*, COS-7 cells were transfected with FLAG-PLCε and HA-Siah1 wild-type or Siah1 TM. After serum starvation, the cells were pretreated with MG132 (10 μM) and then treated with EGF (100 ng/ml) for 60 min. PLCε was immunoprecipitated with α-FLAG antibody, and the immunocomplexes were subjected to immunoblotting with α-HA antibody. *C*, COS-7 cells were transfected with FLAG-PLCε and HA-Siah1 wild-type or Siah1 TM. After serum starvation for 24 h, cells were pretreated with cycloheximide (10 μg/ml) for 1 h and then treated with EGF (100 ng/ml) for the indicated times. Cell lysates were prepared and subjected to immunoblotting with α-PLCε antibody. The remaining PLCε level after EGF stimulation for the indicated time was quantified and expressed as a percentage of the PLCε level of unstimulated control cells. Multiple bands below PLCε position are PLCε degradation products.

The RA2 Domain Contains Siah Binding Regions Distinct from the Ras Binding Region—The RA domains of PLCε are composed of RA1 and RA2 domains. Both domains have similar ubiquitin-like folds, but only the RA2 domain can associate with activated Ras (36). In the present study, we explored the binding region of the RA domains in detail. Glutathione *S*-transferase-pulldown analysis revealed that the RA2 domain is responsible for the interaction with Siah2, and further deletion analysis enabled us to narrow this down to several amino acids (Fig. 2*A*). We mutated several evolutionally conserved amino acids and examined their ability to interact with Siah2. In particular, the mutation of Lys-2186 into Ala led to the 85% inhibition of the interaction between PLCε and Siah2, whereas mutation of VLK (2173–2175) into AAA had little effect on the interaction with Siah2. In addition, the Ras binding-deficient mutant of PLCε (K2151E/K2153E) had almost the same affinity for Siah2 as did the wild type (Fig. 2*B*). According to previous structural analysis of the PLCε RA domain (36), the Siah binding region of PLCε corresponds to the loop between β3 and β4 of the RA2 domain, which is removed from the Ras binding region (β1, β2, and the α1–β3 loop of RA2). In fact, the K2186A mutant showed no difference in binding affinity for the constitutively active H-RasV12 mutant (Fig. 2*C*). Taken together, these results support the possibility that the RA2 domain has a specific Siah binding region that is quite distinct from the Ras binding region.

EGF Stimulation Induced the Interaction between Siah2 and PLCε—EGF stimulation evokes PLCε activity through Ras-dependent translocation to the plasma membrane (8). In the present study, we investigated whether EGF treatment affects the interaction between Siah and PLCε. Interestingly, EGF treatment induced an association between PLCε and Siah2 in COS-7 cells (Fig. 3*A*). The interaction was initially detected at 5 min after EGF stimulation and was most prominent at 30 min after stimulation. We then investigated whether Ras-dependent activation of PLCε is required for the EGF-dependent association between Siah2 and PLCε. To this end, PLCε activation was blocked by the expression of a dominant negative mutant, RasN17. The expression of the RasN17 mutant abolished EGF-dependent Siah2 binding with PLCε (Fig. 3*B*), which indicates that PLCε activation is required for the association with Siah2. Our findings demonstrate that Siah2 associates with activated PLCε after EGF stimulation.

EGF Stimulation Induced the Siah-dependent Degradation of PLCε—Siah proteins function as E3 ubiquitin ligases and mediate the ubiquitination of various substrate molecules by direct interaction (17–22). Thus, we investigated whether the EGF-dependent association between Siah proteins and PLCε can lead to the ubiquitination of PLCε. We found that PLCε was efficiently ubiquitinated in COS-7 cells upon EGF stimulation (Fig. 4*A*). Many Siah substrates are known to be subjected to degradation by the ubiquitin-proteasome pathway (17–22), and in the present study, the expression of Siah2 also led to the EGF-dependent degradation of PLCε, which was blocked by co-incubation with MG132 (a proteasomal inhibitor), indicating that Siah-mediated ubiquitination leads to the proteasomal degradation of PLCε (Fig. 4*B*). The PLCε K2186A mutant was not ubiquitinated by EGF stimulation (Fig. 4*C*). Concomitantly, the PLCε K2186A mutant was not efficiently degraded by EGF stimulation (Fig. 4*D*), which demonstrates that the interaction between PLCε and Siah2 is required for the EGF-induced PLCε degradation. These results suggest that PLCε is subjected to Siah-dependent ubiquitination and degradation upon EGF stimulation.

The EGF-dependent Activation of Src Leads to the Phosphorylation of Siah Proteins—The growth factor-dependent ubiquitination of substrate molecules by Siah proteins has not been reported to date. We attempted to identify the upstream mediator responsible for the activation of Siah proteins upon EGF stimulation. It was recently reported that Siah1 is phosphorylated on tyrosine residues by a JNK-dependent pathway after camptothecin treatment, and that this tyrosine phosphorylation plays an important role in the stability and function of Siah1 (37). Thus, we examined whether EGF treatment could induce the tyrosine phosphorylation of Siah proteins. Interestingly, both Siah1 and Siah2 were found to be phosphorylated on tyrosine residues after EGF treatment in COS-7 cells (Fig. 5*A*). We tested several pharmacological inhibitors to identify poten-

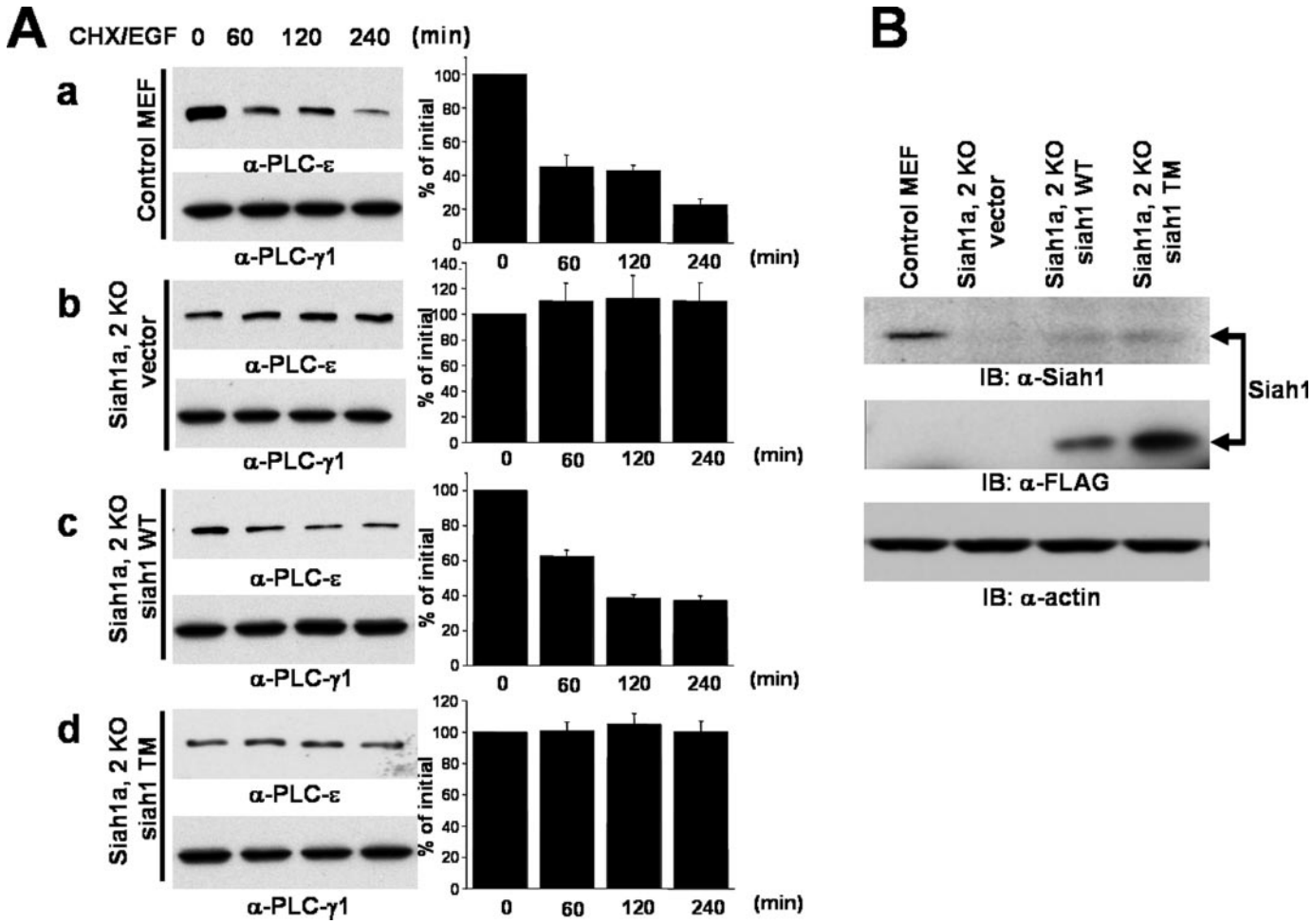

FIGURE 7. **PLCε degradation in Siah-deficient MEF cells.** *A*, MEF cells derived from a Siah1a/Siah2 double knockout mouse (*b*) or a wild-type mouse (*a*) were prepared. A FLAG-Siah1 wild-type (*c*) or a Siah1 TM mutant (*d*) was introduced into the Siah1a/Siah2 double knockout cells by lentivirus-mediated infection. The cells were serum-starved and stimulated with EGF (100 ng/ml) and cycloheximide (10 μg/ml) for the indicated times. Cell lysates were prepared, and the level of PLCε was measured by immunoblotting with α-PLCε antibody. The remaining PLCε level after EGF stimulation for the indicated time was quantified and expressed as a percentage of the PLCε level of unstimulated control cells. *B*, MEF cells were treated with MG132 (10 μM) for 12 h, and then cell lysates were prepared. Endogenous and exogenous expression of Siah1and Siah1 constructs in MEF cells was detected by immunoblotting with α-Siah1 antibody or α-FLAG antibody.

tial upstream kinases responsible for the EGF-induced phosphorylation of Siah proteins. Src tyrosine kinase inhibitor PP2 blocked the EGF-induced phosphorylation of Siah2 (Fig. 5*B*), whereas the EGF-dependent phosphorylation of Siah2 was unaffected by SP600125 (a JNK inhibitor), which suggested a novel phosphorylation-dependent means of Siah protein regulation. There are five conserved tyrosine residues in Siah1 and Siah2, and Tyr-100 and Tyr-126 of Siah1 have been reported to be phosphorylated by camptothecin treatment. Thus, we investigated whether Tyr-100 and Tyr-126 of Siah1 are also phosphorylated by EGF stimulation. Mutation of both residues to phenylalanines had no effect on the EGF-dependent phosphorylation of Siah1 (Fig. 5*C*). Thus, we substituted Tyr-47, Tyr-199, and Tyr-223 for phenylalanine and re-examined EGF-induced phosphorylation. Individual mutations of these tyrosine residues resulted in a partial reduction of the tyrosine phosphorylation of Siah1, but the mutation of all three tyrosine residues completely blocked this phosphorylation (Fig. 5*C*). These results imply that Siah is phosphorylated at multiple tyrosine residues via a Src-dependent pathway after EGF stimulation.

Siah Phosphorylation Is Required for the EGF-induced Degradation of PLCε—To elucidate the role of the Src-dependent tyrosine phosphorylation of Siah proteins, we pretreated cells with Src tyrosine kinase inhibitor PP2 before EGF stimulation and examined the ubiquitination of PLCε in these cells. Src inhibition led to the suppression of the EGF-dependent ubiquitination of PLCε, whereas pretreatment of SP600125 had no effect on PLCε ubiquitination (Fig. 6*A*). To further confirm the role of Siah phosphorylation, we utilized a phosphorylation-deficient mutant of Siah1 (Siah1 TM) that had phenylalanine substitutions at Tyr-47, Tyr-199, and Tyr-223. Siah1 TM did not effectively induce the ubiquitination of PLCε after EGF treatment, whereas wild-type and the Siah1 Y100F/Y126F mutant efficiently ubiquitinated PLCε (Fig. 6*B*). Concomitantly, the EGF-dependent degradation of PLCε was impaired in the cells transfected with Siah1 TM (Fig. 6*C*), as compared with the cells transfected with wild-type Siah1. Taken together, these results show that the Src-dependent phosphorylation of Siah is required for the EGF-induced ubiquitination and degradation of PLCε.

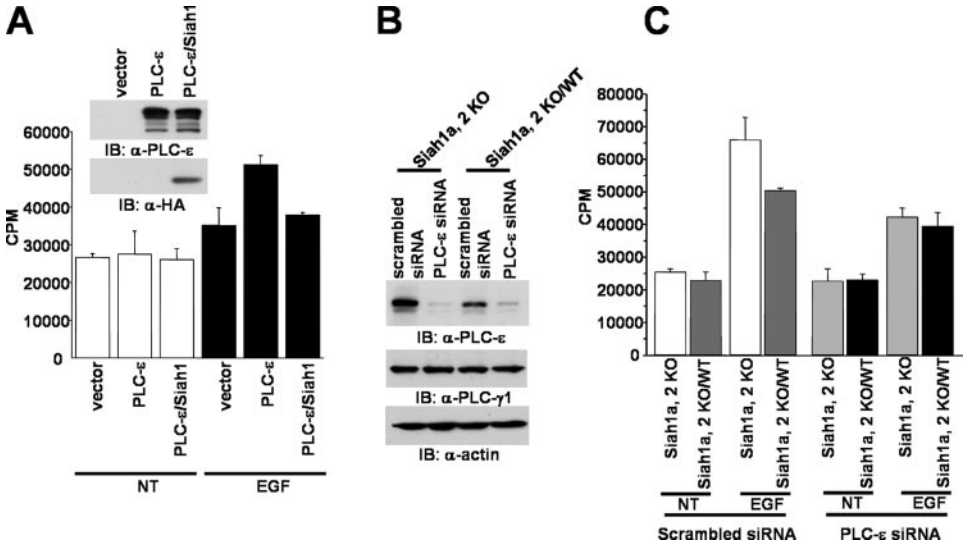

FIGURE 8. **Siah-dependent suppression of cell growth.** *A*, HEK293 cells were transfected with FLAG-PLCε in the presence or absence of HA-Siah1. After serum starvation for 24 h, the cells were treated with EGF (100 ng/ml) for 24 h. EGF-dependent cell growth was measured by thymidine incorporation analysis. Results are shown as the means ± S.D. ($n = 3$). Expression of PLCε and Siah1 was detected 24 h after transfection. *B*, MEF cells were transfected with PLCε siRNA or control scrambled siRNA. After 48-h incubation, cell lysates were prepared and subjected to immunoblotting with α-PLCε antibody. *C*, MEF cells were transfected with PLCε siRNA or control scrambled siRNA. After serum starvation for 24 h, the cells were treated with EGF (100 ng/ml) for 24 h. EGF-dependent cell growth was measured by thymidine incorporation analysis. Results are shown as the means ± S.D. ($n = 3$).

Siah Is Required for the EGF-dependent Degradation of PLCε in MEFs—We attempted to determine whether the EGF-dependent degradation of PLCε is impaired in the absence of the Siah proteins. To this end, we utilized Siah1a/Siah2 double knockout MEF cells. PLCε was subjected to EGF-dependent degradation in MEF cells derived from wild-type mice (Fig. 7*A*, *panel a*), but the degradation of PLCε was blocked in the absence of Siah1a and Siah2 (Fig. 7*A*, *panel b*). To clarify the involvement of Siah, we expressed wild-type Siah1 or Siah1 TM in knockout cells by lentiviral infection (Fig. 7*B*). Add-back of wild-type Siah1 restored the EGF-dependent degradation of PLCε (Fig. 7*A*, *panel c*), however, the add-back of Siah1 TM did not (Fig. 7*A*, *panel d*). These results confirm the role of endogenous Siah proteins in the EGF-dependent degradation of PLCε.

Siah Suppressed PLCε-induced Cell Growth—Various reports suggest that PLCε can promote cell growth (12, 13), and our previous results revealed that EGF-dependent cell growth was enhanced by PLCε. Thus, we speculated that the Siah-dependent degradation of PLCε can contribute to the negative regulation of EGF-dependent cell growth. To test this hypothesis, we first examined whether Siah1 can inhibit the EGF-dependent cell growth potentiated by PLCε expression. As shown in Fig. 8*A*, PLCε expression enhanced EGF-dependent cell growth in HEK293 cells and co-expression of Siah1 abolished the PLCε-dependent enhancement of cell growth. We then measured the EGF-dependent cell growth in Siah1a/Siah2-double knockout cells and in Siah1-add-back cells. The add-back of Siah1 in double knockout cells suppressed EGF-dependent cell growth (Fig. 8*C*). We then reduced PLCε in Siah1a/Siah1-double knockout cells and in Siah1-add-back cells to investigate whether Siah-dependent growth inhibition is attributable to PLCε degradation (Fig. 8*B*). PLCε reduction in double knockout cells led to EGF-dependent growth inhibition, and notably, the suppression of Siah-dependent cell growth was abolished by PLCε knockdown (Fig. 8*C*). Taken together, these results suggest that Siah inhibits EGF-dependent cell growth by removing PLCε.

DISCUSSION

Although the activation mechanisms and physiological functions of PLCε have been largely revealed, little is known about the negative regulation of PLCε. The present study provides evidences that growth factor-activated PLCε is subjected to proteasomal degradation. Siah proteins interact with the RA2 domain of PLCε and promote PLCε ubiquitination in the process. Our findings demonstrate that the RA2 domain of PLCε plays a role in the inactivation of PLCε as well as in the activation of PLCε after growth factor stimulation. The RA2 domain of PLCε binds with activated Ras or Rap, and this binding is critical for the growth factor-dependent translocation to the membrane and the activation of PLCε (8, 36). It is interesting to note that the blockade of the Ras-dependent activation of PLCε led to the inhibition of the EGF-dependent interaction between Siah2 and PLCε, which implies that PLCε activation may be a prerequisite for the EGF-induced ubiquitination and degradation of PLCε. In addition, the EGF-dependent interaction between Siah2 and PLCε is most prominent at 30 min after EGF stimulation (Fig. 3*A*), whereas the EGF-induced translocation of PLCε into the membrane for activation reportedly begins at 5 min after stimulation and ends at 40 min after stimulation (8), which indicates that the Siah-induced ubiquitination of PLCε is induced after the activation of PLCε. Thus, we speculate that activated PLCε is subjected to Siah-dependent ubiquitination and degradation to terminate PLCε downstream signaling. This is the first study to explore the molecular mechanism underlying the negative regulation of PLCε.

Several studies have reported that PLCε promotes cell growth. For example, carcinogen-induced tumor formation was inhibited in PLCε-deficient mice (12), and the overexpression of PLCε in BaF3 cells potentiated platelet-derived growth factor-dependent cell growth (13). Consistent with previous reports, we found that PLCε knockdown in MEFs led to the suppression of EGF-dependent cell growth. We conclude that it is necessary to control the level of PLCε in cells to prevent aberrant cell growth. Many reports have implicated Siah proteins in cell growth control. Siah is a transcriptional target of p53 and contributes to p53-induced apoptosis and tumor suppression (23–25). Moreover, β-catenin degradation by genotoxic stress was mediated by Siah1, which leads to the suppres-

sion of cancer cell growth (26, 27). Siah1 was also found to induce growth arrest by inhibiting cytokinesis via the degradation of kinesin (Kid) (28). The involvement of Siah proteins in the regulation of growth factor-dependent cell growth was newly revealed in the present study. PLCε was found to be a novel substrate of Siah proteins, and it was degraded by Siah proteins after EGF stimulation. We demonstrated that Siah proteins contribute to the negative regulation of growth factor-induced MEF cell growth by mediating PLCε degradation. The fact that PLCε reduction abolished Siah-dependent growth inhibition (Fig. 7) suggests that PLCε is a major substrate of Siah proteins, which needs to be degraded to regulate cell growth in MEF cells.

The present study suggests a novel regulatory mechanism for Siah-mediated substrate degradation. We found that the Src-dependent phosphorylation of Siah is required for the EGF-induced degradation of PLCε. Whether Src directly phosphorylates Siah proteins or activates other protein kinases remains unclear. Previous reports have shown that Siah proteins are phosphorylated in different environments. For example, Siah2 was phosphorylated on Thr-24 and Ser-29 by p38 MAPK under hypoxia, and this was found to be important for the degradation of PHD-3 (38). Greene *et al.* (37) reported that Siah1 is phosphorylated on Tyr-100 and Tyr-126 upon camptothecin treatment via the activation of the JNK pathway. This phosphorylation increased the stability of Siah1 and its association with the adaptor protein POSH. Our analysis revealed that Siah1 is phosphorylated on Tyr-47, Tyr-199, and Tyr-223 via Src activation. We speculate that the EGF-induced phosphorylation of Siah proteins contributes to the binding of proteins with PLCε and the subsequent degradation of PLCε, because pretreatment with the Src tyrosine kinase inhibitor PP2 reduced the EGF-induced interaction between PLCε and Siah proteins as well as the ubiquitination of PLCε (data not shown). Thus, Siah phosphorylation appears to be an important regulatory mode that mediates various interactions with substrate molecules or adaptor proteins in different cellular contexts.

In summary, we identified Siah1 and Siah2 as negative regulators of PLCε. PLCε is subjected to EGF-dependent degradation via Siah-induced ubiquitination and proteasomal degradation processes. Siah is phosphorylated by EGF stimulation, and this phosphorylation is required for PLCε degradation. Physiologically, the Siah-induced degradation of PLCε contributes to the negative regulation of the EGF-dependent cell growth potentiated by PLCε.

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Siah Proteins Induce the Epidermal Growth Factor-dependent Degradation of Phospholipase C?

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