Activation mechanism of PCTAIRE kinase 3 (PCTK3)

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Abbreviation

PCTK3	PCTAIRE kinase 3
CDK	cyclin dependent kinase
Rb	retinoblastoma protein
PKA	cAMP-dependent kinase
FAK	focal adhesion kinase
ECM	extracellular matrix
GAP	GTPase-activating protein
GEF	guanine nucleotide exchanging factor
GDI	guanine nucleotide dissociation inhibitors
ROCK	rho-associated kinase
MLC	myosin light chain
LIMK	LIM-domain kinase

Chapter 1. Abstract

PCTAIRE kinase 3/cyclin dependent kinase 18 (PCTK3/CDK18) is a member of serine/threonine protein kinase that belongs to the cyclin dependent kinase (CDK) family. The CDK family controls various cellular functions, such as cell proliferation, differentiation, and motility. However, the physiological function of PCTK3 has been unknown because of unidentified activator(s). In this study, I revealed the activation mechanism of PCTK3, and which is involved in the regulation of actin cytoskeleton. First, I identified cyclin A2 and cyclin E1 as interacting proteins of PCTK3. The PCTK3 activity toward retinoblastoma protein (Rb) was increased by cyclin A2, while cyclin E1 did not activate PCTK3. I also found that cytoplasmic cyclin A2 stability was increased in the presence of PCTK3. Furthermore, PCTK3 contains some putative PKA phosphorylation sites. *In vivo* and *In vitro* kinase assay revealed that PKA directly phosphorylated PCTK3 at Ser^{12}, Ser^{66}, and Ser^{109}. The activity of phosphomimic PCTK3 S12D mutant was significantly increased even in the absence of cyclin A2, and which was comparative to that of CDK2/cyclin A2 complex in the presence of cyclin A2. Next, I examined the physiological function of PCTK3 using RNA interference. PCTK3-knockdown in HEK293T cells induced morphological change and polymerized actin accumulation in the leading edge. Additionally, I demonstrated that phosphorylation of cofilin, an actin depolymerizing factor, was increased in PCTK3-knockdown cells. Because cofilin depolymerizing activity is regulated by Rho GTPases such as RhoA and Rac1, I investigated whether PCTK3 affects the activities of RhoA and Rac1. PCTK3 knockdown led to RhoA activation and Rac1 inactivation in HEK293T cells, indicating that PCTK3 modulates actin cytoskeleton via the regulation of RhoGTPase activity. Finally, I found that PCTK3 knockdown also promoted the activation of focal adhesion kinase (FAK), suggesting that PCTK3 might act as a negative regulator of FAK. Taken together, my results provide the evidence that PCTK3 activity is regulated by cyclin A2 and PKA in the cytoplasm, and that PCTK3 controls actin dynamics through negatively modulating the RhoA and FAK activities.

Chapter 2. General Introduction

2.1. PCTAIER Kinase 3/Cyclin-Dependent Kinase 18

In mammal, cyclin dependent kinases (CDKs), a family of serine/threonine protein kinase, play an important role in the regulation of cellular functions such as cell proliferation, transcription and neural function [1]. Their activities are regulated by association with different cyclins and phosphorylation at specific sites by protein kinases [2]. Since cyclins are differentially expressed and degraded by ubiquitin mediated proteolysis at specific phase of cell cycle, the CDK activity is oscillated during the cell cycle transition [3]. Canonical CDKs contain the PSTAIRE helix implicated in binding to cyclins in the kinase domain [1]. The PCTAIRE kinase (PCTK) subfamily of the CDK family consists of three members, PCTK1/CDK16, PCTK2/CDK17 and PCTK3/CDK18, and contains the PCTAIRE sequence instead of the PSTAIRE sequence. PCTK family members show high sequence homology in their central kinase domain, while the structures of their N-terminal and C-terminal regions are different from each other. The PCTK genes are conserved in higher eukaryotes from Caenorhabditis elegans to human, but there are no PCTK orthologous in the yeast. The PCTK subfamily is widely expressed in mammalian tissues and is relatively more abundant in post-mitotic cells, suggesting that they possibly function in higher eukaryotes and exert the different biological function from cell cycle [4]. PCTK1, the best-characterized member of this kinase family, regulates neurite outgrowth in the Neuro2A neuroblastoma cell line [5], and is involved in the membrane trafficking through the early secretory pathway via phosphorylation of N-ethylmaleimide-sensitive fusion protein [6, 7]. Recent studies have been reported that PCT-1, the C. elegans ortholog of PCTK1, is complexed with CYY-1 (orthologous to mammalian cyclin Y, a novel membrane-associated cyclin) and also is necessary for targeting presynaptic components to the axon [8]. Furthermore, human PCTK1 has been shown to be activated by cyclin Y and be essential for spermatogenesis [9]. PCTK2 is associated with Trap (tudor repeat associator with PCTAIRE 2) and ik3-1/cables, the adaptor that functionally connects c-abl and CDK5 to support neurite growth [10, 11]. The third member of this family PCTK3 is the least well-studied. Although exogenously expressed PCTK1 and PCTK2

phosphorylate myelin basic protein and histone H1 in vitro, PCTK3 kinase activity has been not detected. To elucidate regulatory mechanism of PCTK3 activity, it is necessary for identifying activators of PCTK3.

2.2. Cyclin A

Cyclin A consists of two isoforms, cyclin A1 and cyclin A2, in mammal. Cyclin A1 is expressed predominantly in germ cells, and is required for the entry of germ cells into the first meiotic division in male mouse [12]. In contract, cyclin A2 is ubiquitously expressed in somatic cells, and it has a major role in progression of S phase and transition of G2/M phase through activation of CDK2 and CDK1, respectively. Although cyclin A predominantly localizes in the nucleus during S phase, it also shuttles between the nucleus and cytoplasm and is degraded by ubiquitin-mediated proteolysis during G2 phase. It was reported that cyclin A/CDK2 was transiently maintained by SCAPER, S phase cyclin A-associated protein, in the cytoplasm [13]. On the other hand, a recent study showed cyclin A2 was involved in the regulation of cell migration and invasiveness through direct interaction with RhoA, suggesting a novel function of cyclin A in a CDK1- and CDK2-independent manner. [14]

2.3. cAMP/PKA Signaling Pathway

cAMP, an intracellular second messenger, is involved in the regulation of various cell functions including blood glucose level, immune function, and neural function. cAMP is synthesized by hormone-mediated adenylate cyclase activation. Increased intracellular cAMP levels lead to activation of its target proteins, such as cAMP-dependent kinase (PKA) [15, 16], while synthesized cAMP is eventually degraded to adenosine monophosphate by phosphodiesterase [17]. PKA is constituted by two catalytic subunits (C) and two regulatory subunits (R). cAMP stimulates the dissociation of regulatory subunits and catalytic subunits, resulting in the PKA activation (Fig. 3). Activated PKA exerts phosphorylation activity toward the following consensus sequence Arg–Arg–X–Ser/Thr (X represents any amino acid) [18]. PKA also plays an important role in maintaining meiotic arrest [19].

In Xenopus oocyte, PKA phosphorylates and inactivates Cdc25C phosphatase, whereas Wee1B kinase activity is enhanced by PKA [20, 21]. The activity of cyclin B/CDK1 complex is controlled by Wee1 kinases and Cdc25C phosphatases [22, 23]. PKA regulates the activity of cyclin B/CDK1 complex via dual regulation of the Cdc25 phosphatase and Wee1B kinase [20]. On the other hand, PKA phosphorylates PCTK1 at Ser^{119} and Ser^{153}, and phosphorylation of PCTK1 on Ser^{153} inhibits the interaction between PCTK1 and cyclin Y, that is to say, PKA inactivates PCTK1 activity [5, 9]. Thus, PKA regulates the activity of CDK in a direct or indirect manner.

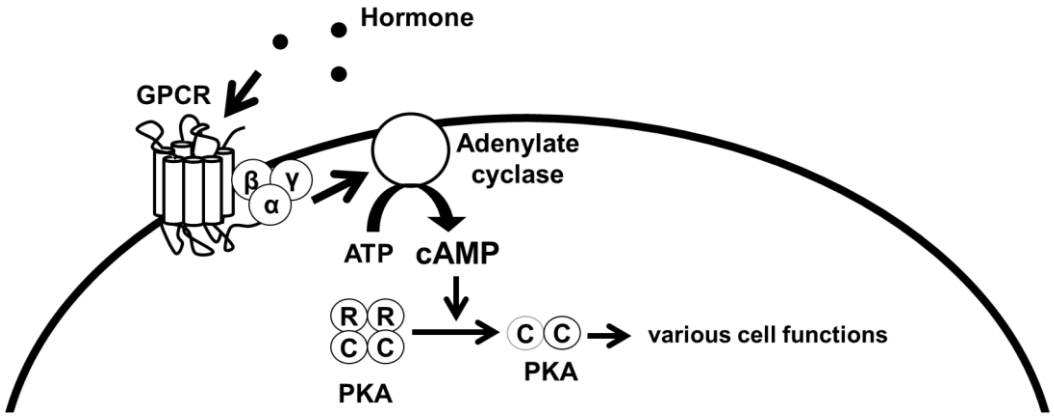

Figure 1. PKA signaling pathway

2.4. Rho GTPases Regulate Actin Cytoskeleton

Cell migration is a typical cell function common to eukaryotic cells, which is associated with variously physiological phenomena, such as organogenesis, neural network formation, cancer invasion and metastasis [24, 25]. Cell migration is generally achieved by three processes: (1) protrusion of pseudopodia, including lamellipodia and filopodia, at cell front (2) adhesion of pseudopodia to surrounding tissues or extracellular matrix (ECM), and (3) traction of the cell body toward the front [26(3)]. These processes are controlled by cytoskeleton including actin fiber, microtubule, and intermediate filament [27]. The distribution of cytoskeleton is substantially altered in response to the extracellular stimulation or cell cycle progression, giving mechanical strength and contractile force to cell body [28]. Actin cytoskeleton is constituted by the actin filament and actin binding proteins and controlled by cross-talk between actin binding proteins and the Rho GTPase family, such as RhoA,

Rac1, and Cdc42 [29]. The Rho GTPase family regulates the fundamental processes including cell movement, polarity, and division in eukaryotic cells [30]. Conversion between active GTP-bound state and inactive GDP-bound state of Rho GTPase is controlled by guanine nucleotide exchanging factor (GEF) [31], GTPase-activating protein (GAP) [32], and guanine nucleotide dissociation inhibitors (GDI) [33] (Fig. 1). Active GTP-bound Rho GTPase leads to the assembly of stress fiber and characteristic membrane protrusion, such as lamellipodia and filopodia, through coordinating their effector proteins [29]. A number of effector proteins of Rho GTPases have been identified so far, including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins [34]. Rho-associated kinase (ROCK) is one of the effector proteins of RhoA, which promotes the actin crosslinking activity of myosin II via phosphorylating myosin light chain (MLC) [35]. ROCK is also involved in the regulation of actin polymerization through modulating the LIM-domain kinase (LIMK)/cofilin signal transduction [36]. Thus, RhoA/ROCK signaling pathway plays important roles in the regulation of actin cytoskeleton and cell motility.

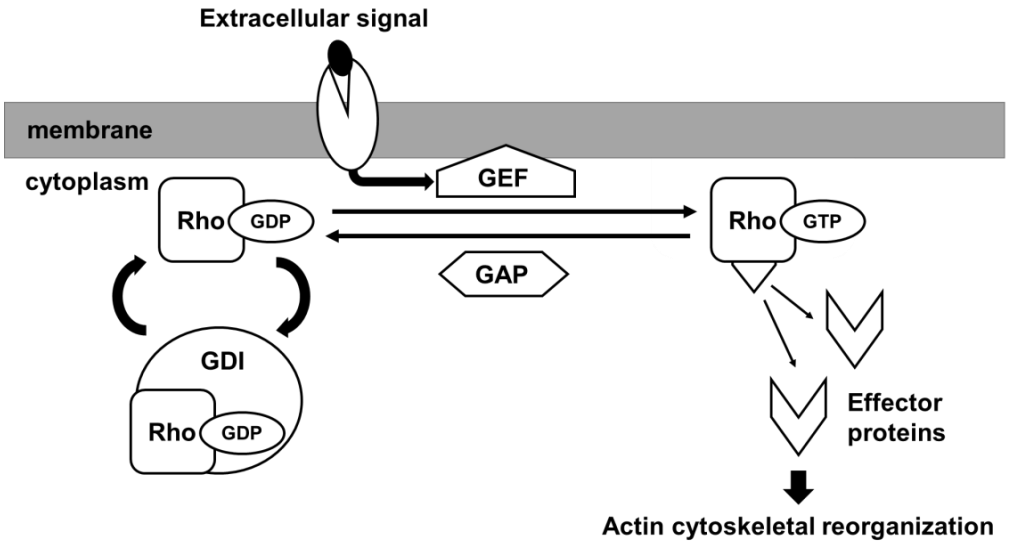

Figure 2. Basic model of Rho GTPase regulation

2.5. Focal Adhesion

In multicellular organism, cells are attached to surrounding ECM via adhesion receptors [37]. ECM is constituted by filament proteins, proteoglycan, or polysaccharide, which is essential for the

regulation of cell survival, differentiation, proliferation, and motility [38]. Cells are attached to ECM through focal adhesion which mediates the mechanical force to intracellular signal [39]. Focal adhesion is also connected with the actin cytoskeletal network including actin binding proteins and Rho GTPases [40]. Actin cytoskeletal networks give the physical strength to cells, maintain the cell shape, and translate the mechanical force to intracellular organelles [41]. The balance of focal adhesion assembly and disassembly is important for cell spreading and migration. During the early stages of cell adhesion, minute dot-like structures, called focal complex, are appeared at lamellipodia or filopodia. Focal complex is disassembled in short term (within several minutes), or grows to focal adhesion. Focal adhesion is eventually matured to stable assembly, called fibrillar adhesion (Fig. 2). These adhesion assembles (focal complex, focal adhesion, and fibrillar adhesion) are constituted by different molecules, respectively [42]. Focal adhesion is constituted by various proteins, such as ECM-interacting proteins (for instance, integrin, which is the heterodimer consisted of α-chain and β-chain), integrin-actin crosslinking proteins (talin, α-actinin, and tensin), tyrosine kinases (focal adhesion protein kinase (FAK) and Src), and substrates of tyrosine kinases (paxilin and p130Cas) [39]. These proteins transduce their signal to downstream molecules in response to the binding of ECM to integrin, resulting in the regulation of cell proliferation, differentiation, and motility [43].

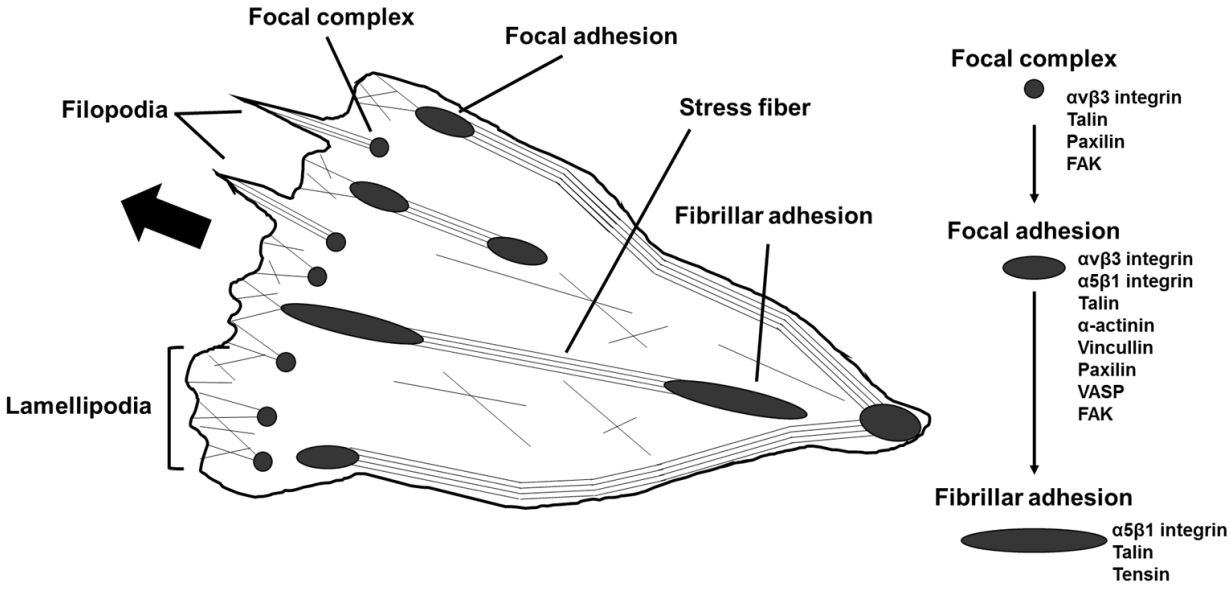

Figure 3. Formation and development of focal adhesion

Chapter 3. Introduction

PCTAIRE kinase 3 (PCTK3)/cyclin dependent kinase 18 (CDK18) is a member of the CDK family [44]. Although the PCTK3 is relatively more abundant in post-mitotic cells, their enzymatic characteristic and physiological function have remained unknown because of unidentified its activator(s) [4]. In this study, I demonstrated that PCTK3 is activated by association with cyclin A2 and phosphorylates retinoblastoma protein (Rb) in vitro. Additionally, I noted that PCTK3 contains some putative PKA phosphorylation sites (Arg-Arg-X-Ser/Thr), and that the phosphorylation of PCTK3 by PKA resulted in an increase in its catalytic activity, even in the absence of the cyclin A2. Furthermore, I also found that PCTK3 is involved in the regulation of actin cytoskeleton via negatively regulating FAK and RhoA activities. These findings may provide insight into the function of PCTK3.

Chapter 4. Experimental Procedures

4.1. Antibodies and Materials

Antibody against cyclin A was purchased from BD Biosciences, and anti-cyclin B1, anti-cyclin D1, anti- cyclin E1, anti-cyclin E2, anti-cyclin H, anti-CDK1, anti-CDK2, anti-phospho-Rb (Ser^{795}) and ($Ser^{807/811}$), anti-phospho-cofilin (Ser^{3}) and anti-phospho-PKA substrate (RRXS/T), anti-phospho-FAK (Tyr^{397}), anti-FAK, anti-phospho-Src family (Tyr^{416}), anti-phospho-Src (Try^{527}) and anti-Src antibodies were from Cell Signaling Technology. Anti-PCTK3, anti-α-tubulin and anti-β-actin antibodies were from Santa Cruz Biotechnology. Anti-FLAG M2 antibody was form Sigma-Aldrich. Anti-GST antibody was from Wako Pure Chemical Industries. Anti-Strep antibody was from Qiagen. Alexa-555 conjugated-phalloidin was from Cytoskeleton.

4.2. Plasmid Construction

cDNAs encoding mouse full-length PCTK1, PCTK2, PCTK3, CDK2, PFTK1, cyclin A2, cyclin E1, cyclin K, and cyclin Y and human retinoblastoma protein (779-928 amino acid; Rb C) were cloned by PCR using the respective specific primers. PCR products were cloned into TA-cloning vector pGEM-T Easy (Promega), and the inserted DNA sequences were confirmed by DNA sequencing. The inserted DNAs were subcloned in frame into the different mammalian expression vectors including Strep-tagged expression vector pEXPR-IBA105 (IBA GmbH), glutathione S-transferase (GST) fusion expression vector pEBG and FLAG-tagged expression vector pFLAG-CMV-2 (Sigma-Aldrich). A cDNA encoding for human Rb C was subcloned into the pMAL vector (New England Biolabs) of the bacterial maltose binding protein (MBP) fusion expression system. The expression plasmid pFLAG-PKA-C encoding the catalytic subunit α isoform of mouse PKA was described previously [45]. Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the manufacturer's instructions. The mutation was confirmed by DNA sequencing analysis.

4.3. Expression and Purification of Rb Recombinant Protein

The bacterial expression plasmid of Rb, pMAL-Rb C, was introduced into the bacterial strain BL21 Star DE3 (Life Technologies). An overnight culture in LB medium was diluted fresh LB medium and incubated at 37 °C in a shaking incubator for 1 hour. After isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.2 mM, the culture was incubated at 30 °C for an additional 6 hours. The cells were resuspended in ice-cold soluble buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mg/ml aprotinin and 10 mM leupeptin). After freezing and thawing, suspended cells were sonicated on ice in short bursts. The lysate was cleared by centrifugation at 10,000 x g for 10 minutes at 4 °C. The supernatant was then incubated with amylose resin (New England Biolabs) for overnight at 4 °C. The beads were washed five times with ice-cold soluble buffer and incubated with 10 mM maltose at 4 °C to elute fusion protein from the beads. After centrifugation, the supernatant was dialyzed against phosphate-buffered saline (PBS). The purified proteins were electrophoresed on SDS-PAGE and visualized by Coomassie brilliant blue staining, and the protein concentration was determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

4.4. Cell Culture and Transfection

COS-7, HeLa and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO2. Cells were transfected with various expression vectors using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

4.5. Subcellular Fractionations

HEK293T cells were washed twice with ice-cold PBS, harvested by scraping, and lysed in a buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 10 μg/ml leupeptin and 10 μg/ml aprotinin). After incubation on ice for 15 minutes, the cells were added with 0.5% NP-40 and

homogenized by vortex for 10 seconds. The homogenate was centrifuged at 2500 rpm for 3 minutes to sediment the nuclei. The supernatant was then centrifuged at a 10,000 rpm for 10 minutes, and the resulting supernatant formed the cytoplasm fraction. The nuclear pellet was washed three times with buffer A to remove any contamination from cytoplasmic proteins. To extract nuclear proteins, the isolated nuclei were resuspended in buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin and 10 μg/ml aprotinin), and the mixture was incubated on ice for 20 minutes. Nuclear lysates were collected after centrifugation at 12,000 rpm for 15 minutes at 4 °C. The purity of nuclear and cytoplasm fractions was confirmed using anti-lamin B1 antibody (MBL) as a nuclear marker and α-tubulin antibody as a cytoplasmic marker, respectively.

4.6. Pull-down and Co-immunoprecipitation Assays

Pull-down and co-immunoprecipitation assays were performed as previously described [46]. Briefly, cells were scraped in an ice-cold TNE buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40 and 1 mM EDTA) supplemented with protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin). The cell extracts were centrifuged at 10,000 x g for 10 minutes at 4 °C prior to immunoprecipitation or immunoblotting. Equal protein amounts of the lysates were analyzed for protein expression. For pull down assay, lysates were incubated with Strep-Tactin Sepharose (IBA GmbH) or glutathione Sepharose (GE Healthcare) for overnight at 4 °C. For immunoprecipitation, lysates were incubated with anti-FLAG M2 or anti-PCTK3 antibody in the presence of protein G Sepharose (GE Healthcare) for overnight h at 4 °C. The beads were washed forth with wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40 and 1 mM EDTA). Precipitated proteins were subjected to *in vitro* kinase assay or immunoblot analysis. Protein expression and phosphorylation were determined by immunoblot of immunoprecipitates or total cell lysate. The RhoA and Rac1 activities were measured by RhoA Pull-down Activation Assay Biochem Kit or Rac1 Pull-down Activation Assay Biochem Kit (Cytoskeleton) according to the manufacture's instruction. Cells were washed with PBS and lysed in ice-cold Cell Lysis Buffer (50 mM Tris-HCl, pH7.5, 10 mM $MgCl_2$, 40

mM NaCl, 62 μg/ml Leupeptin, 62 μg/ml Pepstatin A, 14 mg/ml Benzamidine and 12 mg/ml tosyl arginine methyl ester). Cell lysates were centrifuged at 10,000 g, 4 °C for 1min. Equal protein amounts of the lysates (300 – 800 μg) were incubated with Rhotekin/ or PAK-RBD Protein beads at 4 °C on a rotator for 2 hours. After incubation, the beads were washed with wash buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl and 2 % Igepal). The amount of bound RhoA or Rac1 were analyzed by immunoblot and normalized to the total RhoA of Rac1. Quantitation was performed using Image J.

4.7. Transfection of Small Interference RNA

The synthetic small interfering RNA (siRNA) oligonucleotides were purchased from Sigma-Aldrich. siRNAs were targeting human PCTK3 on nucleotides 530-549 in NM_ 002596 for PCTK3 siRNA#1 (ID# SASI_Hs02_00334101), on nucleotides 1286-1305 for PCTK3 siRNA#2 (ID# SASI_Hs01_00015475) and on nucleotides 692-710 for PCTK3 siRNA#3 (ID# SASI_Hs01_00015476). A MISSION siRNA Universal Negative Control #1 was used as negative control. The transfection of siRNA was performed using Lipofectamine 2000 according to the manufacturer's instructions.

4.8. *In Vitro* Kinase Assay

In vitro kinase assay was carried out as previously described [46]. For *in vitro* kinase assay of PCTK3, cell lysates were incubated with a glutathione Sepharose or immunoprecipitated with anti-PCTK3 antibody and protein G Sepharose overnight at 4 °C by rotation. The beads were washed three times with wash buffer and twice with 50 mM Tris-HCl, pH7.5. The kinase reaction was carried out by resuspending the complexes in 100 μl of kinase buffer [50 mM Tris-HCl pH 7.5, 20 mM magnesium acetate, 20 μM or 50μM ATP and phosphatase inhibitor cocktail (Nacalai Tesque)] including 5 μg/ml purified MBP-Rb C in the absence or presence of 2 μCi [γ-^{32}P] ATP and incubating for 30 minutes at 30 °C. Phosphorylated MBP-Rb C was separated by SDS-PAGE and visualized with a BAS-1500 Bioimaging Analyzer (Fuji Film) or subjected to immunoblot analysis using

anti-phospho-Rb (Ser^{795}). To assay the phosphorylation of PCTK3 by PKA, cell lysate were immunoprecipitated with anti-FLAG for overnight at 4 °C by rotation. The kinase reaction was carried out by resuspending the complexes in 100 µl of kinase buffer with 2 µCi [γ-^{32}P] ATP and incubating for 30 minutes at 30 °C. Immunocomplexes were eluted by heating at 95 °C in 6 x SDS loading buffer, subjected to SDS-PAGE, and visualized by BAS-1500 Bioimaging Analyzer. Quantitative densitometric analysis was performed using Image J software.

4.9. *In Vivo* Kinase Assay

HEK293T cells were treated with 10 µM forskolin for 30 minutes and scraped in an ice-cold TNE buffer supplemented with phosphatase inhibitor cocktail. The cell extracts were centrifuged at 10,000 x g for 10 minutes at 4 °C to remove cellular debris, and the supernatants were immunoprecipitated with anti-PCTK3 antibody with protein G-Sepharose for overnight at 4 °C by rotation. The beads were washed with wash buffer, and immunocomplexes were eluted by heating at 95 °C in 6 x SDS loading buffer, subjected to SDS-PAGE, and immunoblotted using anti-phospho-RRXS/T antibody.

4.10. Immunofluorescence Analysis

Immunofluorescence analysis was performed as previously described [46]. In brief, HEK293T cells grown on poly-L-lysine-coated chamber slides were cotransfected with FLAG-PCTK3 and Myc-cyclin A2. At 24 hours post-transfection, cells were washed twice with PBS and fixed for 20 minutes in 3.7% formaldehyde. Following sequential washes with PBS, cells were permeabilized for 5 minutes in 0.1% Triton X-100, washed three times with PBS, and then treated with 5% bovine serum albumin for 30 minutes. Cells were subsequently incubated with mouse anti-FLAG M2 IgG or rabbit anti-Myc polyclonal antibody for overnight at 4 °C. Following three washes with PBS, cells were incubated for 1 hour with goat anti–mouse IgG directly conjugated to Alexa Fluor 488 or goat anti–rabbit IgG directly conjugated to Alexa Fluor 555 (Life Technologies). The slides were washed

thoroughly with PBS and mounted in fluorescent mounting medium Vectashield (Vector Laboratories). A confocal laser-scanning microscope (Leica TCS-SP5) was used to obtain staining profiles.

Chapter 5. Results

5.1. Identification of Cyclin A2 as an Activator of PCTK3

A recent report revealed that PCTK1, a member of the PCTK subfamily, was activated by membrane-associated cyclin Y [9, 47]. I hypothesized that PCTK3 is also activated by cyclin family members. To identify activators of PCTK3, I expressed a Strep-tagged mouse PCTK3 in human embryonic kidney HEK293T cells and conducted Strep pull-down experiments. Proteins coprecipitated with PCTK3 were analyzed by an immunoblot assay using anti-cyclin A, B1, D1, E1, E2, and H antibodies. Immunoblot analysis revealed that PCTK3 interacts with cyclin A and cyclin E1, but not cyclin B1, D1, E2 and H, in HEK293T cells (Fig. 4A). To confirm the interaction between PCTK3 and cyclin A2 or E1, GST pull-down experiment with lysates from COS-7 cells expressing GST-fused PCTK3 and FLAG-tagged cyclins was performed. Additionally, I also examined whether cyclin K and cyclin Y binds to PCTK3, because PCTK3 was found to interact with cyclin K in a large-scale interaction study [48]. As shown in Fig. 4B, GST-PCTK3 is associated with FLAG-cyclin A2 and cyclin E1, but not with cyclin K and cyclin Y.

Subsequently, to examine whether PCTK3 activity is regulated by binding of cyclin A2 or cyclin E1, I carried out in vitro kinase assay using retinoblastoma protein (Rb) as a substrate. Rb is phosphorylated by some CDKs in a cell cycle-dependent manner, and PFTAIRE kinase 1 (PFTK1), which is activated by cyclin D3 and cyclin Y, also phosphorylates Rb [49, 50]. The C-terminus of Rb contains a cluster of seven candidate CDK phosphorylation sites (Ser/Thr-Pro motifs; Ser^{780}, Ser^{788}, Ser^{795}, Ser^{807}, Ser^{811}, Thr^{821}, and Thr^{826}) [51]. For in vitro kinase assay, I produced recombinant maltose-binding protein (MBP)-fused Rb C (amino acids 779–928) in *E. coli*. COS-7 cells were cotransfected with GST-PCTK3 in the presence or absence of FLAG-cyclin A2 or E1, and cell lysates were subjected to glutathione-Sepharose pull-down. An *in vitro* kinase analysis with [γ-^{32}P] ATP demonstrated that MBP-Rb C was phosphorylated by PCTK3 in the presence of cyclin A2, whereas cyclin E1 did not activate PCTK3 (Fig. 4C). Furthermore, an *in vitro* kinase assay using myelin basic protein (MyBP) and histone H1 as substrates was performed. As shown in Fig. 4D, cyclin A2/CDK2

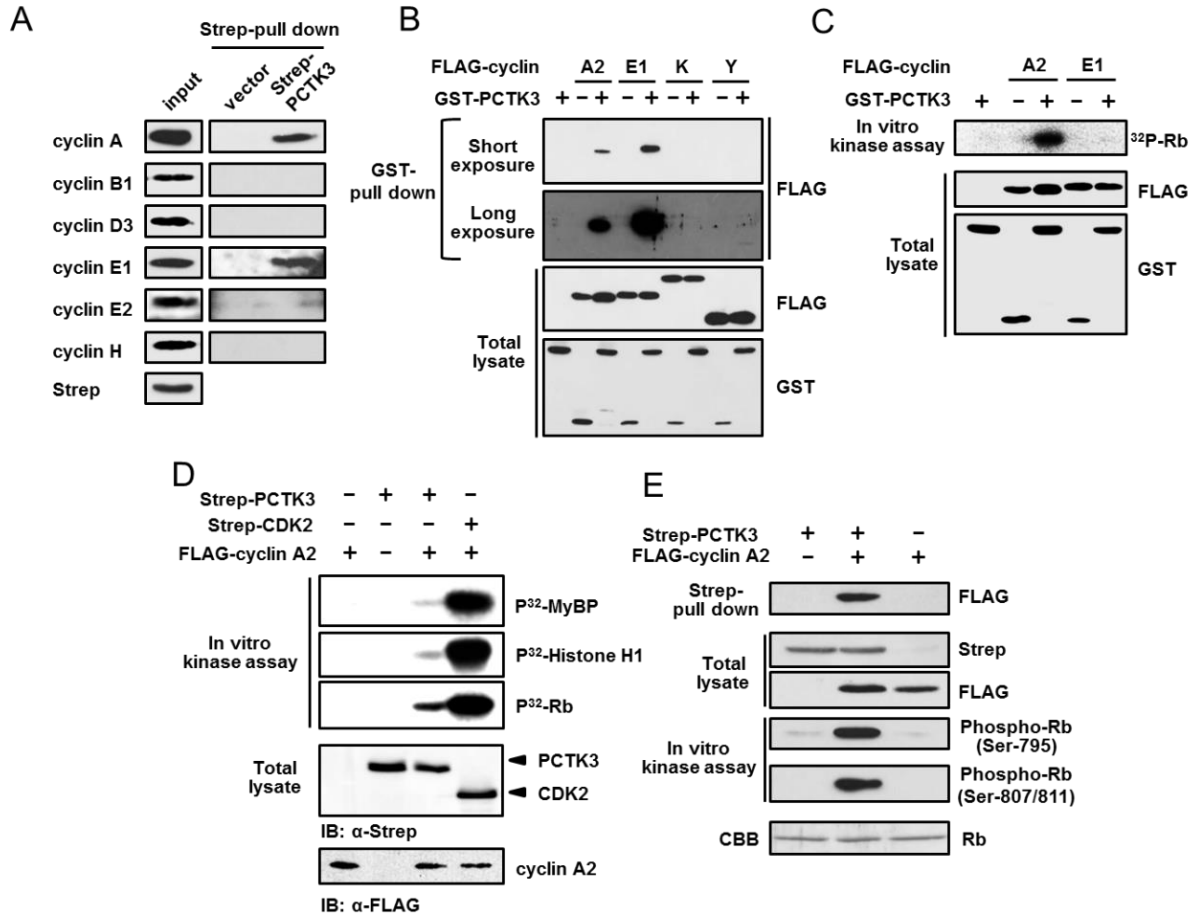

Figure 4. Identification of cyclin A2 as an activator of PCTK3. *A*, HEK293T cells were transfected with Strep-PCTK3. After the Strep pull down assay, the precipitated proteins were subjected to immunoblot analysis with anti-cyclin A, anti-cyclin B1, anti-cyclin D3, anti-cyclin E1, anti-cyclin E2, and anti-cyclin H antibodies. Total cell lysates were used as the input samples and positive control. *B*, GST-PCTK3 was expressed in COS-7 cells with either FLAG-cyclin A2, FLAG-cyclin E1, FLAG-cyclin K or FLAG-cyclin Y. In the control experiment (GST-PCTK3 (-)), GST alone was expressed instead of GST-PCTK3. The GST pull-down precipitates were immunoblotted with anti-FLAG antibody. Protein expression was confirmed by immunoblotting total cell lysates. *C*, GST and GST-PCTK3 was expressed with either FLAG-cyclin A2 or FLAG-cyclin E1 in COS-7 cells. After the GST pull down, the precipitated samples were incubated in a kinase buffer containing [γ-^{32}P] ATP and MBP-Rb C for 30 minutes. The supernatants containing MBP-Rb C were separated on SDS-PAGE, after which the gel was analyzed by bioimaging analyzer. The expression level of GST-fused and FLAG-tagged proteins was confirmed by immunoblotting with anti-FLAG and anti-GST antibody, respectively. *D*, COS-7 cells were transfected with Strep-PCTK3 and FLAG-cyclin A2. GST pull downed proteins were incubated in a kinase buffer containing MBP-Rb C for 30 minutes. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) and (Ser$^{807/811}$) antibodies, respectively. *D*, COS-7 cells were transfected with Strep-PCTK3, Strep-CDK2 and FLAG-cyclin A2. Strep pull-downed proteins were incubated in a kinase buffer containing MyBP, MBP-Rb C or Histone H1 for 30 minutes. The supernatants were analyzed by SDS-PAGE and bioimaging analyzer. *E*, Strep-PCTK3 was expressed in COS7 cells with FLAG-cyclin A2. After the Strep pull-down, bound proteins were incubated in a kinase buffer containing MBP-Rb C for 30 minutes. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) and (Ser$^{807/811}$) antibodies, respectively.

complex strongly phosphorylated all three substrates tested (Rb, MyBP and histone H1). On the other hand, catalytic activity of PCTK3 was much less than that of CDK2 even in the presence of cyclin A2. Among three substrates tested, Rb was most efficiently phosphorylated by cyclin A2/PCTK3 complex. Additionally, phosphorylated MBP-Rb C was detected using antibodies against phospho-Rb (Ser^{795}) and phospho-Rb ($Ser^{807/811}$) (Fig. 4E). Both antibodies detected Rb phosphorylation only when PCTK3 was complexed with cyclin A2, indicating that cyclin A2/PCTK3 complex phosphorylates Rb at least at Ser^{795} and $Ser^{807/811}$ *in vitro*. These results revealed that PCTK3 is activated through association with cyclin A2.

5.2. Cyclin A2 Specifically Activates PCTK3

Cyclin A2 has been shown to interact with CDK1 and CDK2 during cell cycle [52]. To evaluate whether the phosphorylation of Rb is dependent on PCTK3 or other kinases coprecipitated with cyclin A2, I performed GST-pull down experiments. As shown in Fig. 5A, endogenous CDK1 and CDK2 were specifically coprecipitated with GST-cyclin A (lane 4), while GST-PCTK3, GST-PCTK3/FLAG-cyclin A2 complex, and FLAG-cyclin A2 did not coprecipitate endogenous CDK1 and CDK2 (lane1-3). Furthermore, I designed PCTK3 inactive mutant based on amino acids conserved between various members of the CDK family. The analogous K194R mutation at the putative ATP binding site of PCTK1 has been reported to generate kinase dead mutants [5]. A kinase-dead mutant of PCTK3 in which the putative ATP binding site was replaced by arginine (PCTK3 K150R) was created and tested by in vitro kinase assay. Although both PCTK3 wild-type and K150R were interacted with cyclin A2, PCTK3 K150R activity was almost completely lost (Fig. 5B). These results indicated that the phosphorylation of Rb is dependent on PCTK3 but not other kinases, and strongly supported that cyclin A2 is an activator of PCTK3.

Furthermore, I examined whether other members of the PCTK subfamily, PCTK1 and PCTK2, is activated by cyclin A2, because activators of PCTK2 have not yet been identified. Interestingly, although PCTK1 and PCTK2 were interacted with cyclin A2, only PCTK3 was activated by cyclin A2

(Fig. 5C). On the other hand, PFTK1 with high homology to PCTK3 was not even associated with cyclin A2, and was also unable to phosphorylate Rb C (Fig. 5D). However, GST-pull down and *in vitro* kinase assays showed that CDK2 had a greater affinity for cyclin A2 than PCTK3, and that catalytic activity of PCTK3 was much less than that of CDK2 in the presence of cyclin A2 (Fig. 5D). These results demonstrated that cyclin A2 specifically activates PCTK3, and implied the existence of other activators of PCTK3 besides cyclin A2.

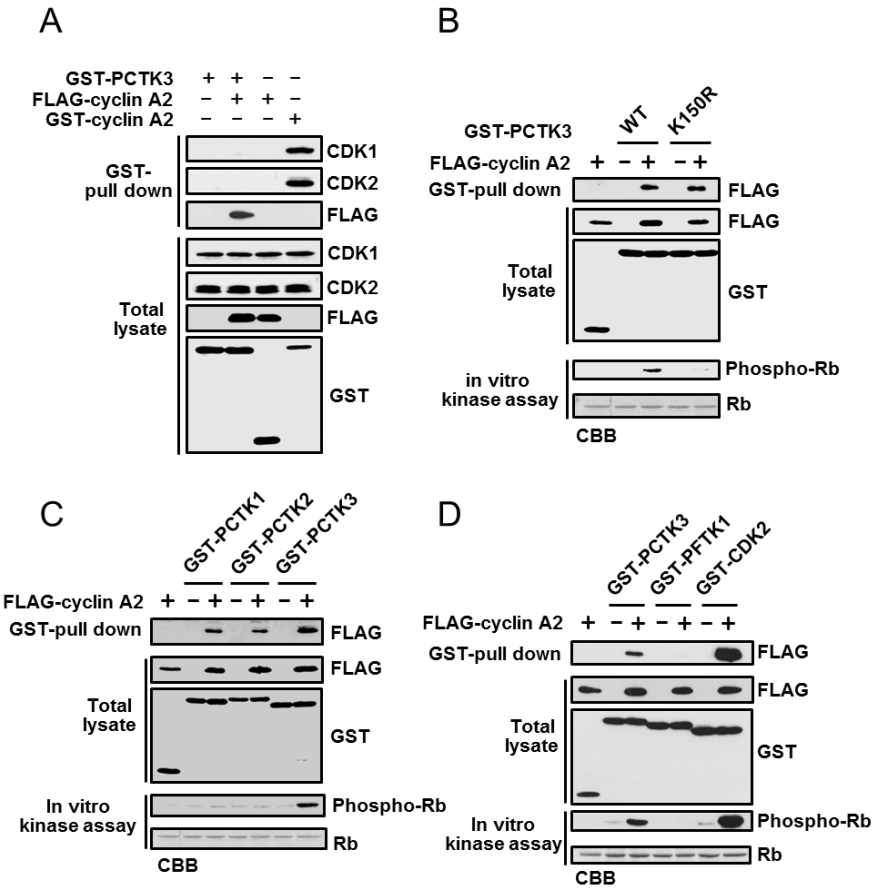

Figure 5. Cyclin A2 specifically activates PCTK3. *A*, COS-7 cells were transfected with GST-PCTK3, FLAG-cyclin A2 and GST-cyclin A2. In the control experiment, GST alone was transfected instead of GST-PCTK3 or GST-cyclin A2. The GST pull-down precipitates were subjected to immunoblotting with anti-FLAG, anti-CDK1, and anti-CDK2 antibodies. *B*, GST-PCTK3 wild type (WT) or K150R mutant was expressed in COS-7 cells with FLAG-cyclin A2. In the control experiment, GST alone was expressed instead of GST-PCTK3. After the GST pull down, the bound proteins were incubated in a kinase buffer with MBP-Rb C. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) antibodies, respectively. *C,* FLAG-cyclin A2 was expressed in COS-7 cells with GST, GST-PCTK1, GST-PCTK2 or GST-PCTK3. The GST pull-down precipitates were subjected to *in vitro* kinase assay using anti-phospho-Rb (Ser^{795}) antibody. *D,* GST, GST-PCTK3, GST-PFTK1, or GST-CDK2 was expressed in COS-7 cells with FLAG-cyclin A2. The GST pull downed proteins were subjected to *in vitro* kinase assay.

5.3. Endogenous Interaction between PCTK3 and Cyclin A2 in Cytoplasm

Next, I examined the endogenous interaction between PCTK3 and cyclin A2. A previous study reported that PCTK3 protein is expressed in some cell lines including HEK293 cells [6]. Thus, I confirmed whether PCTK3 expression was detectable in some cell lines. As shown in Fig. 6A, anti-PCTK3 antibody detected a specific protein band with the predicted molecular mass of PCTK3 in HEK293T cells, while its expression was very low or absent in human breast carcinoma MCF7cells and human cervical carcinoma HeLa cells. PCTK3 expression in HEK293T cells was confirmed by knock down analysis using siRNA. As shown in Fig. 6B, PCTK3 expression was efficiently reduced by each of three PCTK3 siRNA, whereas negative control siRNA had no effect on PCTK3 expression.

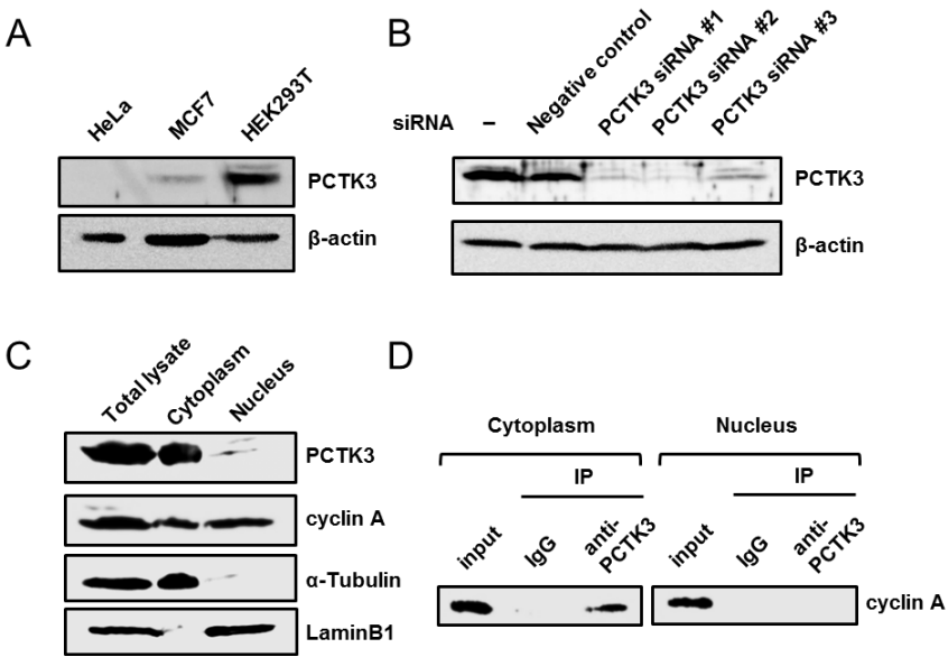

Figure 6. Endogenous interaction between PCTK3 and cyclin A in the cytoplasm. *A,* Immunoblot analysis of total proteins prepared from HeLa, MCF7 and HEK293T human cell lines. β-actin was used as a loading control. *B,* siRNA knockdown of PCTK3 in HEK293T cells. Cells were transfected with three different siRNAs against PCTK3. Forty-eight hours after transfection, cell extracts were immunoblotted with anti-PCTK3 antibody. Expression of β-actin was used as a loading control. *C,* Subcellular localization of endogenous PCTK3 and cyclin A. HEK293T cells were separated into nuclear and cytoplasmic fractions as described in EXPERIMENTAL PROCEDURES. Aliquots of each fraction were immunoblotted for PCTK3 and cyclin A. The purity of nuclear and cytoplasmic fractions was confirmed using anti-lamin B1 antibody as a nuclear marker and α-tubulin antibody as a cytoplasmic marker, respectively. *D,* Endogenous interaction between PCTK3 and cyclin A in HEK293T cells. The fractionated lysates from HEK293T cells were immunoprecipitated using anti-PCTK3 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-cyclin A antibody. Normal rabbit IgG (*IgG*) was used as a control.

Furthermore, I examined the distribution of PCTK3 and cyclin A between nucleus and cytoplasm using a simple cell fractionation. PCTK3 protein appeared in the cytoplasm, while cyclin A was localized to both the cytoplasm and nucleus (Fig. 6C). I then immunoprecipitated PCTK3 from cytoplasmic and nuclear fractions of HEK293T cells using anti-PCTK3 antibody, and immunoprecipitates were assayed by immunoblotting with anti-cyclin A antibody. Cyclin A was detected in the cytoplasmic PCTK3 immunoprecipitates but not in the nuclear (Fig. 6D), indicating that PCTK3 indeed interacts with cyclin A *in vivo*.

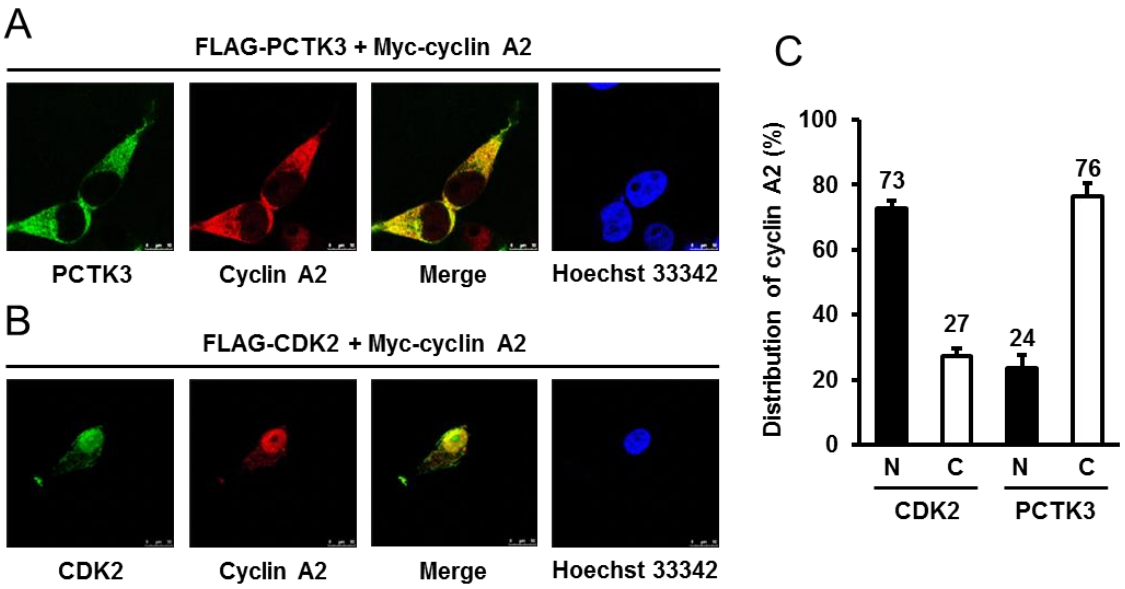

Figure 7. Subcellular localization of PCTK3 and cyclin A2. *A and B,* HEK293T cells were cotransfected with Myc-cyclin A2 together with either FLAG-PCTK3 (*A*) or FLAG-CDK2 (*B*). Cells were fixed and incubated with mouse anti-FLAG and rabbit anti-Myc antibodies. The primary antibody was visualized with Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-rabbit IgG, followed by confocal microscopy. Fluorescence for FLAG-PCTK3 or FLAG-CDK2 (*green*) and Myc-cyclin A2 (*red*) is shown with the merged images (merge is in *yellow*). Hoechst nuclear staining is represented in *blue*. *C,* Fluorescence intensities in the nuclear (*N, closed bar*) and cytoplasmic (*C, open bar*) regions were quantified using Image J software. The data shown are the means ± S.E. ($n \geq 5$).

To visualize the subcellular distribution of PCTK3 and cyclin A2, FLAG-PCTK3 and Myc-cyclin A2 were cotransfected into HEK293T cells and examined by immunofluorescence analysis using confocal laser-scanning microscope. FLAG-PCTK3 was present at the cytoplasm and Myc-cyclin A2 was distributed in the cytoplasm and nucleus (Fig. 7A), consistent with a simple cell fractionation

experiment (Fig. 6C). The merged data showed significant overlap between FLAG-PCTK3 and Myc-cyclin A (Fig. 7B), indicating that cyclin A is colocalized with PCTK3 in the cytoplasm. On the other hand, cyclin A was colocalized with CDK2 in the nucleus (Fig. 7B). Interestingly, cyclin A expression in the cytoplasm was enhanced by coexpression of PCTK3 compared with that of CDK2 (Fig.7C), suggesting that PCTK3 regulates the localization and stability of cyclin A2 in the cytoplasm. Together, these findings demonstrate that PCTK3 is interacted with cyclin A in the cytoplasm.

5.4. PCTK3 Protects Cyclin A2 against Degradation in Cytoplasm

It is known that cyclin A2 is degraded in prometaphase by anaphase-promoting complex/cyclosome and its process is necessary for mitosis progression [53(59)]. I hypothesized that cyclin A2 is protected from degradation by PCTK3 in cytoplasm. Thus, to analyze whether PCTK3 regulates the stability of cyclin A2, I investigated the expression level of cyclin A2 in PCTK3

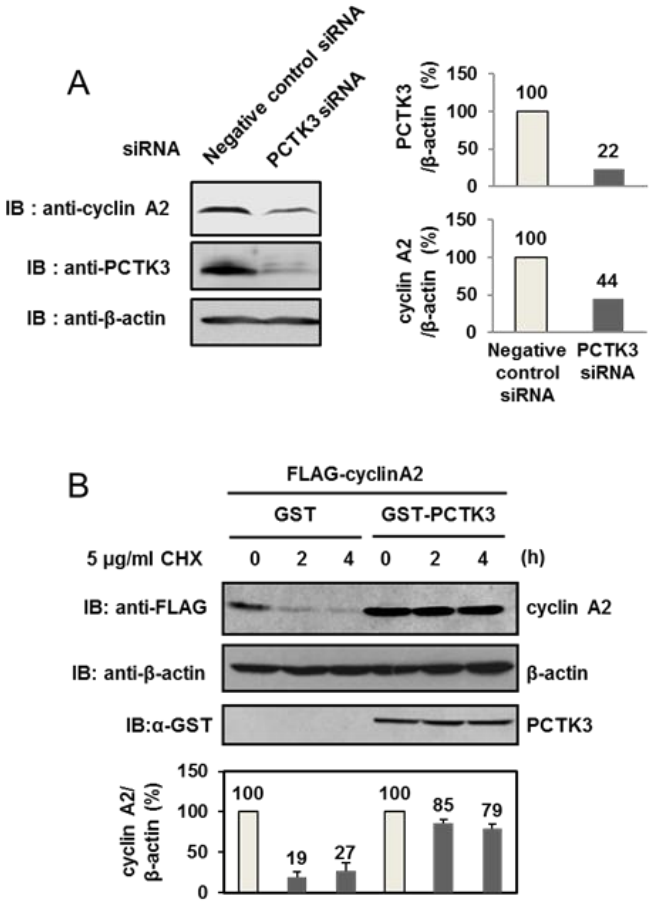

Figure 8. PCTK3 protects cyclin A2 against degradation in the cytoplasm. *A.* HEK293T cells were transfected with PCTK3 siRNA. The cell lysates were subjected to immunoblotting with anti-PCTK3 and anti-cyclin A antibodies. Expression of α-tubulin was used as a loading control. *B,* HEK293T cells were cotransfected with GST or GST-PCTK3, together with FLAG-cyclin A2. After 24 hours, cycloheximide (*CHX*) (5 μg/ml) was added to the cell culture. Cells were harvested at indicated times after cycloheximide treatment and FLAG-cyclin A2 expression levels were determined by immunoblotting (IB: anti-FLAG). β-actin was used as a loading control (IB: anti-β-actin). FLAG-cyclin A2 expression levels were quantified by densitometric analysis and represented in a graph.

knockdown HEK293T cells. As expected, PCTK3 knockdown cells show a reduction in the expression level of cyclin A2 relative to negative control siRNA-treated cells (Fig. 8A). Furthermore, to verify the protective effect of PCTK3 against degradation of cyclin A2, HEK293T cells cotransfected with FLAG-cyclin A2 and GST-PCTK3 were treated with cycloheximide, a protein synthesis inhibitor. The cells were harvested at indicated times after treatment, and total cell lysates were subjected to immunoblot analysis. As shown in Fig. 8B, the expression level of cyclin A2 in the absence of GST-PCTK3 was clearly decreased, whereas cyclin A2 expression in the presence of GST-PCTK3 was slightly decreased. These results suggest that PCTK3 may protect the cyclin A2 from degradation.

5.5. PCTK3 is Phosphorylated by PKA *in Vitro* and *in Vivo*

PKA is an essential factor which is concerned with many of the various physiological functions, including cytoskeletal organization, cell motility, and signal transduction [54]. PKA has been reported to preferentially phosphorylate serine/threonine residues within the consensus sequence Arg-Arg-X-Ser/Thr [18]. A previous report demonstrated that PKA inactivates PCTK1 via phosphorylation at Ser^{153} [5]. Amino acid sequence analysis revealed that mouse and human PCTK3 contain four putative phosphorylation sites ($RRLS^{12}$, $RRFS^{66}$, $RRAS^{109}$ and $RRQS^{449}$ in mouse and $RRFS^{14}$, $RRFS^{89}$, $RRAS^{162}$ and $RRQS^{502}$ in human) for PKA, respectively, suggesting that PCTK3 is a substrate for PKA. To examine whether endogenous PCTK3 is phosphorylated by PKA, HEK293T cells were treated with an adenylate cyclase activator, forskolin. Cell lysates were immunoprecipitated with anti-PCTK3 antibody, and immunoblot analysis using an anti-phospho RRXS/T antibody was examined. As shown in Fig. 9A, PCTK3 protein was specifically precipitated by anti-PCTK3 antibody and was efficiently phosphorylated by treatment with forskolin. Next, I examined whether PKA directly phosphorylates PCTK3. COS-7 cells were cotransfected with FLAG-PCTK3 and FLAG-PKA catalytic subunit, and in vitro kinase assay was carried out. As shown in Fig. 9B, PKA directly phosphorylated PCTK3. Furthermore, to identify phosphorylation sites of PCTK3 by PKA, four nonphosphorylatable mutants of mouse PCTK3 in which each of the putative

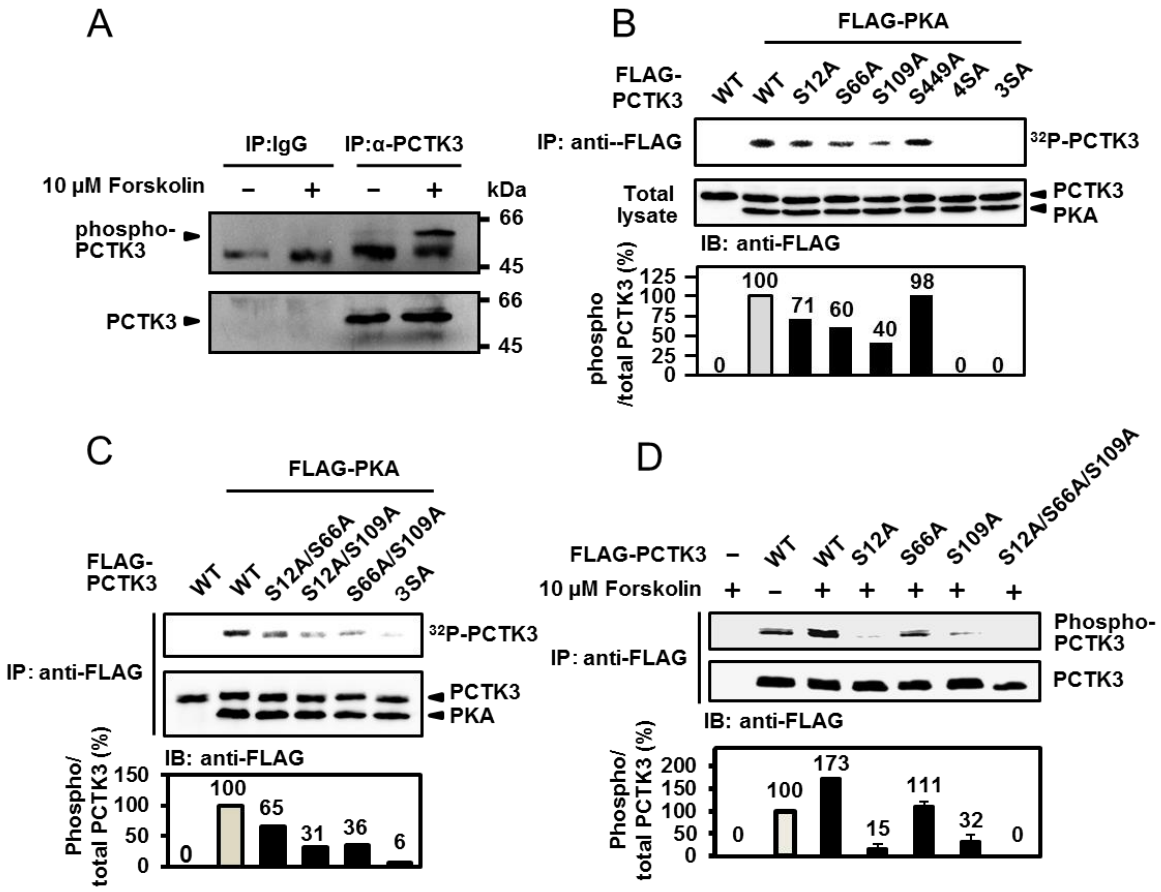

Figure 9. PCTK3 is phosphorylated by PKA *in vitro* and *in vivo*. *A*, Endogenous PCTK3 is phosphorylated by PKA. HEK293T cells were treated with 10 μM forskolin for 30 minutes. Cell lysates were immunoprecipitated with anti-PCTK3 antibody. Immunoprecipitates were analyzed by immunoblot analysis using anti-phospho-RRXS/T or anti-PCTK3 antibodies. The bands of phosphorylated and total PCTK3 were indicated by arrows. *B*, FLAG-PKA C, FLAG-PCTK3 wild type (*WT*), and mutants (S12A, S66A, S109, S449, S12A/S66A/S109A/S449A (*4SA*), and S12A/S66A/S109A (*3SA*)) were expressed in COS-7 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with kinase buffer containing [γ-^{32}P] ATP. Equal amounts of cell lysates were analyzed by immunoblotting with anti-FLAG antibody. The graph indicates the phosphorylation rate of PCTK3. The phosphorylation rate of wild-type PCTK3 was taken as 100%. *C*, The double or triple mutants of PCTK3 (S12A/S66A, S12A/S109A, S66A/S109A, and 3SA) were subjected to *in vitro* kinase assay using PKA. *D*, HeLa cells were transfected with FLAG-PCTK3 wild type (*WT*) and mutants (S12A, S66A, S109A, and 3SA). After 24 hours, transfected cells were treated with 10 μM forskolin at 30 minutes. Cell extracts were subjected to immunoprecipitated with anti-FLAG antibody (IP: anti-FLAG). Immunoprecipitates were subsequently analyzed by immunoblotting with anti-phospho-RRXS/T or anti-FLAG antibody.

PKA phosphorylation sites was replaced by alanine (S12A, S66A, S109A and S449A) were constructed and tested by the in vitro and in vivo kinase assay. Phosphorylation of S12A, S66A and S109A mutants was reduced compared with that of wild-type PCTK3, whereas the phosphorylation intensity of S449A mutant was almost equal to that of wild-type PCTK3 (Fig. 9B). Although PKA phosphorylated S12A/S66A, S66A/S109A, and S12A/S109A mutants of PCTK3 (Fig. 9C), it failed to phosphorylate S12A/S66A/S109A mutant (Fig. 9B and C). These results identified three serine residues (Ser^{12}, Ser^{66} and Ser^{109}) as potential phosphorylation sites of mouse PCTK3 by PKA. Furthermore, phosphorylation sites of PCTK3 by PKA were confirmed by an in vivo kinase assay using an anti-phospho RRXS/T antibody. HeLa cells transiently transfected with FLAG-tagged PCTK3 were treated with 10μM forskolin. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and then immunoblotted with anti-phospho RRXS/T antibody. As expected, forskolin treatment resulted in a 1.7-fold increase of PCTK3 phosphorylation, while phosphorylation of the S12A, S66A and S109A mutants was reduced compared with that of wild-type PCTK3 (Fig. 9D). These findings provide evidence that PCTK3 is phosphorylated by PKA at three sites ($RRLS^{12}$, $RRFS^{66}$ and $RRAS^{109}$ in mouse) *in vitro* and *in vivo*.

5.6. PCTK3 Activity Is Regulated via Phosphorylation at Ser^{12} by PKA

I then investigated the effects of PKA phosphorylation of PCTK3 on its kinase activity. PCTK3 activity was determined by GST-pull down and *in vitro* kinase assays using Rb C as the substrate. Three phospho-mimic PCTK3 mutants (PCTK3 S12D, S66D, and S109D), in which the PKA phosphorylation sites were replaced by aspartic acid, were generated, and the kinase activities of these phospho-mimic mutants were examined. As shown in Fig. 10A, the PCTK3 S12D mutant had relatively high activity for Rb phosphorylation, even in the absence of cyclin A2. Furthermore, the catalytic activity of the cyclin A2/S12D mutant complex was significantly increased (> 2-fold) as compared with that of cyclin A2/wild-type PCTK3, although the ability of S12D mutant to bind to cyclin A was not changed. By comparison, the PCTK3 S66D and S109D mutants had no effect on

kinase activity. On the other hand, the activity of the phospho-null mutant S12A was obviously decreased as compared with that of wild-type PCTK3 (Fig. 10B), strongly supporting that the phosphorylation at Ser^{12} influences the catalytic activation. In addition, the kinase activity of the PCTK3 S12D mutant was compared with that of the cyclin A2/CDK2 complex. Although cyclin A2/wild-type PCTK3 activity was six times less than that of cyclin A2/CDK2, the cyclin A2/S12D mutant had strong activity and reached a level comparable with the kinase activity of cyclin A2/CDK2 (Fig. 10C).

I also determined whether endogenous PCTK3 was activated by PKA. First, to determine endogenous PCTK3 activity, I immunoprecipitated PCTK3 from HEK293T cells transfected with negative control siRNA or PCTK3 siRNA and determined *in vitro* kinase activity using Rb as the substrate. As shown in Fig. 10D, significantly high Rb phosphorylation activity was detected in immunoprecipitates from cells transfected with negative control siRNA as compared with those from cells transfected with PCTK3 siRNA, suggesting that this activity was dependent on endogenous PCTK3 activity. Therefore, I assessed the effects of forskolin treatment on the kinase activity of PCTK3. Rb phosphorylation was significantly increased after forskolin treatment. These data demonstrated that PCTK3 is activated via phosphorylation at Ser^{12} by PKA.

Several members of the CDK family preferentially phosphorylate the canonical motif Ser/Thr–Pro–X–Lys/Arg/His (where X represents any amino acid). Because PCTK3 phosphorylated Rb *in vitro*, I investigated whether Rb was phosphorylated by PCTK3 *in vivo*. HEK293T cells were transfected with FLAG-tagged PCTK3 wild type or S12D mutant, after which cell lysates were subjected to immunoblotting using anti-phospho-Rb (Ser^{795}). Both PCTK3 wild type and S12D failed to phosphorylate endogenous Rb, although Rb phosphorylation was detected in cyclin A2/CDK2 transfected cells (Fig. 10E). This suggested that Rb is a poor substrate for PCTK3 *in vivo*, possibly due to its different subcellular localization.

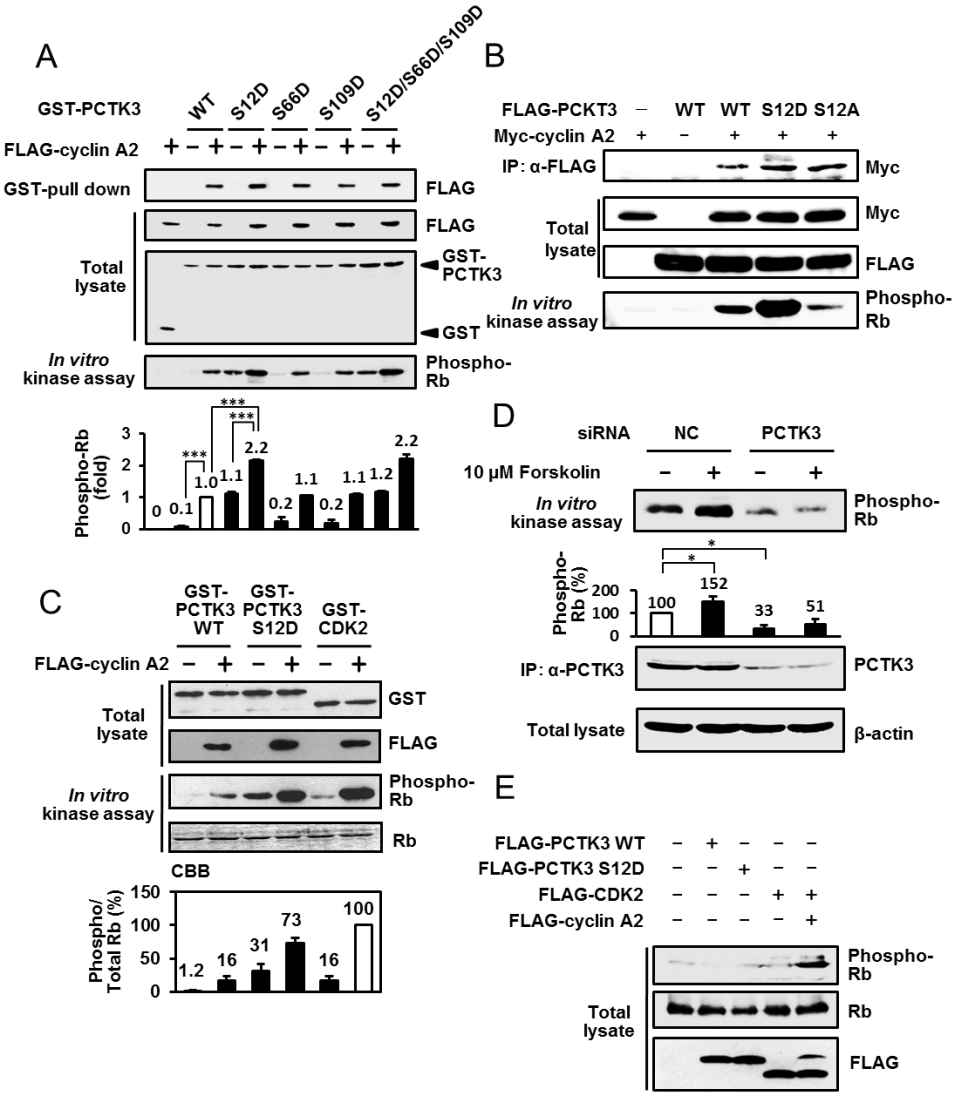

Figure 10. PCTK3 activity is regulated via phosphorylation at Ser[12] by PKA. *A*, COS-7 cell lysates expressing GST-PCTK3 mutants (S12D, S66D, S109D, and S12D/S66D/S109D) and FLAG-cyclin A2 were used in a kinase assay using Rb as the substrate. The relative kinase activity of PCTK3 was quantified by densitometric analysis. The activity of wild-type PCTK3 complexed with FLAG-cyclin A2 was taken as 1. Results are expressed as means ± S.E. from three independent experiments. Statistical significance was determined by ANOVA. ***, $p < 0.001$. *B*, FLAG-PCTK3 wild type (*WT*), S12D, S12A mutants were expressed with Myc-cyclin A2 in COS-7 cells. The cell lysates were immunoprecipitated with anti-FLAG antibody (IP: anti-FLAG), and an *in vitro* kinase assay was performed using MBP-Rb C as the substrate. *C*, GST-PCTK3 wild type (*WT*), S12D mutant and GST-CDK2 was expressed in COS-7 cells with or without FLAG-cyclin A2. The protein complexes were pulled down by glutathione-Sepharose beads, and an *in vitro* kinase assay was performed using MBP-Rb C protein as the substrate. The graph shows the average of three independent experiments. *D*, Expression of PCTK3 in HEK293T cells was knocked down by siRNA. PCTK3 knockdown cells were treated with 10 μM forskolin at 30 minutes. Cell lysates were immunoprecipitated with anti-PCTK3 antibody. Immunoprecipitates were subjected to *in vitro* kinase assay using Rb as the substrate. The activity of endogenous PCTK3 in negative control (*NC*) knockdown cells was taken as 100%. Experiments were performed three times independently. Results are expressed as means ± S.E. Statistical significance was determined by ANOVA. *, $p < 0.05$. *E*, HEK293T cells were transfected with FLAG-PCTK3 wild type and S12D mutant, FLAG-CDK2, and FLAG-cyclin A2. After 24 hours, cell extracts were analyzed by immunoblotting with anti-phospho-Rb (Ser[795]), anti-Rb, and anti-FLAG antibodies.

5.7. Suppression of PCTK3 Induces Actin Cytoskeletal Change through Phosphorylation of Cofilin

Furthermore, I explored possible physiological functions of PCTK3 using a RNA interference approach. I noted that PCTK3 knockdown in HEK293T cells induced morphological changes, cell spreading, and extensions (Fig. 11A and B). To investigate whether this is responsible for alteration of the actin cytoskeleton, F-actin was visualized by staining cells with phalloidin. Control HEK293T cells exhibited a relatively weak cortical distribution of F-actin (Fig. 11B, upper panel). In PCTK3 knockdown cells, lamellipodial extensions were induced, and F-actin was concentrated at these lamellipodia. PCTK3 knockdown also stimulated the appearance of stress fibers (Fig. 11B, lower panel). Furthermore, the effects of PCTK3 overexpression on actin filament dynamics were examined. Constitutively active mutant of PCTK3 (PCTK3 S12D) suppressed formation of actin stress fibers, whereas wild-type PCTK3 and S12A mutant did not.

Actin filament dynamics are regulated by actin-depolymerizing factor/cofilin proteins, and cofilin is inactivated through the phosphorylation at Ser^3 [55, 56]. Therefore, I investigated the phosphorylation states of cofilin in PCTK3 knockdown HEK293T cells by immunoblotting using anti-phospho-cofilin (Ser^3). As shown in Fig. 11D, cofilin phosphorylation in PCTK3 knockdown HEK293T cells was markedly increased as compared with that in control cells. In contrast, overexpression of PCTK3 S12D effectively suppressed cofilin phosphorylation without affecting total cofilin protein levels, although wild-type PCTK3 did not affect (Fig. 11E). The phospho-null S12A mutant rather slightly increased. These data suggested that PCTK3 was involved in the actin cytoskeleton organization.

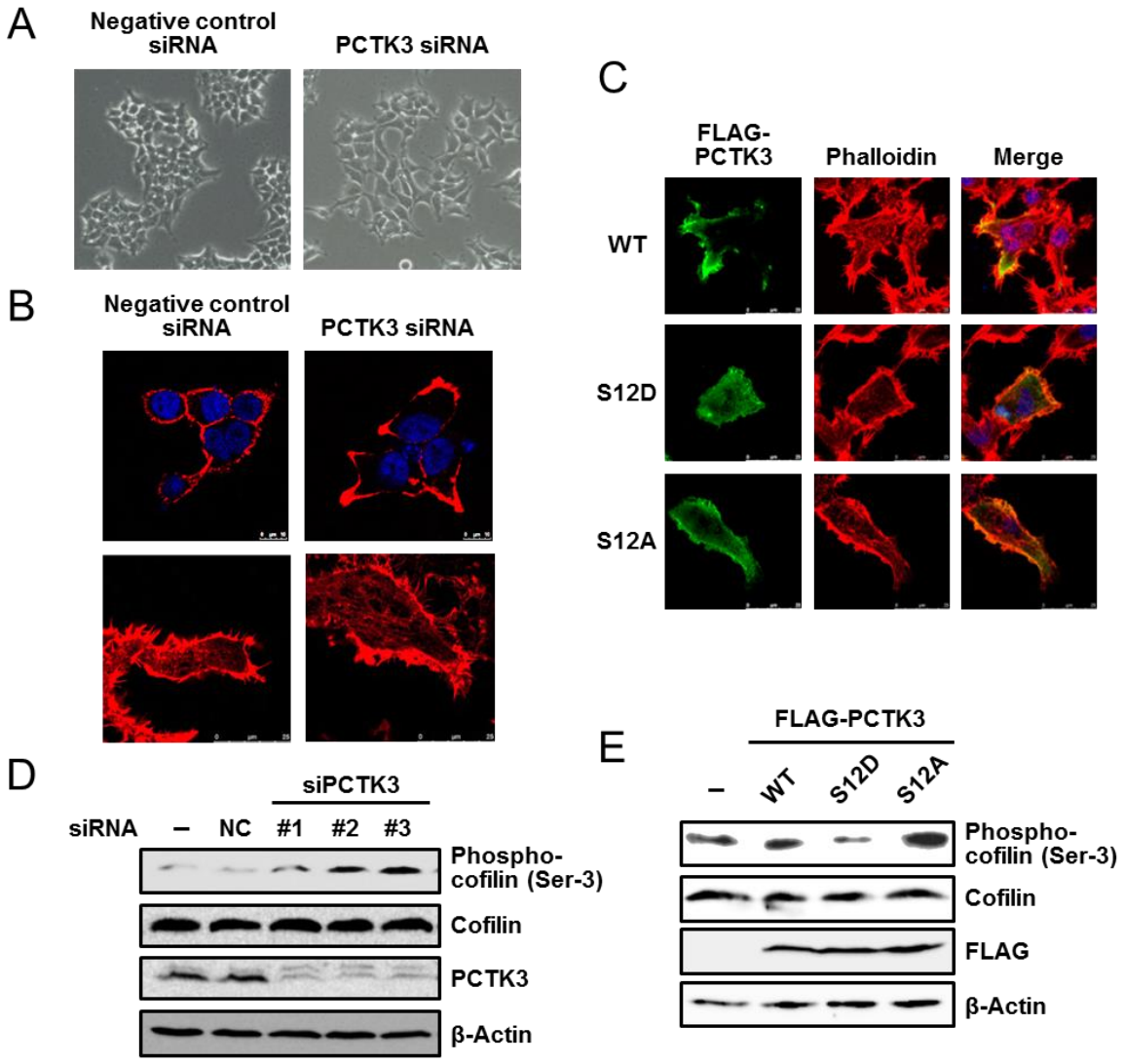

Figure 11. Suppression of PCTK3 induces actin cytoskeletal changes through cofilin phosphorylation. *A* and *B*, HEK293T cells were transfected with negative control siRNA or PCTK3 siRNA. After 48 hours, cells were fixed and incubated with Alexa 555-conjugated phalloidin (*B*, *red*). Hoechst nuclear staining is represented in *blue*. *C*, HEK293T cells were transfected with PCTK3 siRNA for 48 hours and then transfected with FLAG-tagged mouse PCTK3 wild type (*WT*), S12D or S12A mutants. After 24 hours, cells were fixed and incubated with mouse anti-FLAG antibody. The primary antibody was visualized with Alexa Fluor 488-conjugated anti-mouse IgG, followed by confocal microscopy. Fluorescence for FLAG-PCTK3 (*green*) and F-actin (Alexa 555-conjugated phalloidin staining, *red*) are shown with merged images (merge is in *yellow*). Hoechst nuclear staining is represented in *blue*. *D*, Cell lysates of PCTK3 knock-down HEK293T cells were subjected to immunoblotted with anti-PCTK3, anti-cofilin, and anti-phospho-cofilin (Ser^3) antibodies. Expression of β-actin was used as a loading control. *E*, HEK293T cells were transfected with FLAG-PCTK3 (wild-type (*WT*), S12D or S12A mutant). After 24 hours, cell lysates were subjected to immunoblotting using anti-phospho-cofilin (Ser^3), anti-cofilin, and anti-FALG antibodies.

5.8. PCTK3 Negatively Regulates the FAK and RhoA

Cofilin phosphorylation is controlled by RhoA/ROCK signaling pathway, which also induces stress fiber formation [29]. Thus, I investigated the RhoA and Rac1 activities by using Rhotekin/ or PAK RBD pull down methods. As shown Fig. 12A, PCTK3 knockdown led to RhoA activation and Rac1 inactivation in HEK293T cells, indicating that PCTK3 regulates the balance between the RhoA and Rac1 activities. To gain the insight into the mechanisms of RhoA regulation by PCTK3, I explored the signal transduction pathway between PCTK3 and RhoA by using various anti-phospho antibodies. As shown in Fig. 12B, immunoblot analysis revealed that phospho-Focal adhesion kinase (FAK) (Tyr^{397}) and phospho-Src family (Tyr^{416}) are increased in PCTK3-knockdown cells, while the expression level of FAK1 and Src showed no change. In addition, PCTK3-knockdown did not affect the phosphorylation of Src at Try^{527} which is the CSK (C-terminal Src kinase) phosphorylation site, indicating that PCTK3 does not implicate the CSK regulation. Interestingly, I observed that phosphorylated FAK and phosphorylated Src were co-localized with F-actin at lamellipodia in PCTK3-knockdown cells, whereas phosphorylated FAK and phosphorylated Src did not detected in control cells (Fig. 12C and 12D). FAK plays a central role in the focal adhesion assembly and activating various cytoskeletal-associated proteins including RhoA and Rac1. Taken together, PCTK3 controls actin cytoskeleton through negatively regulating the FAK and RhoA activities.

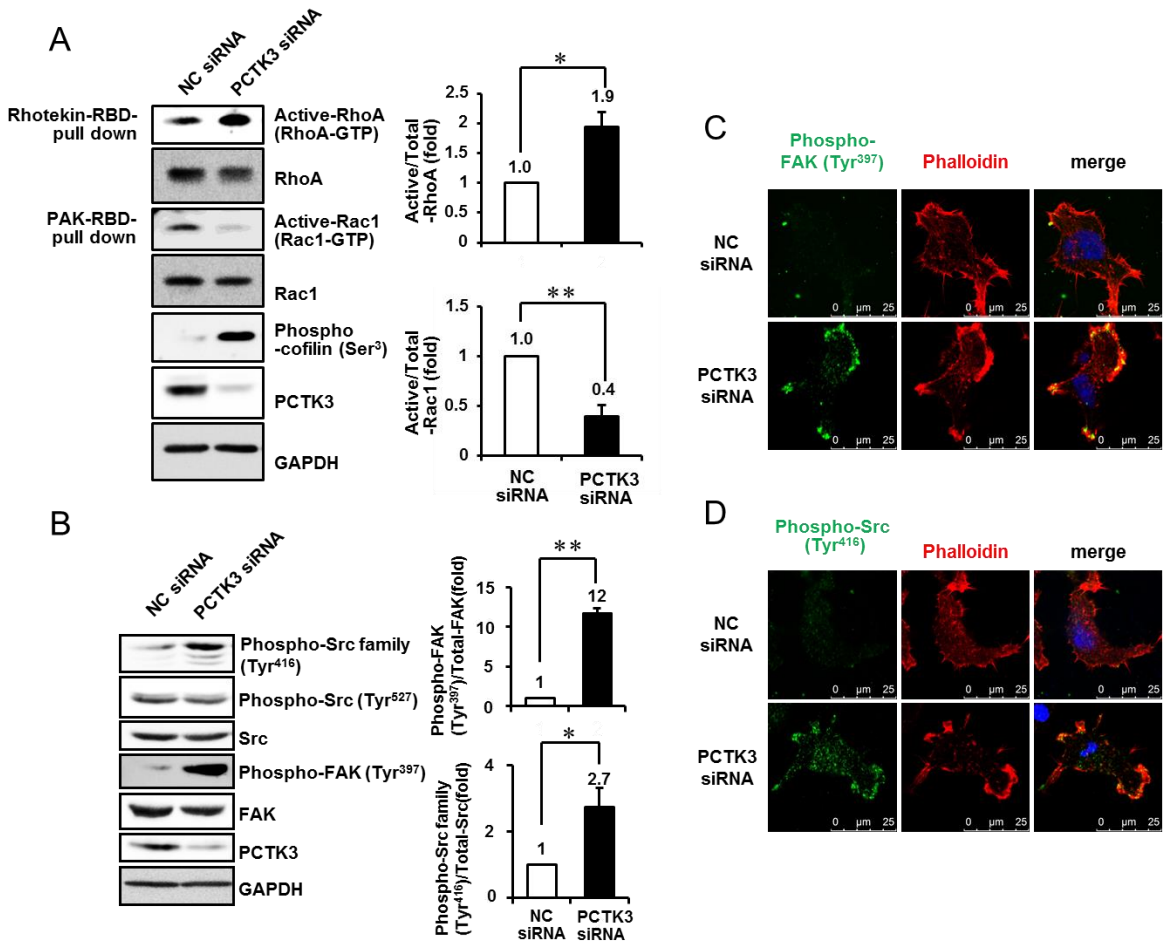

Figure 12. PCTK3-knockdwon activates the FAK and RhoA activities. *A,* PCTK3-knockdown cell lysates were incubated with Rhotekin/ or PAK-RBD beads. The avtive-RhoA or active-Rac1 bound to the beads were subjected to immunoblot analysis using anti-RhoA or anti-Rac1 antibodies. Expression of GAPDH was used as a loading control. Relative activity of RhoA and Rac1 were normalized by the RhoA or Rac1 levels in total cell lysates. The activity of RhoA and Rac1 in NC siRNA cells were taken as 1. Results are expressed as means ± S.E. from three independent experiments. Statistical significance was determined by student T test. **, $p < 0.01$. *, $p < 0.05$. *B,* PCTK3-knockdown cell lysates were subjected to immunoblot analysis using anti-FAK, anti-Src, anti-phospho-Src (Tyr^{416}), anti-phospho-Src (Tyr^{527}), anti-phospho-FAK (Tyr^{397}), and anti-PCTK3 antibodies. Immunoblot intensity of GAPDH was used as a loading control. Relative phosphorylation of FAK and Src were normalized by the FAK or Src levels in total cell lysates. The phosphorylation of FAK and Src in NC siRNA cells were taken as 1. Results are expressed as means ± S.E. from three independent experiments. Statistical significance was determined by student T test. **, $p < 0.01$. *, $p < 0.05$. *C and D,* PCTK3-knockdown cells were subjected to immunofluorescent staining using anti-phospho-FAK (Tyr^{397}) and anti-phospho-Src (Tyr^{416}) antibodies. Fluorescence for phosphor-FAK (Tyr^{397}) and anti-phospho-Src (Tyr^{416}) (*green*) and F-actin (Alexa 555-conjugated phalloidin staining, *red*) are shown with merged images (merge is in *yellow*). Hoechst nuclear staining is represented in *blue*.

Chapter 6. Discussion

In this study, these findings provide the information about an activation mechanism of PCTK3, which activity is regulated by two step pathway. One step is the binding to cyclin A2, which induce the weakly activation of PCTK3. Cyclin A2 enhance the catalytic activity of CDK2 by causes the conformational changes in the PSTAIRE helix and T-loop region of the kinase [57]. The kinase domain of PCTK is highly conserved between isoforms (more than 80 % similarity) and displays high sequence homology with the kinase domain of CDK2 (52 % identity for human PCTK3 and human CDK2 kinase domains) [4]. The crystal structure of PCTK3 has been still remained to be determined, while that of PCTK1 is available from the Protein Date bank (PDB ID code 3MTL). By comparison with the crystal structure of CDK2/cyclin A2 complex and PCTK1, two residues Lys^{225} and Glu^{253} of cyclin Y have been identified as the important residues for the binding to PCTK1 1 [47]. The Lys–Glu pair is found in different cyclins and likely is a conserved structural feature in CDK/cyclin complexes, suggesting that PCTK1 is activated by cyclin Y in a manner similar to which cyclin A2 activates CDK2. Additionally, although crystal structure analysis of PCTK1 also demonstrated that the PCTAIRE sequence forms β-strand instead of helix, it has been reported that the PCTAIRE sequence and its peripheral region of PCTK1 are important for the interaction with cyclin Y [9]. Accordingly, these results suggest that PCTAIRE sequence of PCTK is important for the binding to cyclins. Furthermore, as shown Fig.5C, all of three PCTK isoforms interacted with cyclin A2 with an equal affinity, suggesting that cyclin A2 binds to PCTK3 thorough the common region of PCTK isoforms, such as the PCTAIRE sequence. On the other hand, Fig.5A showed that PCTK3 is not complex with CDK1 and CDK2, that is, PCTK3 is competed with CDK1 and CDK2 for the interaction with cyclin A2. Furthermore, I found that binding of cyclin A2 with PCTK3 is weakly than that of with CDK2 in the proliferating-cells, suggests that cyclin A2 predominantly binding to CDK2 or CDK1 during cell cycle.

On the other hand, I also observed that cyclin E1 has a greater affinity for PCTK3 than cyclin A2, but it was not affected the catalytic activity of PCTK3. The best studied function of cyclin E1 is

activated in the late G1 phase of the cell cycle via complex with CDK2 and is implicated to promoting S-phase entry [58]. However, recent study has suggested that cyclin E inhibits the activity of CDK5 by competing with p35 for CDK5 binding [59]. These results suggest that cyclin E1 inactivates PCTK3 by dissociating cyclin A2 form PCTk3. In order to obtain the more detail activation mechanism of PCTK3 by cyclin A2 and cyclin E1, it would be necessary to perform the crystal structure analysis.

Although cyclin A2 has no nuclear localization signal (NLS), it shuttles between the nucleus and the cytoplasm because of its binding partners [60, 61]. However, the translocation mechanism of cyclin A2 remains unrevealed because of the fact that CDK2 also has no NLS signal. Recently, S phase cyclin A associated protein residing in the endoplasmic reticulum (SCAPER) was identified and characterized as a substrate of cyclin A2/CDK2 which is localized to cytoplasm exclusively [13]. The function of SCAPER remains unknown although it was suggested that SCAPER might regulate the cell cycle progression and act as buffer the cytoplasmic pool of cyclin A2. PCTK3 is also cytoplasmic protein and colocalized with cyclin A2 in the cytoplasm. Furthermore, I observed that PCTK3 bound to cyclin A2 in the cytoplasm and promoted the transition of cyclin A2 from nuclear to cytoplasm (Fig.6D, Fig.7). These results demonstrated that PCTK3 is the cytoplasmic binding partner of cyclin A2 and cyclin A2 alter its subcellular localization by binding to different partners.

The expression levels of cyclin A2 are tightly controlled by transcription and ubiquitin-mediated degradation in a temporal manner. Cyclin A2 begins to be degraded in early prometaphase and is completed at metaphase. The major ubiquitin ligase in mitosis is the anaphase-promoting complex/cyclosome (APC/C) [53]. However, the proteolysis mechanism of cytoplasmic cyclin A2 is still unrevealed. Because the expression levels of cyclin A2 is decreased in PCTK3 knock-down cells, I hypothesized that PCTK3 protects cyclin A2 from proteolysis. To verify this hypothesis, I investigated the regulation of cyclin A2 degradation. As shown Fig. 8B, although free cyclin A2 is readily degraded by the proteasome, degradation of cyclin A2 is suppressed in the presence of PCTK3. These observations strongly supported the physiologically significance of the association of PCTK3 with cyclin A2.

In addition to activation by binding to cyclin A2, PCTK3 is also activated by PKA via phosphorylation at Ser^{12}, which induce further activation of PCTK3. Endogenous PCTK3 is phosphorylated and activated by forskolin induced PKA activation in HEK293T cells. Again, point mutation analysis also revealed that PKA phosphorylates Ser^{12}, Ser^{66} and Ser^{109} of PCTK3 in vitro and in vivo. These results demonstrated that PCTK3 is the bona fide substrate of PKA. I also observed that the activity of phosphor-mimicked Ser^{12} is comparative to the activity of CDK2 in the presence of cyclin A2, suggesting that not only binding to cyclin A2, but also the phosphorylation at Ser^{12} of PCTK3 is require for the fully activation of PCTK3. Although phospho-mimicked Ser^{12} cause an increase of the PCTK3 activity, phospho-mimicked Ser^{66} and Ser^{109} (homologous to Ser^{119} and Ser^{153} of PCTK1, respectively) indicated no effect against the catalytic activity of PCTK3. Recently, Mikolcevic et al. also reported that the PKA plays an inhibitory role in interaction between PCTK1 and cyclin Y by phosphorylation on Ser^{153} of PCTK1 [9]. Furthermore, phosphorylation at Ser^{153} of PCTK1 also creates the binding motif of 14-3-3 proteins and transfers PCTK1 from the cell membrane to cytoplasm [9]. 14-3-3 proteins consist of 7 type isoform and have the ability to interact with various proteins [62]. A previous report showed that PCTK3 is interacted with 14-3-3ζ [63]. Consisted with this, I found that PCTK3 binds to 14-3-3 β, γ, η, ε, τ and ζ (date not shown). Therefore, phosphorylation of Ser^{66} and Ser^{109} of PCTK3 may mediate the interaction with 14-3-3 proteins and alter the subcellular localization. PKA also involves in the regulation of cellular structure through phosphorylates the cytoskeletal proteins including microtubules, intermediate filaments and actin [64, 65]. Furthermore, PKA regulates the actin filament turnover by modulates the activity of LIM domain kinase 1 (Limk1) [66]. Additionally, recent study reported that cyclin A2 also play a role in cytoskeletal reorganization via associated with RhoA, a member of the small GTPase family. Suppression of cyclin A2 by shRNA showed an increase in cell size and d cortical localization of F-actin independent of cell cycle and displays an increased cell migration and invasion [14]. Similary with cyclin A2-deficient phenotype, PCTK3 knock-down HEK293T cells display an increase of cell size and aggregation of F-actin at edge of cell membrane and increased the autophosphorylation of

FAK at Tyr^{397}. Phosphorylation of FAK at Tyr^{397} is the major event related to the FAK activation. Activated FAK transmit the signal to actin cytoskeleton by forming focal adhesion with Src family and focal adhesion proteins such as paxillin, vinculin or α-actinin [67]. Because FAK is also interacted with GEFs and GAPs, and modulates the GDP/GTP-exchanging activities by phosphorylating their tyrosine residues, the balance of Rho small GTPases activities are regulated by FAK [68]. Therefore, these data suggest that PCTK3 involves the regulation of the cell morphology, motility via negatively modulating the FAL activity.

Although CDKs were originally identified as enzymes that controlled cell cycle events, some members of the CDK family are involved in other cellular processes [69]. For example, CDK5 is not considered to play a significant role in cell cycle regulation, however, it has an important function in the control of neurogenesis, including neurite outgrowth, axon guidance, and cell migration. CDK5 activated by neuron-specific regulator p35 inhibits p21-activated kinase 1 (PAK1) activity through phosphorylation at Thr^{212} (Pro–Val–Thr^{212}–Pro–Thr–Arg–Asp) [70]. PAK1, an effector of Rac, has a pivotal role in cell morphology and motility via the LIMK/cofilin pathway [71]. Phosphorylation of PAK1 by p35/CDK5 is likely to be implicated in the dynamics of the actin cytoskeleton reorganization. CDK5 also controls neural migration and focal adhesion assembly via directly phosphorylating focal adhesion proteins, such as FAK at Ser^{732} and paxillin at Ser^{244} [72, 73]. PCTK3 may also phosphorylate focal adhesion proteins, and control actin dynamics.

In summary, I demonstrated that cyclin A2 is a specific activator of PCTK3, and that phosphorylation at Ser12 of PCTK3 significantly enhances its kinase activity in the absence of cyclin A2. These findings shed light on the activation mechanism of not only PCTK3 but also other uncharacterized members of the CDK family, such as PCTK2. Further investigations will be needed to clarify the physiological functions and pathological roles of PCTK3.

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Chapter 9 Appendix

PCTAIRE Kinase 3/Cyclin-dependent Kinase 18 Is Activated through Association with Cyclin A and/or Phosphorylation by Protein Kinase A

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PCTAIRE Kinase 3/Cyclin-dependent Kinase 18 Is Activated through Association with Cyclin A and/or Phosphorylation by Protein Kinase A*

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Background: PCTK3 is an uncharacterized serine/threonine kinase that belongs to the cyclin-dependent kinase family.
Results: The activity of PCTK3 is increased via interaction with cyclin A2 and phosphorylation by PKA. PCTK3 knockdown induces actin polymerization.
Conclusion: PCTK3 is activated by cyclin A2 and PKA and is involved in actin dynamics.
Significance: This study provides clues to the physiological function of PCTK3.

PCTAIRE kinase 3 (PCTK3)/cyclin-dependent kinase 18 (CDK18) is an uncharacterized member of the CDK family because its activator(s) remains unidentified. Here we describe the mechanisms of catalytic activation of PCTK3 by cyclin A2 and cAMP-dependent protein kinase (PKA). Using a pulldown experiment with HEK293T cells, cyclin A2 and cyclin E1 were identified as proteins that interacted with PCTK3. An *in vitro* kinase assay using retinoblastoma protein as the substrate showed that PCTK3 was specifically activated by cyclin A2 but not by cyclin E1, although its activity was lower than that of CDK2. Furthermore, immunocytochemistry analysis showed that PCTK3 colocalized with cyclin A2 in the cytoplasm and regulated cyclin A2 stability. Amino acid sequence analysis revealed that PCTK3 contained four putative PKA phosphorylation sites. *In vitro* and *in vivo* kinase assays showed that PCTK3 was phosphorylated by PKA at Ser^{12}, Ser^{66}, and Ser^{109} and that PCTK3 activity significantly increased via phosphorylation at Ser^{12} by PKA even in the absence of cyclin A2. In the presence of cyclin A2, PCTK3 activity was comparable to CDK2 activity. We also found that PCTK3 knockdown in HEK293T cells induced polymerized actin accumulation in peripheral areas and cofilin phosphorylation. Taken together, our results provide the first evidence for the mechanisms of catalytic activation of PCTK3 by cyclin A2 and PKA and a physiological function of PCTK3.

In mammals, the cyclin-dependent kinases (CDKs),[2] a family of serine/threonine protein kinases, play an important role in regulating cellular functions such as cell proliferation, transcription, and neuron function (1). Their activities are regulated by associations with different cyclins and phosphorylation at specific sites by protein kinases (2). Because cyclins are differentially expressed and degraded by ubiquitin-mediated proteolysis at specific cell cycle phases, CDK activity oscillates during cell cycle transitions (3). Canonical CDKs contain the PSTAIRE helix, which has been implicated in binding to cyclins in the kinase domain (4). The PCTAIRE kinase (PCTK) subfamily of CDKs includes three members, PCTK1/CDK16, PCTK2/CDK17, and PCTK3/CDK18, which contain the PCTAIRE sequence instead of the PSTAIRE sequence. PCTK family members have high sequence homology in their central kinase domains, whereas the structures of their N-terminal and C-terminal regions differ. PCTK genes are conserved in higher eukaryotes, from *Caenorhabditis elegans* to humans. However, there are no PCTK orthologs in yeast. The PCTK subfamily is widely expressed in mammalian tissues and is relatively more abundant in post-mitotic cells, suggesting that they may function in higher eukaryotes and have different biological functions from those during the cell cycle (5). PCTK1 is the best-characterized member of this kinase family and regulates neurite outgrowth in the Neuro2A neuroblastoma cell line (6). It is also involved in membrane trafficking through the early secretory pathway via phosphorylation of *N*-ethylmaleimide-sensitive fusion protein (7, 8). A recent study reported that PCT-1, the *C. elegans* ortholog of PCTK1, is complexed with CYY-1 (ortholog of mammalian cyclin Y, a novel membrane-associated cyclin) and is also necessary for targeting presynaptic components to the axons (9). Furthermore, human PCTK1 is activated by cyclin Y and is essential for spermatogenesis (10). PCTK2 is associated with Trap (tudor repeat associator with PCTAIRE 2) and ik3–1/cables, an adaptor that functionally connects c-abl and CDK5 to support neurite growth (11, 12). The third member of this family, PCTK3, has been the least studied. Although exogenously expressed PCTK1 and PCTK2 phosphorylate myelin basic protein and histone H1 *in vitro*, PCTK3 kinase activity has not been detected. To characterize PCTK3 activity and eluci-

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[2] The abbreviations used are: CDK, cyclin-dependent kinase; PCTK, PCTAIRE kinase; PFTK, PFTAIRE kinase; PKA, protein kinase A; Rb, retinoblastoma protein; MBP, maltose-binding protein; MyBP, myelin basic protein; PAK1, p21-activated kinase 1; SCAPER, S phase cyclin A-associated protein residing in the endoplasmic reticulum.

date its regulatory mechanism, it is necessary to identify activators for PCTK3.

Mammalian cyclin A consists of two isoforms: cyclin A1 and cyclin A2. In male mice, cyclin A1 is predominantly expressed in germ cells and is required for the entry of germ cells into the first meiotic division (13). In contrast, cyclin A2 is ubiquitously expressed in somatic cells and has a major role in S-phase progression and the G_2/M phase transition by activating CDK2 and CDK1, respectively. Although cyclin A is predominantly localized in the nucleus during S phase, it also shuttles between the nucleus and cytoplasm and is degraded by ubiquitin-mediated proteolysis during the G_2 phase. Cyclin A/CDK2 is transiently maintained by SCAPER (S phase cyclin A-associated protein residing in the endoplasmic reticulum), an S phase cyclin A-associated protein, in the cytoplasm (14). However, a recent study showed cyclin A2 is involved in the regulation of cell migration and invasiveness through its direct interaction with RhoA, suggesting a novel function of cyclin A in a CDK1- and CDK2-independent manner (15).

Protein kinase A (PKA) phosphorylates numerous proteins involved in various cellular phenomena and preferentially to phosphorylates a serine or threonine within the following consensus sequence: Arg-Arg-*X*-Ser/Thr (*X* represents any amino acid) (16). PKA also plays an important role in maintaining meiotic arrest (17). In *Xenopus* oocytes, PKA phosphorylates and inactivates Cdc25C phosphatase, whereas Wee1B kinase activity is enhanced by PKA (18, 19). The activity of the cyclin B-CDK1 complex is controlled by Wee1 kinase and Cdc25C phosphatase (20, 21). PKA regulates the activity of the cyclin B-CDK1 complex via dual regulation of the Cdc25 phosphatase and Wee1B kinase (18). On the other hand, PKA inhibits the interaction between PCTK1 and cyclin Y via phosphorylation of PCTK1 at Ser^{153}, resulting in PCTK1 inactivation (6, 10). Thus, PKA regulates the activities of CDKs in either a direct or an indirect manner.

In this study we demonstrated that PCTK3 is activated by its association with cyclin A2 and phosphorylates retinoblastoma protein (Rb) *in vitro*. In addition, we found that PCTK3 contains four putative PKA phosphorylation sites (Arg-Arg-*X*-Ser/Thr) and that PCTK3 phosphorylation by PKA results in an increase in its catalytic activity even in the absence of cyclin A2. These findings provide some insights into the functions of PCTK3.

EXPERIMENTAL PROCEDURES

Antibodies and Materials—Antibody against cyclin A was purchased from BD Biosciences, and anti-cyclin B1, anti-cyclin D1, anti- cyclin E1, anti-cyclin E2, anti-cyclin H, anti-CDK1, anti-CDK2, anti-Rb, anti-phospho-Rb (Ser^{795}) and ($Ser^{807/811}$), anti-cofilin, anti-phospho-cofilin (Ser^{3}), and anti-phospho-PKA substrate (RR*X*(S/T)) antibodies were from Cell Signaling Technology. Anti-PCTK3, anti-α-tubulin, and anti-β-actin antibodies were from Santa Cruz Biotechnology. Anti-FLAG M2 antibody was form Sigma. Anti-GST antibody was from Wako Pure Chemical Industries. Anti-Strep antibody was from Qiagen. Alexa-555 conjugated-phalloidin was from Cytoskeleton.

Plasmid Construction—cDNAs encoding mouse full-length PCTK1, PCTK2, PCTK3, CDK2, PFTAIRE kinase 1 (PFTK1), cyclin A2, cyclin E1, cyclin K, and cyclin Y and human retinoblastoma protein (779–928 amino acid; Rb C) were cloned by PCR using the respective specific primers. PCR products were cloned into TA-cloning vector pGEM-T Easy (Promega), and the inserted DNA sequences were confirmed by DNA sequencing. The inserted DNAs were subcloned in-frame into the different mammalian expression vectors including Strep-tagged expression vector pEXPR-IBA105 (IBA GmbH), glutathione *S*-transferase (GST) fusion expression vector pEBG, and FLAG-tagged expression vector pFLAG-CMV-2 (Sigma). A cDNA encoding for human Rb C was subcloned into the pMAL vector (New England Biolabs) of the bacterial maltose-binding protein (MBP) fusion expression system. The expression plasmid pFLAG-PKA-C encoding the catalytic subunit α isoform of mouse PKA was described previously (22). Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara Bio) according to the manufacturer's instructions. The mutation was confirmed by DNA sequencing analysis.

Expression and Purification of Rb Recombinant Protein—The bacterial expression plasmid of Rb, pMAL-Rb C, was introduced into the bacterial strain BL21 Star DE3 (Invitrogen). An overnight culture in LB medium was diluted in fresh LB medium and incubated at 37 °C for 1 h with continuous shaking. After isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.2 mM, the culture was incubated at 30 °C for an additional 6 h. The cells were resuspended in ice-cold extraction buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After freezing and thawing, suspended cells were sonicated on ice in short bursts. The lysate was cleared by centrifugation at 10,000 $\times$ *g* for 10 min at 4 °C. The supernatant was then incubated with amylose resin (New England Biolabs) overnight at 4 °C. The beads were washed 5 times with ice-cold extraction buffer A and incubated with 10 mM maltose at 4 °C to elute fusion protein from the beads. After centrifugation, the supernatant was dialyzed against PBS. The purified protein was electrophoresed on SDS-PAGE and visualized by Coomassie Brilliant Blue staining, and the protein concentration was determined by Bradford assay (Bio-Rad) using BSA as a standard.

Cell Culture and Transfection—COS-7, HeLa, and HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Cells were transfected with various expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Subcellular Fractionations—HEK293T cells were washed twice with ice-cold PBS, harvested by scraping, and lysed in a buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 15 min, the cells were mixed with 0.5% Nonidet P-40 and homogenized by vortex for 10 s. The homogenate was centrifuged at 2500 rpm for 3 min to sediment the nuclei. The supernatant was then centrifuged at a 10,000 rpm for 10 min, and the resulting supernatant was used as the cytoplasm fraction. The

nuclear pellet was washed three times with buffer A to remove any contamination from cytoplasmic proteins. To extract nuclear proteins, the isolated nuclei were resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and the mixture was incubated on ice for 20 min. Nuclear lysates were collected after centrifugation at 12,000 rpm for 15 min at 4 °C. The purity of nuclear and cytoplasm fractions was confirmed by immunoblotting using an anti-lamin B1 antibody (MBL) as a nuclear marker and α-tubulin antibody as a cytoplasmic marker, respectively.

Pulldown and Co-immunoprecipitation Assays—Pulldown and co-immunoprecipitation assays were performed as previously described (23). Briefly, cells were scraped in an ice-cold TNE buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA) supplemented with protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin). The cell extracts were centrifuged at 10,000 × *g* for 10 min at 4 °C before immunoprecipitation or immunoblotting. Equal protein amounts of the lysates were analyzed for protein expression. For pulldown assays, lysates were incubated with Strep-Tactin Sepharose (IBA GmbH) or glutathione Sepharose (GE Healthcare) overnight at 4 °C. For immunoprecipitation, lysates were incubated with anti-FLAG M2 or anti-PCTK3 antibody in the presence of protein G Sepharose (GE Healthcare) overnight at 4 °C. The beads were washed 4 times with wash buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM EDTA). Precipitated proteins were subjected to *in vitro* kinase assay or immunoblot analysis. Protein expression and phosphorylation were determined by immunoblotting of immunoprecipitates or total cell lysates.

Transfection of Small Interference RNA—The synthetic small interfering RNA (siRNA) oligonucleotides for PCTK3 (PCTK3 siRNA#1 (ID# SASI_Hs02_00334101), PCTK3 siRNA#2 (ID# SASI_Hs01_00015477), and PCTK3 siRNA#3 (ID# SASI_Hs02_00374212)) were purchased from Sigma. A MISSION siRNA Universal Negative Control #1 was used as the negative control. The transfection of siRNA was performed using Lipofectamine 2000 according to the manufacturer's instructions.

In Vitro Kinase Assay—*In vitro* kinase assays were carried out as previously described (23). For *in vitro* kinase assay of PCTK3, cell lysates were incubated with a glutathione-Sepharose or immunoprecipitated with anti-PCTK3 antibody and protein G Sepharose overnight at 4 °C by rotation. The beads were washed 3 times with wash buffer and twice with 50 mM Tris-HCl, pH 7.5. The kinase reaction was carried out by resuspending the complexes in 100 μl of kinase buffer (50 mM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 20 or 50 μM ATP and phosphatase inhibitor mixture (Nacalai Tesque)) including 5 μg/ml purified MBP-Rb C in the absence or presence of 2 μCi of [γ-^{32}P]ATP and incubating for 30 min at 30 °C. Phosphorylated MBP-Rb C was separated by SDS-PAGE and visualized with a BAS-1500 Bioimaging Analyzer (Fuji Film) or subjected to immunoblot analysis using anti-phospho-Rb (Ser^{795}) antibody. To assay the phosphorylation of PCTK3 by PKA, cell lysate were immunoprecipitated with anti-FLAG antibody overnight at 4 °C by rotation. The kinase reaction was carried out by resuspending the complexes in 100 μl of kinase buffer with 2 μCi [γ-^{32}P]ATP and incubating for 30 min at 30 °C. Immunocomplexes were released by heating at 95 °C in SDS sample buffer and subjected to SDS-PAGE, and phosphorylated proteins were visualized by BAS-1500 Bioimaging Analyzer. Quantitative densitometric analysis was performed using Image J software.

In Vivo Kinase Assay—HEK293T cells were treated with 10 μM forskolin for 30 min in the presence of 10% FBS and scraped in an ice-cold TNE buffer supplemented with phosphatase inhibitor mixture. The cell extracts were centrifuged at 10,000 × *g* for 10 min at 4 °C to remove cellular debris, and the supernatants were immunoprecipitated with anti-PCTK3 antibody with protein G-Sepharose overnight at 4 °C by rotation. The beads were washed with wash buffer, and immunocomplexes were released by heating at 95 °C in SDS sample buffer, subjected to SDS-PAGE, and immunoblotted using anti-phospho-RR*X*(S/T) antibody.

Immunofluorescence Analysis—Immunofluorescence analysis was performed as previously described (23). In brief, HEK293T cells grown on poly-L-lysine-coated chamber slides were cotransfected with Myc-cyclin A2 together with either FLAG-PCTK3 or FLAG-CDK2. At 24 h post-transfection, cells were washed twice with PBS and fixed for 20 min in 3.7% formaldehyde. After sequential washing with PBS, cells were permeabilized for 5 min in 0.1% Triton X-100, washed 3 times with PBS, and then treated with 5% BSA for 30 min. Cells were subsequently incubated with mouse anti-FLAG M2 IgG or rabbit anti-Myc polyclonal antibody overnight at 4 °C. After 3 washes with PBS, cells were incubated for 1 h with goat anti-mouse IgG directly conjugated to Alexa Fluor 488 or goat anti-rabbit IgG directly conjugated to Alexa Fluor 555 (Invitrogen). The slides were washed thoroughly with PBS and mounted in fluorescent mounting medium Vectashield (Vector Laboratories). A confocal laser-scanning microscope (Leica TCS-SP5) was used to obtain staining profiles.

RESULTS

Identification of Cyclin A2 as An Activator of PCTK3—PCTK1, a member of the PCTK subfamily, is activated by membrane-associated cyclin Y (10, 24). We hypothesized that PCTK3 was also activated by cyclin family members. To identify PCTK3 activators, we expressed Strep-tagged mouse PCTK3 in HEK293T cells and conducted Strep pulldown experiments. The proteins that coprecipitated with PCTK3 were analyzed by immunoblot assay using anti-cyclin A, B1, D1, E1, E2, and H antibodies. Immunoblot analysis revealed that PCTK3 interacted with cyclin A and cyclin E1, but not cyclin B1, D1, E2, or H, in HEK293T cells (Fig. 1*A*). To confirm the interaction between PCTK3 and cyclin A2 or E1, we performed GST pulldown experiments using lysates prepared from COS-7 cells expressing GST-fused PCTK3 and FLAG-tagged cyclins. We also examined whether cyclin K and cyclin Y bound to PCTK3, because PCTK3 was found to interact with cyclin K in a large-scale interaction study (25). As shown in Fig. 1*B*, GST-PCTK3 associated with FLAG-cyclin A2 and cyclin E1 but not with cyclin K or cyclin Y.

Subsequently, to determine whether PCTK3 activity was regulated by binding to cyclin A2 or cyclin E1, we performed an *in*

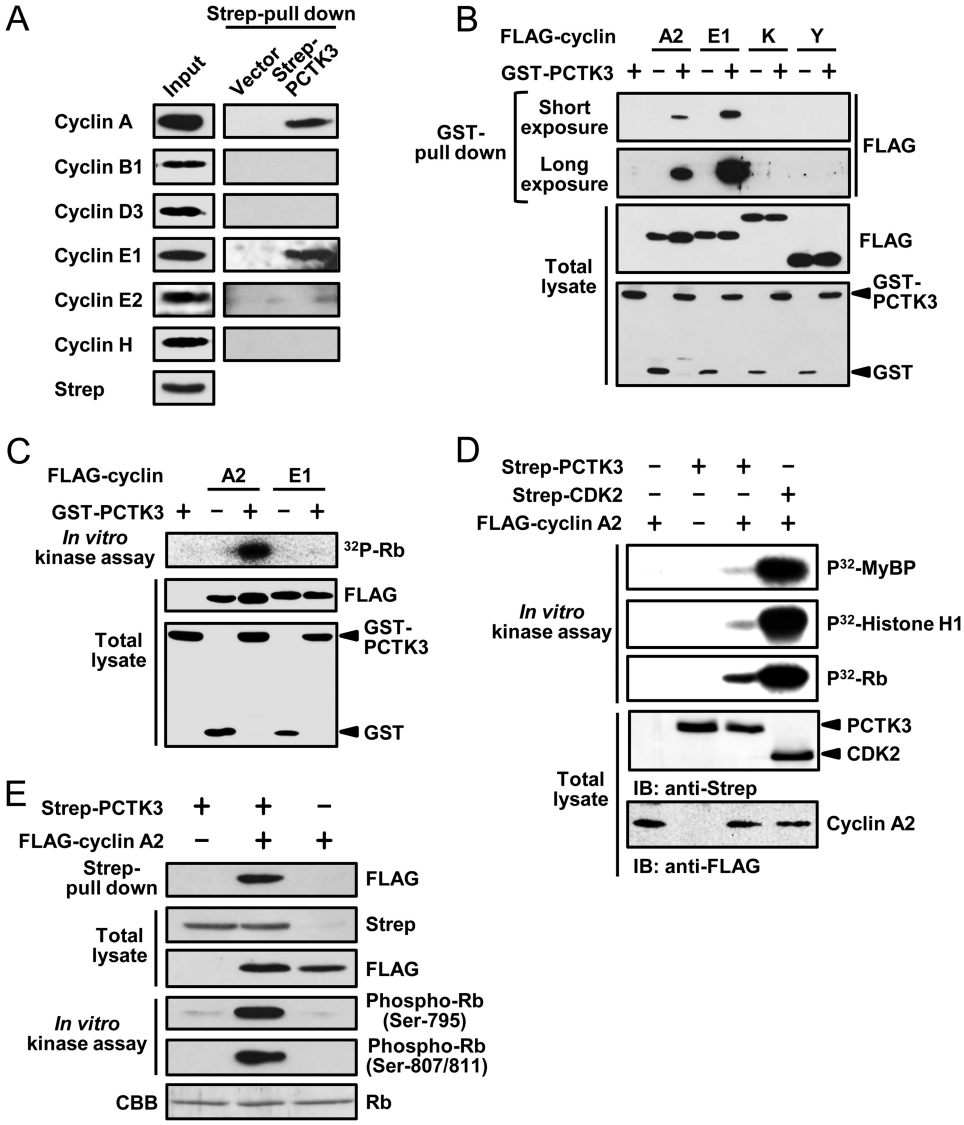

FIGURE 1. **Identification of cyclin A2 as an activator of PCTK3.** *A*, HEK293T cells were transfected with Strep-PCTK3. After the Strep pulldown assay, the precipitated proteins were subjected to immunoblot analysis with anti-cyclin A, anti-cyclin B1, anti-cyclin D3, anti-cyclin E1, anti-cyclin E2, and anti-cyclin H antibodies. Total cell lysates were used as the input samples and positive control. *B*, GST-PCTK3 was expressed in COS-7 cells with either FLAG-cyclin A2, FLAG-cyclin E1, FLAG-cyclin K, or FLAG-cyclin Y. In the control experiment (GST-PCTK3 (−)), GST alone was expressed instead of GST-PCTK3. The GST pulldown precipitates were immunoblotted with anti-FLAG antibody. Protein expression was confirmed by immunoblotting total cell lysates. *C*, GST and GST-PCTK3 was expressed with either FLAG-cyclin A2 or FLAG-cyclin E1 in COS-7 cells. After the GST pulldown, the precipitated samples were incubated in a kinase buffer containing [γ-^{32}P]ATP and MBP-Rb C for 30 min. The supernatants containing MBP-Rb C were separated on SDS-PAGE, after which the gel was analyzed by bioimaging analyzer. The expression level of GST-fused and FLAG-tagged proteins was confirmed by immunoblotting with anti-FLAG and anti-GST antibody, respectively. *D*, COS-7 cells were transfected with Strep-PCTK3 and FLAG-cyclin A2. GST pull downed proteins were incubated in a kinase buffer containing MBP-Rb C for 30 min. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) and (Ser$^{807/811}$) antibodies, respectively. *D*, COS-7 cells were transfected with Strep-PCTK3, Strep-CDK2, and FLAG-cyclin A2. Strep pull-downed proteins were incubated in a kinase buffer containing MyBP, MBP-Rb C, or histone H1 for 30 min. The supernatants were analyzed by SDS-PAGE and bioimaging analyzer. *IB*, immunoblot. *E*, Strep-PCTK3 was expressed in COS7 cells with FLAG-cyclin A2. After the Strep pulldown, bound proteins were incubated in a kinase buffer containing MBP-Rb C for 30 min. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) and (Ser$^{807/811}$) antibodies, respectively. *CBB*, Coomassie Brilliant Blue.

vitro kinase assay using retinoblastoma protein (Rb) as the substrate. Rb is phosphorylated by some CDKs in a cell cycle-dependent manner, and PFTK1, which is activated by cyclin D3 and cyclin Y, also phosphorylates Rb (26, 27). The C terminus of Rb contains a cluster of seven candidate CDK phosphorylation sites (Ser/Thr-Pro motifs: Ser^{780}, Ser^{788}, Ser^{795}, Ser^{807}, Ser^{811}, Thr^{821}, and Thr^{826}) (28). For this *in vitro* kinase assay, we produced recombinant MBP-fused Rb C (amino acids 779–928) in *Escherichia coli*. COS-7 cells were cotransfected with GST-PCTK3 in the presence or absence of FLAG-cyclin A2 or E1, after which cell lysates were subjected to glutathione-Sepharose pulldown. In an *in vitro* kinase analysis with [γ-^{32}P]ATP, MBP-Rb C was phosphorylated by PCTK3 in the presence of cyclin A2, whereas cyclin E1 did not activate PCTK3 (Fig. 1*C*). We also performed an *in vitro* kinase assay using myelin basic protein (MyBP) and histone H1 as substrates. As shown in Fig. 1*D*, cyclin A2-CDK2 complex strongly phosphorylated all three of the substrates tested (Rb, MyBP, and histone H1). However, the catalytic activity of PCTK3 was much lower than that of CDK2 even in the presence of cyclin A2. Among the three sub-

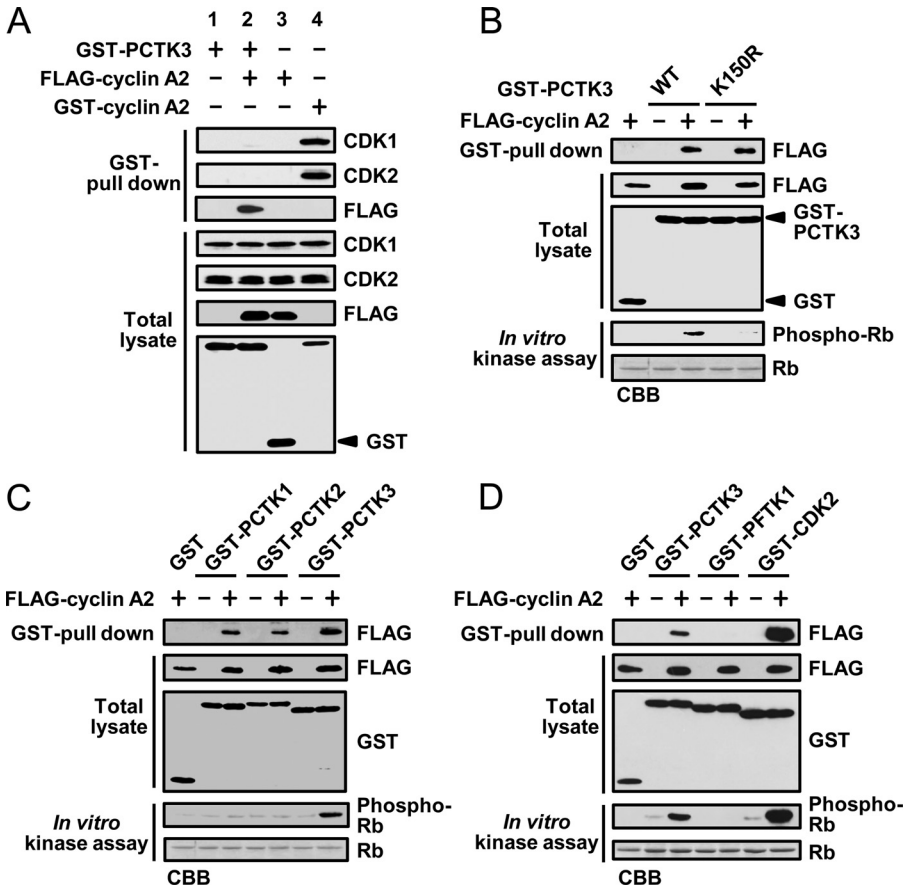

FIGURE 2. **Cyclin A2 specifically activates PCTK3.** *A*, COS-7 cells were transfected with GST-PCTK3, FLAG-cyclin A2, and GST-cyclin A2. In the control experiment, GST alone was transfected instead of GST-PCTK3 or GST-cyclin A2. The GST pulldown precipitates were subjected to immunoblotting with anti-FLAG, anti-CDK1, and anti-CDK2 antibodies. *B*, GST-PCTK3 wild type (*WT*) or K150R mutant was expressed in COS-7 cells with FLAG-cyclin A2. In the control experiment, GST alone was expressed instead of GST-PCTK3. After the GST pulldown, the bound proteins were incubated in a kinase buffer with MBP-Rb C. The precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting with anti-FLAG and anti-phospho-Rb (Ser^{795}) antibodies, respectively. *C*, FLAG-cyclin A2 was expressed in COS-7 cells with GST, GST-PCTK1, GST-PCTK2, or GST-PCTK3. The GST pulldown precipitates were subjected to *in vitro* kinase assay using anti-phospho-Rb (Ser^{795}) antibody. *D*, GST, GST-PCTK3, GST-PFTK1, or GST-CDK2 was expressed in COS-7 cells with FLAG-cyclin A2. The GST pulled-down proteins were subjected to an *in vitro* kinase assay.

strates tested, Rb was the most efficiently phosphorylated by cyclin A2-PCTK3 complex. In addition, phosphorylated MBP-Rb C was detected using antibodies against phospho-Rb (Ser^{795}) and phospho-Rb ($Ser^{807/811}$) (Fig. 1*E*). Both of these antibodies detected Rb phosphorylation only when PCTK3 and cyclin A2 were cotransfected, indicating that cyclin A2-PCTK3 complex phosphorylates Rb at least at Ser^{795} and $Ser^{807/811}$ *in vitro*. These results showed that PCTK3 is activated through its association with cyclin A2.

Cyclin A2 Specifically Activates PCTK3—Cyclin A2 interacts with CDK1 and CDK2 during cell cycle progression (29). We performed GST pulldown experiments to evaluate whether Rb phosphorylation was dependent on PCTK3 or other kinases that coprecipitated with cyclin A2. As shown in Fig. 2*A*, endogenous CDK1 and CDK2 were specifically coprecipitated with GST-cyclin A (*lane 4*), whereas GST-PCTK3, GST-PCTK3/FLAG-cyclin A2 complex, and FLAG-cyclin A2 did not coprecipitate with endogenous CDK1 and CDK2 (*lanes 1–3*). In addition, we designed a PCTK3 inactive mutant on the basis of amino acids that are conserved among various members of the CDK family. An analogous K194R mutation at the putative ATP binding site of PCTK1 generates a kinase dead mutant (6). We created a kinase-dead mutant of PCTK3, in which the putative ATP binding site was replaced by arginine (PCTK3 K150R), and evaluated its catalytic activity with an *in vitro* kinase assay. Although both PCTK3 wild type and K150R interacted with cyclin A2, no kinase activity of PCTK3 K150R was detected (Fig. 2*B*). Thus, Rb phosphorylation is dependent on PCTK3 but not on other protein kinases, and it is highly likely that cyclin A2 is an activator of PCTK3.

We also examined whether other members of the PCTK subfamily, PCTK1 and PCTK2, were activated by cyclin A2, because PCTK2 activators have not yet been identified. Although PCTK1 and PCTK2 interacted with cyclin A2, only PCTK3 was activated by cyclin A2 (Fig. 2*C*). In contrast, PFTK1, with high homology to PCTK3, was not associated with cyclin A2 and did not phosphorylate Rb C (Fig. 2*D*). However, GST pulldown and *in vitro* kinase assays suggested that CDK2 had a higher affinity for cyclin A2 than PCTK3 and that the catalytic activity of PCTK3 was lower than that of CDK2 in the presence of cyclin A2 (Fig. 2*D*). These results demonstrated that cyclin A2 specifically activates PCTK3 and suggested that there are additional mechanisms for PCTK3 activation in addition to its interaction with cyclin A2.

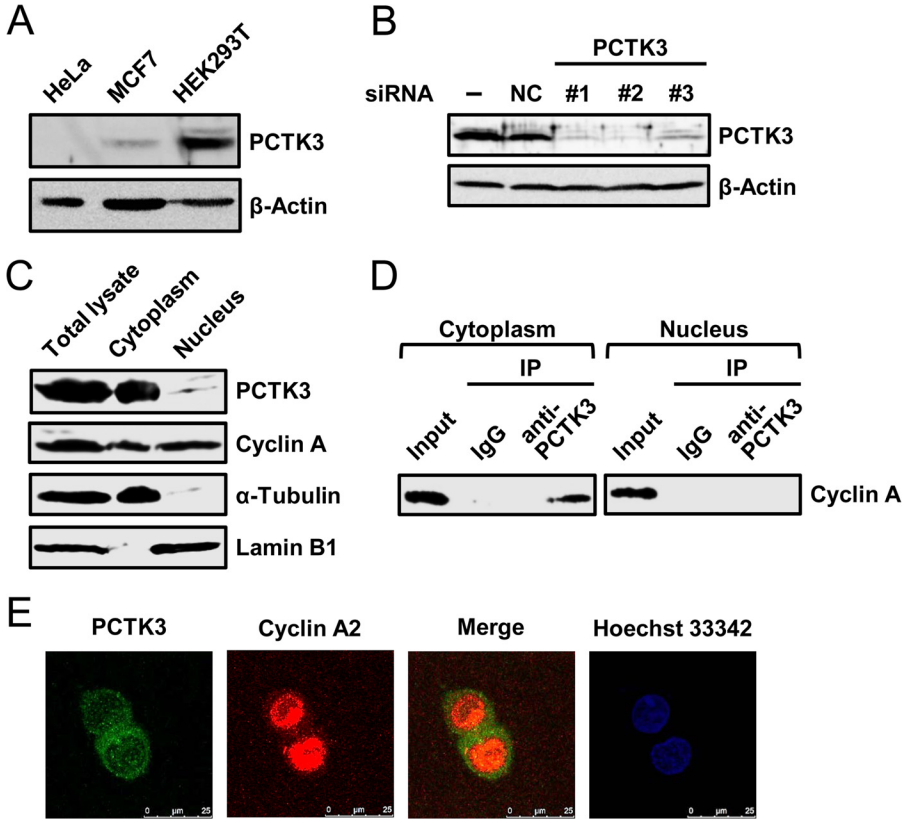

FIGURE 3. **Endogenous interaction between PCTK3 and cyclin A in the cytoplasm.** *A*, immunoblot analysis of total proteins prepared from HeLa, MCF7, and HEK293T human cell lines. *β*-Actin was used as a loading control. *B*, siRNA knockdown of PCTK3 in HEK293T cells. Cells were transfected with three different siRNAs against PCTK3 (*#1–3*) or negative control siRNA (*NC*). Forty-eight hours after transfection, cell extracts were immunoblotted with anti-PCTK3 antibody. Expression of *β*-actin was used as a loading control. *C*, subcellular distributions of endogenous PCTK3 and cyclin A. HEK293T cells were separated into nuclear and cytoplasmic fractions as described under "Experimental Procedures." Aliquots of each fraction were immunoblotted for PCTK3 and cyclin A. The purity of nuclear and cytoplasmic fractions was confirmed using anti-lamin B1 antibody as a nuclear marker and *α*-tubulin antibody as a cytoplasmic marker, respectively. *D*, endogenous interaction between PCTK3 and cyclin A in HEK293T cells. The fractionated lysates from HEK293T cells were immunoprecipitated (*IP*) using anti-PCTK3 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-cyclin A antibody. Normal rabbit IgG (IgG) was used as a control. *E*, intracellular localization of endogenous PCTK3. HEK293T cells were fixed and incubated with rabbit anti-PCTK3 and mouse anti-cyclin A antibodies. The primary antibody was visualized with Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 555-conjugated anti-mouse IgG followed by confocal microscopy. Fluorescence for PCTK3 (*green*) and cyclin A (*red*) is shown with the merged images (merge is in *yellow*). Hoechst nuclear staining is represented in *blue*.

Endogenous Interaction between PCTK3 and Cyclin A in the Cytoplasm—Next, we investigated the endogenous interaction between PCTK3 and cyclin A. A previous study reported that PCTK3 protein is expressed in some cell lines including HEK293 cells (7). Thus, we confirmed whether PCTK3 expression was detectable in some cell lines. As shown in Fig. 3*A*, an anti-PCTK3 antibody detected a specific protein band with the predicted molecular mass of PCTK3 in HEK293T cells, whereas its expression was very low or absent in human breast carcinoma MCF7cells and human cervical carcinoma HeLa cells. We confirmed PCTK3 expression in HEK293T cells by knockdown analysis using siRNA. As shown in Fig. 3*B*, PCTK3 expression was efficiently reduced by each of the three PCTK3 siRNAs used, whereas negative control siRNA had no effect on PCTK3 expression. We also examined the subcellular distributions of PCTK3 and cyclin A by simple cell fractionation. PCTK3 protein was found in the cytoplasm, whereas cyclin A was localized in both the cytoplasm and nucleus (Fig. 3*C*). Subsequently, we investigated cyclin A binding to PCTK3 in the nuclear and cytoplasmic fractions. Immunoprecipitates from each fraction with an anti-PCTK3 antibody were analyzed by immunoblotting with anti-cyclin A antibody. Cyclin A was detected in the cytoplasmic PCTK3 immunoprecipitates but not in the nuclear immunoprecipitates (Fig. 3*D*), indicating that PCTK3 indeed interacts with cyclin A *in vivo*. We also examined the intracellular localization of endogenous PCTK3 and cyclin A in HEK293T cells. Immunofluorescence analysis using confocal laser-scanning microscopy revealed that PCTK3 and cyclin A were mainly found in the cytoplasm and nucleus, respectively, and that they colocalized in the cytoplasm (Fig. 3*E*).

Additionally, we transfected HEK293T cells with FLAG-PCTK3 and Myc-cyclin A2 and performed immunofluorescence analysis. In cells that were singly transfected with either Myc-cyclin A2 or FLAG-PCTK3, cyclin A2 was strongly detected in the nucleus and moderately in the cytoplasm, whereas PCTK3 was found in only the cytoplasm (data not shown), consistent with previous data (30). In cells cotransfected with Myc-cyclin A2 and FLAG-PCTK3, cyclin A2 was translocated from the nucleus to the cytoplasm and colocalized with PCTK3 in the cytoplasm (Fig. 4, *A* and *C*). By comparison, when cyclin A2 was cotransfected with CDK2, it colocalized with CDK2 in the nucleus (Fig. 4, *B* and *C*). These findings suggested that PCTK3 and CDK2 regulate the localization of cyclin A2 in the cytoplasm and the nucleus, respectively.

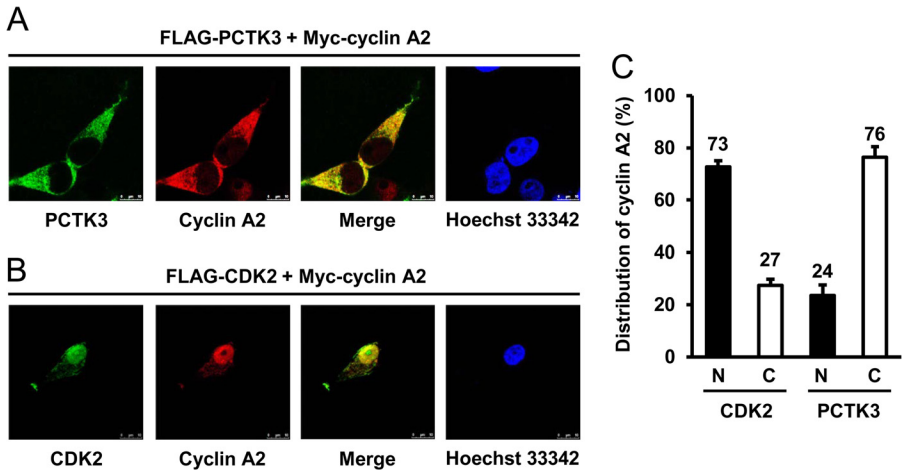

FIGURE 4. **Different intracellular localization of cyclin A2 in PCTK3- and CDK2-overexpressed cells.** *A* and *B*, HEK293T cells were cotransfected with Myc-cyclin A2 together with either FLAG-PCTK3 (*A*) or FLAG-CDK2 (*B*). Cells were fixed and incubated with mouse anti-FLAG and rabbit anti-Myc antibodies. The primary antibody was visualized with Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 555-conjugated anti-rabbit IgG followed by confocal microscopy. Fluorescence for FLAG-PCTK3 or FLAG-CDK2 (*green*) and Myc-cyclin A2 (*red*) is shown with the merged images (*merge* is in *yellow*). Hoechst nuclear staining is represented in *blue*. *C*, the fluorescence intensities of Myc-cyclin A2 in the nuclear (*N, closed bar*) and cytoplasmic (*C, open bar*) regions were quantified using Image J software. The data shown are the means ± S.E. ($n \geq 5$).

PCTK3 Protects Cyclin A2 against Degradation in the Cytoplasm—Cyclin A2 is degraded during prometaphase by anaphase-promoting complex/cyclosome (APC/C), and this degradation is necessary for mitosis progression (31). Cyclin E bound to CDK2 is also protected from degradation by the proteasome (32). We hypothesized that cyclin A2 interacted with PCTK3 is also protected from degradation in the cytoplasm. Thus, to analyze whether PCTK3 regulates cyclin A2 stability, we investigated the protein levels of cyclin A2 in PCTK3 knockdown HEK293T cells. As expected, the protein levels of cyclin A2 in PCTK3 knockdown cells was low compared with that in negative control siRNA-treated cells (Fig. 5*A*). To verify the protective effect of PCTK3 against cyclin A2 degradation, HEK293T cells cotransfected with FLAG-cyclin A2 and GST-PCTK3 were treated with cycloheximide, a protein synthesis inhibitor, after which they were harvested at the indicated times. Cycloheximide chase analysis showed that cyclin A2 was rapidly degraded in the absence of GST-PCTK3, whereas cyclin A2 degradation was remarkably delayed in the presence of GST-PCTK3 (Fig. 5*B*). Furthermore, we examined if PCTK3 sequesters cyclin A2 in the cytoplasm and negatively regulates CDK2 activity in the nucleus. Nuclear fractions of HEK293T cells transfected with FLAG-PCTK3 were immunoprecipitated using anti-CDK2 antibody, and an *in vitro* kinase assay was performed using Rb as the substrate. As shown in Fig. 5*C*, PCTK3 did not regulate CDK2 activity in the nucleus. These results suggest that PCTK3 may regulate the stability of cyclin A2, which is normally degraded in the cytoplasm by ubiquitin-proteasome pathway.

PCTK3 Is Phosphorylated by PKA in Vitro and in Vivo—PKA is an essential factor that has been implicated in various physiological functions, including cytoskeletal organization, cell motility, and signal transduction (16). PKA preferentially phosphorylates serine/threonine residues within the consensus sequence Arg-Arg-*X*-Ser/Thr. A previous report demonstrated that PKA inactivates PCTK1 via phosphorylation at Ser^{153} (6). Amino acid sequence analysis revealed that mouse and human PCTK3 each contained four putative PKA phosphorylation sites, $RRLS^{12}$, $RRFS^{66}$, $RRAS^{109}$, and $RRQS^{449}$ in mouse and $RRFS^{14}$, $RRFS^{89}$, $RRAS^{162}$, and $RRQS^{502}$ in human. This suggested that PCTK3 is a substrate for PKA. To determine whether endogenous PCTK3 was phosphorylated by PKA, HEK293T cells were treated with the adenylate cyclase activator forskolin. We immunoprecipitated cell lysates with an anti-PCTK3 antibody, and followed it by immunoblot analysis using an anti-phospho-RR*X*(S/T) antibody. As shown in Fig. 6*A*, PCTK3 proteins were specifically precipitated by the anti-PCTK3 antibody and were efficiently phosphorylated after treatment with forskolin. Next, we examined whether PKA directly phosphorylates PCTK3. COS-7 cells were cotransfected with FLAG-PCTK3 and FLAG-PKA catalytic subunit, after which an *in vitro* kinase assay was performed. As shown in Fig. 6*B*, PKA directly phosphorylated PCTK3. Furthermore, to identify the PKA phosphorylation sites of PCTK3, we constructed four non-phosphorylatable mutants of mouse PCTK3 in which each of the putative PKA phosphorylation sites was replaced by alanine: S12A, S66A, S109A, and S449A. We tested these using *in vitro* and *in vivo* kinase assays. Phosphorylation of the S12A, S66A, and S109A mutants was reduced as compared with that of wild type PCTK3, whereas the phosphorylation intensity of the S449A mutant was almost equal to that of wild-type PCTK3 (Fig. 6*B*). Although PKA phosphorylated the S12A/S66A, S66A/S109A, and S12A/S109A mutants of PCTK3 (Fig. 6*C*), it failed to phosphorylate the S12A/S66A/S109A mutant (Fig. 6, *B* and *C*). These results identified three serine residues, Ser^{12}, Ser^{66}, and Ser^{109}, as possible PKA phosphorylation sites for mouse PCTK3. Furthermore, the PKA phosphorylation sites of PCTK3 were confirmed by an *in vivo* kinase assay using an anti-phospho-RR*X*(S/T) antibody. HeLa cells transiently transfected with FLAG-tagged PCTK3 were treated with 10 μM forskolin. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then immunoblotted with an anti-phospho-RR*X*(S/T) antibody. As expected, forskolin treatment resulted in a 1.7-fold increase in phosphorylation of

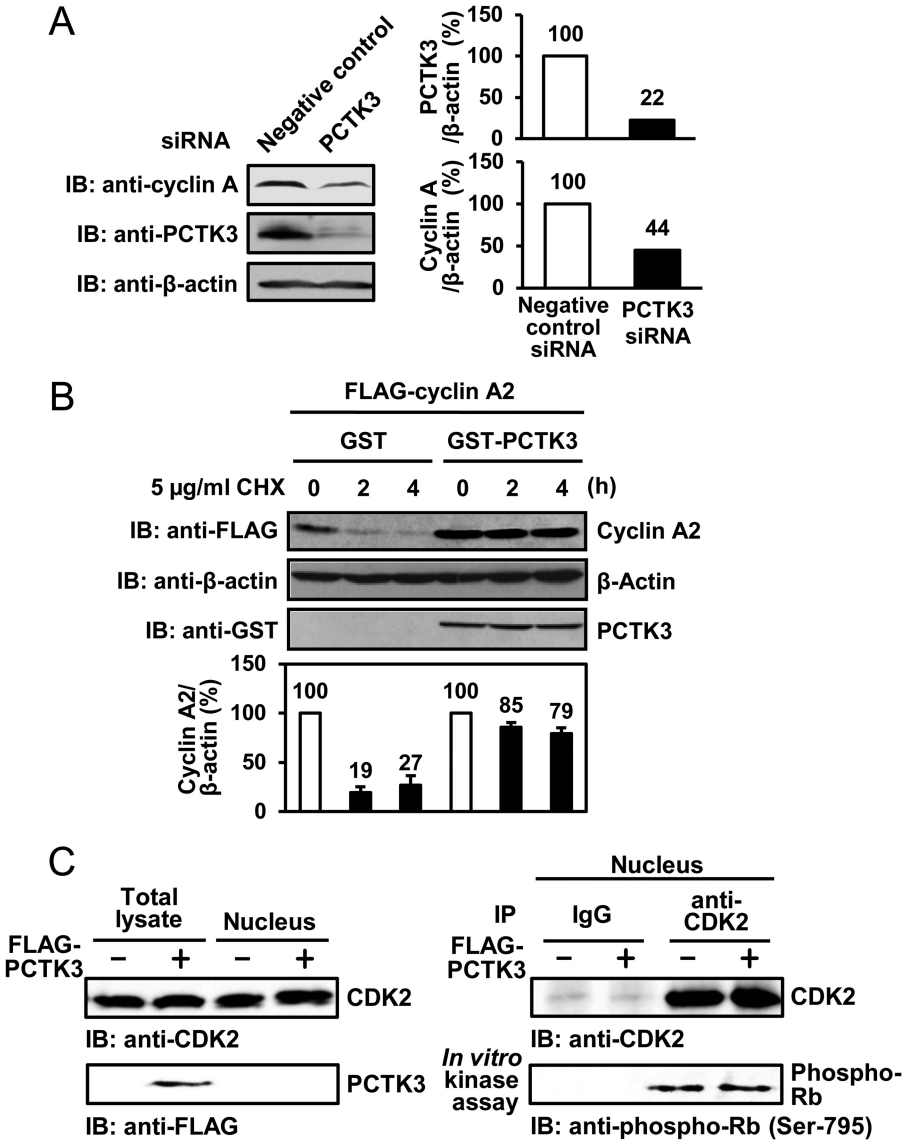

FIGURE 5. **PCTK3 protects cyclin A2 against degradation in the cytoplasm.** *A*, HEK293T cells were transfected with PCTK3 siRNA. The cell lysates were subjected to immunoblotting (*IB*) with anti-PCTK3 and anti-cyclin A antibodies. Expression of α-tubulin was used as a loading control. *B*, HEK293T cells were cotransfected with GST or GST-PCTK3 together with FLAG-cyclin A2. After 24 h, cycloheximide (*CHX*) (5 μg/ml) was added to the cell culture. Cells were harvested at indicated times after cycloheximide treatment and FLAG-cyclin A2 protein levels were determined by immunoblotting (*IB: anti-FLAG*). β-Actin was used as a loading control (IB: *anti-β-actin*). FLAG-cyclin A2 protein levels were quantified by densitometric analysis and represented in a graph. *C*, HEK293T cells were transfected with FLAG-PCTK3 and separated into nuclear and cytoplasmic fractions. The nuclear fractions were immunoprecipitated (*IP*) using anti-CDK2 antibody, and the immunoprecipitates were used in an *in vitro* kinase assay with MBP-Rb C as a substrate. Normal rabbit IgG (*IgG*) was used as a control.

PCTK3 wild type, whereas the phosphorylation of the S12A, S66A, and S109A mutants was reduced as compared with that of wild-type PCTK3 (Fig. 6*D*). These findings provided evidence that PCTK3 is phosphorylated by PKA at three sites (RRLS^{12}, RRFS^{66}, and RRAS^{109} in mouse) both *in vitro* and *in vivo*.

PCTK3 Activity Is Regulated via Phosphorylation at Ser^{12} by PKA—We then investigated the effects of PKA phosphorylation of PCTK3 on its kinase activity. PCTK3 activity was determined by GST pulldown and *in vitro* kinase assays using Rb C as the substrate. Three phosphomimic PCTK3 mutants (PCTK3 S12D, S66D, and S109D), in which the PKA phosphorylation sites were replaced by aspartic acid, were generated, and the kinase activities of these phospho-mimic mutants were examined. As shown in Fig. 7*A*, the PCTK3 S12D mutant had relatively high activity for Rb phosphorylation even in the absence of cyclin A2. Furthermore, the catalytic activity of the cyclin A2-S12D mutant complex was significantly increased (>2-fold) as compared with that of cyclin A2-wild-type PCTK3, although the ability of S12D mutant to bind to cyclin A was not changed. By comparison, the PCTK3 S66D and S109D mutants had no effect on kinase activity. On the other hand, the activity of the phospho-null mutant S12A was obviously decreased as compared with that of wild-type PCTK3 (Fig. 7*B*), strongly supporting that the phosphorylation at Ser^{12} influences the catalytic activation. In addition, the kinase activity of the PCTK3 S12D mutant was compared with that of the cyclin A2-CDK2 complex. Although cyclin A2-wild-type PCTK3 activity was six

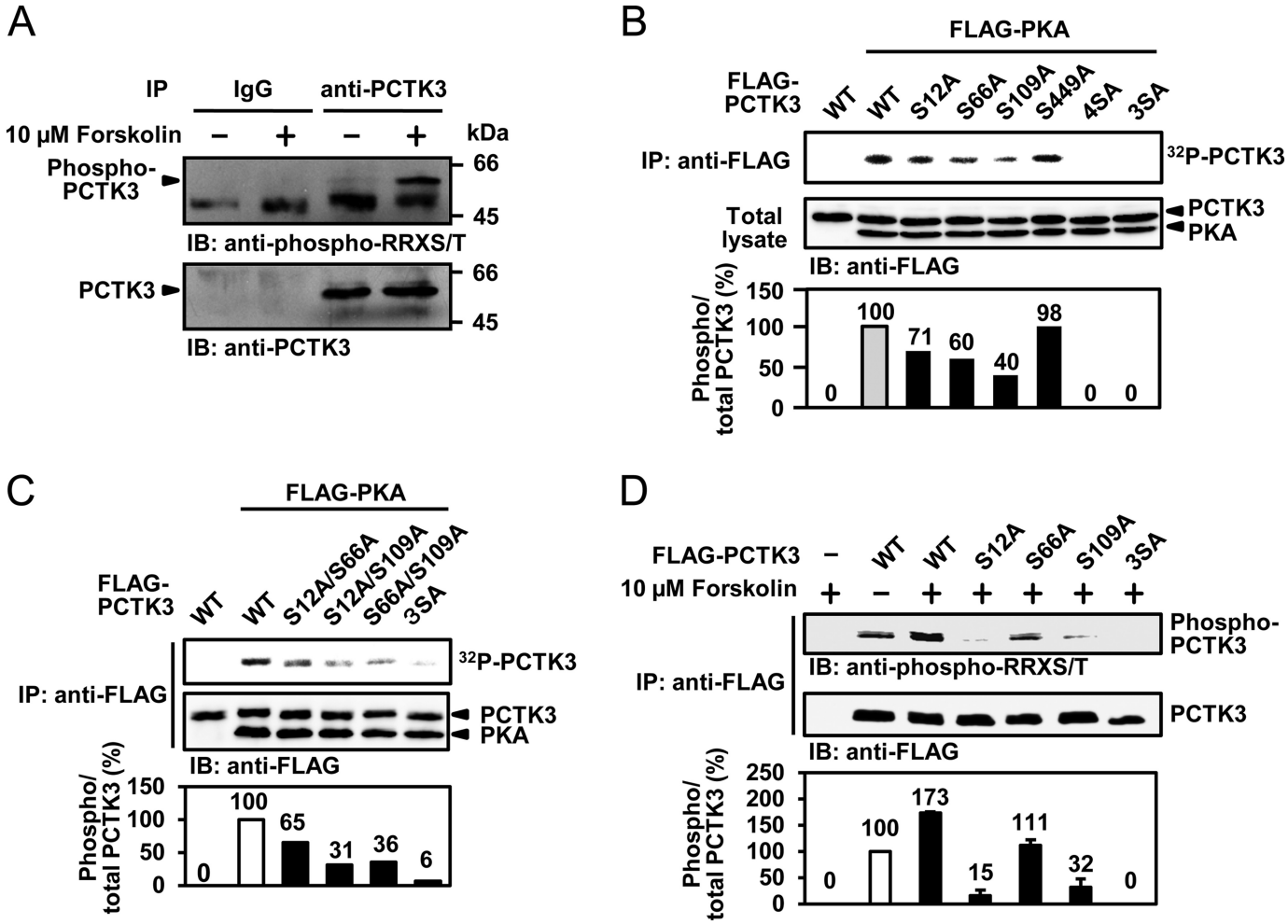

FIGURE 6. **PCTK3 is phosphorylated by PKA *in vitro* and *in vivo*.** *A*, endogenous PCTK3 is phosphorylated by PKA. HEK293T cells were treated with 10 μM forskolin for 30 min in the presence of 10% FBS. Cell lysates were immunoprecipitated (*IP*) with anti-PCTK3 antibody. Immunoprecipitates were analyzed by immunoblot (*IB*) analysis using anti-phospho-RRX(S/T) or anti-PCTK3 antibodies. The bands of phosphorylated and total PCTK3 were indicated by arrows. *B*, FLAG-PKA C, FLAG-PCTK3 wild type (*WT*), and mutants (S12A, S66A, S109, S449, S12A/S66A/S109A/S449A (*4SA*), and S12A/S66A/S109A (*3SA*)) were expressed in COS-7 cells. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with kinase buffer containing [γ-^{32}P]ATP. Equal amounts of cell lysates were analyzed by immunoblotting with anti-FLAG antibody. The graph indicates the phosphorylation rate of PCTK3. The phosphorylation rate of wild-type PCTK3 was taken as 100%. *C*, the double or triple mutants of PCTK3 (S12A/S66A, S12A/S109A, S66A/S109A, and S12A/S66A/S109A (*3SA*)) were subjected to *in vitro* kinase assay using PKA. *D*, HeLa cells were transfected with FLAG-PCTK3 wild type (*WT*) and mutants (S12A, S66A, S109A, and S12A/S66A/S109A (*3SA*)). After 24 h, transfected cells were treated with 10 μM forskolin at 30 min. Cell extracts were subjected to immunoprecipitated with anti-FLAG antibody (*IP: anti-FLAG*). Immunoprecipitates were subsequently analyzed by immunoblotting with anti-phospho-RRX(S/T) or anti-FLAG antibody.

times less than that of cyclin A2-CDK2, the cyclin A2-S12D mutant had strong activity and reached a level comparable with the kinase activity of cyclin A2-CDK2 (Fig. 7*C*).

We also determined whether endogenous PCTK3 was activated by PKA. First, to determine endogenous PCTK3 activity, we immunoprecipitated PCTK3 from HEK293Tcells transfected with negative control siRNA or PCTK3 siRNA and determined *in vitro* kinase activity using Rb as the substrate. As shown in Fig. 7*D*, significantly high Rb phosphorylation activity was detected in immunoprecipitates from cells transfected with negative control siRNA as compared with those from cells transfected with PCTK3 siRNA, suggesting that this activity was dependent on endogenous PCTK3 activity. Therefore, we assessed the effects of forskolin treatment on the kinase activity of PCTK3. Rb phosphorylation was significantly increased after forskolin treatment. These data demonstrated that PCTK3 is activated via phosphorylation at Ser^{12} by PKA.

Several members of the CDK family preferentially phosphorylate the canonical motif Ser/Thr-Pro-*X*-Lys/Arg/His (where *X* represents any amino acid). Because PCTK3 phosphorylated Rb *in vitro*, we investigated whether Rb was phosphorylated by PCTK3 *in vivo*. HEK293T cells were transfected with FLAG-tagged PCTK3 wild type or S12D mutant, after which cell lysates were subjected to immunoblotting using anti-phospho-Rb (Ser^{795}). Both PCTK3 wild type and S12D failed to phosphorylate endogenous Rb, although Rb phosphorylation was detected in cyclin A2-CDK2-transfected cells (Fig. 7*E*). This suggested that Rb is a poor substrate for PCTK3 *in vivo*, possibly due to its different subcellular localization.

Suppression of PCTK3 Induces Actin Cytoskeletal Changes through Cofilin Phosphorylation—In a final set of experiments, we explored possible physiological functions of PCTK3 using a RNA interference approach. We noted that PCTK3 knock-

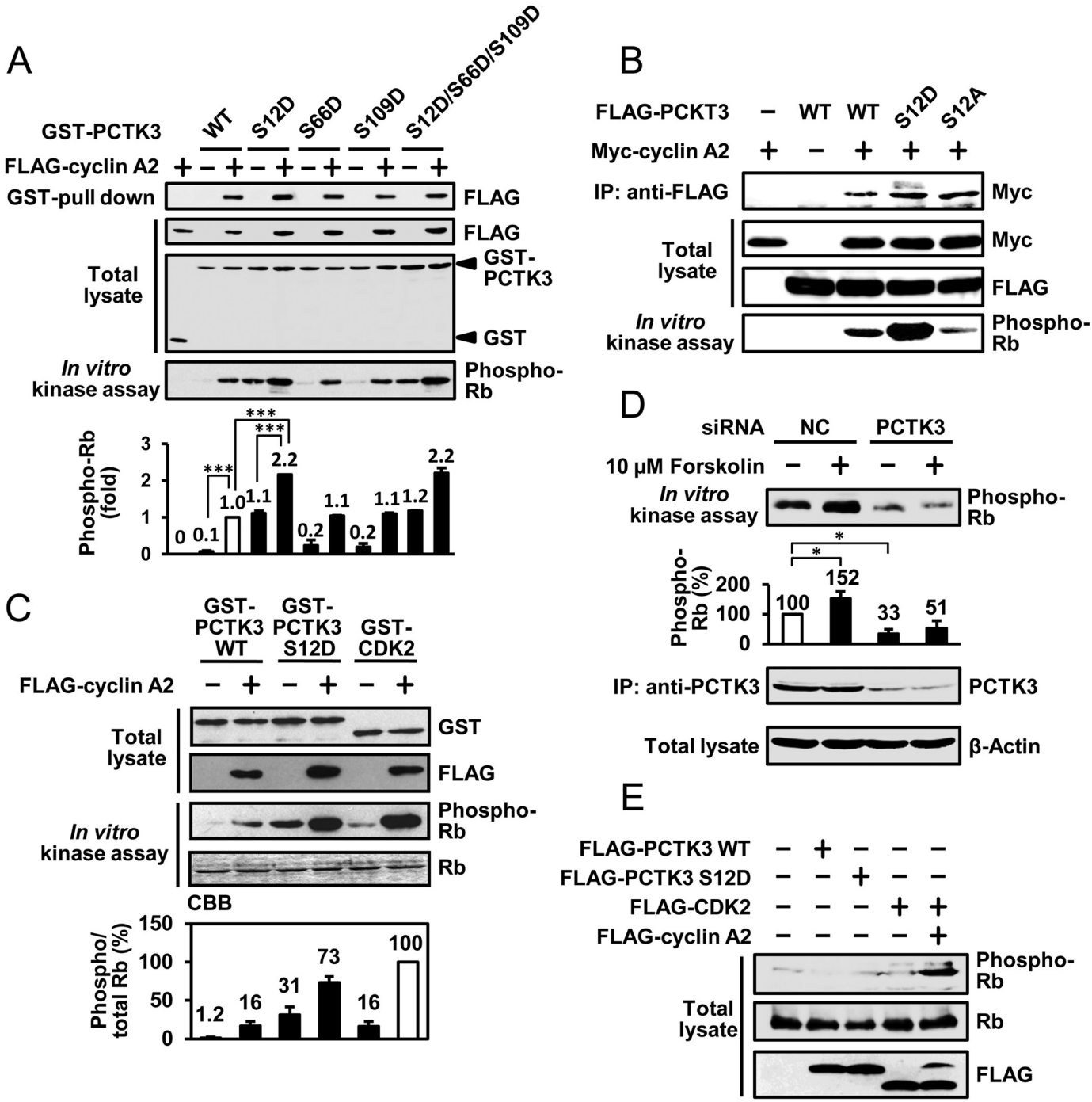

FIGURE 7. **PCTK3 activity is regulated via phosphorylation at Ser[12] by PKA.** *A*, COS-7 cell lysates expressing GST, GST-PCTK3 wild-type (*WT*), or GST-PCTK3 mutants (S12D, S66D, S109D, and S12D/S66D/S109D) and FLAG-cyclin A2 were used in a kinase assay using Rb as the substrate. The relative kinase activity of PCTK3 was quantified by densitometric analysis. The activity of wild-type PCTK3 complexed with FLAG-cyclin A2 was taken as 1. Results are expressed as the means ± S.E. from three independent experiments. Statistical significance was determined by analysis of variance. ***, $p < 0.001$. *B*, FLAG-PCTK3 WT and S12D and S12A mutants were expressed with Myc-cyclin A2 in COS-7 cells. The cell lysates were immunoprecipitated (*IP*) with anti-FLAG antibody (*IP: anti-FLAG*), and an *in vitro* kinase assay was performed using MBP-Rb C as the substrate. *C*, GST-PCTK3 WT, S12D mutant, and GST-CDK2 was expressed in COS-7 cells with or without FLAG-cyclin A2. The protein complexes were pulled down by glutathione-Sepharose beads, and an *in vitro* kinase assay was performed using MBP-Rb C protein as the substrate. The graph shows the average of three independent experiments. *CBB*, Coomassie Brilliant Blue. *D*, expression of PCTK3 in HEK293T cells was knocked down by siRNA. PCTK3 knockdown cells were treated with 10 μM forskolin for 30 min in medium containing 10% FBS. Cell lysates were immunoprecipitated with anti-PCTK3 antibody. Immunoprecipitates were subjected to *in vitro* kinase assay using Rb as the substrate. The activity of endogenous PCTK3 in negative control (*NC*) knockdown cells was taken as 100%. Experiments were performed three times independently. Results are expressed as the means ± S.E. Statistical significance was determined by analysis of variance. *, $p < 0.05$. *E*, HEK293T cells were transfected with FLAG-PCTK3 wild type and S12D mutant, FLAG-CDK2, and FLAG-cyclin A2. After 24 h, cell extracts were analyzed by immunoblotting with anti-phospho-Rb (Ser[795]), anti-Rb, and anti-FLAG antibodies.

down in HEK293T cells induced morphological changes, cell spreading, and extensions (Fig. 8, *A* and *B*). To investigate whether this is responsible for alteration of the actin cytoskeleton, we visualized F-actin by staining cells with phalloidin. Control HEK293T cells exhibited a relatively weak cortical distribution of F-actin (Fig. 8*B*, *upper panel*). In PCTK3 knock-

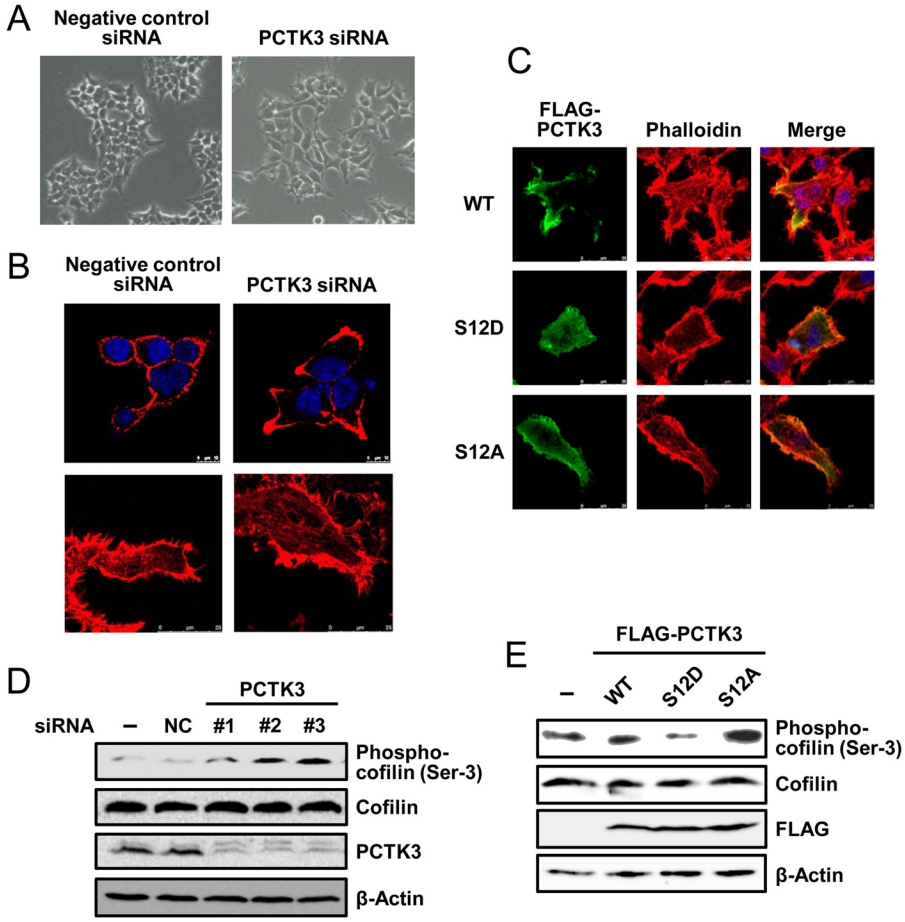

FIGURE 8. **Suppression of PCTK3 induces actin cytoskeletal changes through cofilin phosphorylation.** *A* and *B*, HEK293T cells were transfected with negative control siRNA or PCTK3 siRNA. After 48 h, cells were fixed and incubated with Alexa 555-conjugated phalloidin (*B, red*). Hoechst nuclear staining is represented in *blue*. *C*, HEK293T cells were transfected with PCTK3 siRNA for 48 h and then transfected with FLAG-tagged mouse PCTK3 wild type (*WT*) or S12D or S12A mutants. After 24 h, cells were fixed and incubated with mouse anti-FLAG antibody. The primary antibody was visualized with Alexa Fluor 488-conjugated anti-mouse IgG followed by confocal microscopy. Fluorescence for FLAG-PCTK3 (*green*) and F-actin (Alexa 555-conjugated phalloidin staining, *red*) are shown with merged images (merge is in *yellow*). Hoechst nuclear staining is represented in *blue*. *D*, cell lysates of PCTK3 knock-down HEK293T cells were subjected to immunoblotting with anti-PCTK3, anti-cofilin, and anti-phospho-cofilin (Ser^3) antibodies. Expression of β-actin was used as a loading control. *E*, HEK293T cells were transfected with FLAG-PCTK3 (WT or S12D or S12A mutant). After 24 h, cell lysates were subjected to immunoblotting using anti-phospho-cofilin (Ser^3), anti-cofilin, and anti-FLAG antibodies.

down cells, lamellipodia extensions were induced, and F-actin was concentrated at these lamellipodia. PCTK3 knockdown also stimulated the appearance of stress fibers (Fig. 8*B*, *lower panel*). Furthermore, the effects of PCTK3 overexpression on actin filament dynamics were examined. Constitutively active mutant of PCTK3 (PCTK3 S12D) suppressed formation of actin stress fibers, whereas wild-type PCTK3 and S12A mutant did not (Fig. 8*C*).

Actin filament dynamics are regulated by actin-depolymerizing factor/cofilin proteins, and cofilin is inactivated through the phosphorylation at Ser^3 (33, 34). Therefore, we investigated the phosphorylation states of cofilin in PCTK3 knockdown HEK293T cells by immunoblotting using anti-phospho-cofilin (Ser^3). As shown in Fig. 8*D*, cofilin phosphorylation in PCTK3 knockdown HEK293T cells was markedly increased as compared with that in control cells. In contrast, overexpression of PCTK3 S12D effectively suppressed cofilin phosphorylation without affecting total cofilin protein levels, although wild-type PCTK3 did not affect (Fig. 8*E*). The phospho-null S12A mutant rather slightly increased. These data suggested that PCTK3 was involved in the actin cytoskeleton organization.

DISCUSSION

In this study we provided evidence for the activation mechanisms of PCTK3, the activity of which was regulated by two pathways: binding to cyclin A2 and phosphorylation by PKA. Cyclin A2 is widely expressed in various tissues and has been proposed to regulate the transition from the S phase to the M phase during the mitotic cell cycle. The canonical partners for cyclin A2 are CDK1 and CDK2, and cyclin A2-CDK2 complex has been generally used as a structural model of cyclin-CDK. Crystal structure analysis revealed that cyclin A2 enhanced the catalytic activity of CDK2 through rearrangement of its catalytic core element, including the PSTAIRE helix (35). The kinase domain of PCTK3 is highly conserved among isoforms (>80% identity) and has high sequence homology with that of CDK2 (52% identity in humans) (5). Although the crystal structure of PCTK3 remains to be determined, that of PCTK1 is available from the Protein Data Bank (PDB code 3MTL). By comparison with the crystal structure of the cyclin A2-CDK2 complex, two amino acid residues of cyclin Y, Lys^{225} and Glu^{253}, have been identified as important residues for binding

to PCTK1 (24). This Lys-Glu pair is also found in other cyclins and is likely to be a conserved structural motif in cyclin-CDK complexes. This suggests that the Lys-Glu pair of cyclin A2 is also likely to mediate the interaction with PCTK3. In addition, we demonstrated that all three PCTK isoforms bound to cyclin A2 at nearly equal affinity. These data suggested that PCTK3 may be associated with cyclin A2 through a common region of PCTK isoforms, such as the PCTAIRE sequence. On the other hand, the catalytic activities of PCTK1 and PCTK2 were not affected by binding to cyclin A2. Furthermore, we found that cyclin E1 did not affect the catalytic activity of PCTK3, although it had a greater affinity for PCTK3 than cyclin A2. Several studies have showed that substrate recognition by CDKs is modulated by their associations with different cyclins (36–39). For example, although both cyclin A-CDK2 and cyclin E/CDK2 phosphorylate histone H1, only the cyclin A-CDK2 complex phosphorylates lamin B *in vitro* (39). Furthermore, although both cyclin A and cyclin E associate with CDK1, only cyclin A activates CDK1 kinase activity, indicating that cyclin A and E could modulate the substrate specificity of CDKs. These facts raise the possibility that cyclin E1-PCTK3, cyclin A2-PCTK1, and cyclin A2-PCTK2 complexes may have kinase activity against other substrates but not Rb. On the other hand, cyclin E inhibits the activity of CDK5 by competing with p35 for CDK5 binding (40). This suggests that cyclin E1 may inactivate PCTK3 by dissociating cyclin A2 from PCTK3. To obtain more detailed information regarding the regulatory mechanism of PCTK3 by cyclin A2 and cyclin E1, it will be necessary to perform crystal structure analysis. In an *in vitro* kinase assay, we also found that cyclin A2-PCTK3 efficiently phosphorylates Rb among Rb, MyBP, and histone H1. However, PCTK3, but not CDK2, failed to phosphorylate endogenous Rb. Previous studies indicated that Rb regulates cell proliferation by controlling the activity of various transcription factors in the nucleus (41, 42), suggesting that PCTK3 may not be able to make contact with Rb because of the differences in their subcellular localizations. Taken together, PCTK3 may be involved in other cellular functions independent of the cell cycle via the phosphorylation of its true substrates in the cytoplasm.

Cyclin A2 lacks a nuclear localization signal and is dependent on binding partners for its translocation between the nucleus and the cytoplasm (30, 43). However, the precise translocation mechanism for cyclin A2 remains obscure because CDK2 also has no nuclear localization signal motif. Recently, SCAPER was identified and characterized as a substrate for cyclin A2-CDK2, which is exclusively localized in the cytoplasm (14). Although the function of SCAPER remains unknown, it was suggested that SCAPER may act as a cytoplasmic pool for cyclin A2 and regulate cell cycle progression. On the other hand, we found that PCTK3 bound to cyclin A2 in the cytoplasm and promoted the translocation of cyclin A2 from the nucleus to the cytoplasm. These results demonstrated that PCTK3 is a cytoplasmic binding partner of cyclin A2 and that cyclin A2 alters its subcellular localization by binding to different partners.

The expression level of cyclin A2 is tightly controlled by transcription and ubiquitin-mediated degradation. Cyclin A2 degradation begins in early prometaphase and is completed by metaphase. The major ubiquitin ligase required for mitosis is APC/C (anaphase-promoting complex/cyclosome) (31). Because the level of cyclin A2 protein was greatly reduced in PCTK3 knockdown cells as compared with that in control cells, we hypothesized that PCTK3 protects cyclin A2 from proteolytic degradation. As expected, cycloheximide chase analysis showed that cyclin A2 degradation is suppressed in the presence of PCTK3, whereas free cyclin A2 is readily degraded. A previous study showed that cyclin E bound to CDK2 is also protected from degradation by the proteasome (32). These observations strongly support the physiological significance of the association of between PCTK3 and cyclin A2.

Furthermore, PCTK3 was also activated by PKA via phosphorylation at Ser^{12}, independently of its interaction with cyclin A2. Endogenous PCTK3 in HEK293T cells was phosphorylated and activated by the PKA activator forskolin. In addition, site-directed mutagenesis analysis revealed that PKA phosphorylates Ser^{12}, Ser^{66}, and Ser^{109} of PCTK3. These results indicate that PCTK3 is a *bona fide* substrate of PKA. Furthermore, the Rb phosphorylation activity of a phosphomimic mutant of Ser^{12} (S12D) was comparable with the activity of CDK2 in the presence of cyclin A2, suggesting that the complete activation of PCTK3 is required for not only binding to cyclin A2 but also for its phosphorylation at Ser^{12}. Although the S12D mutant caused an increase in PCTK3 activity, phosphomimic mutations of Ser^{66} and Ser^{109} (homologous to Ser^{119} and Ser^{153} of PCTK1, respectively) had no effects on the Rb phosphorylation activity of PCTK3. Recently, Mikolcevic *et al.* (10) reported that PKA plays an inhibitory role in the interaction between PCTK1 and cyclin Y by phosphorylation at Ser^{153} of PCTK1. On the other hand, phosphorylation at Ser^{153} of PCTK1 creates the binding motif of 14-3-3 proteins and transfers PCTK1 from the cell membrane to the cytoplasm. The 14-3-3 protein family includes seven members (β, γ, ϵ, σ, ζ, τ, and η) in mammals, which are highly conserved and ubiquitously expressed proteins (44). These 14-3-3 proteins can interact with various proteins, usually by recognizing phosphoserine or phosphothreonine motifs, and alter the activity, subcellular localization, and interacting partners of their target proteins, which in turn modulate diverse biological processes. A previous report showed that PCTK3 interacts with 14-3-3ζ (45). Consistent with this, we found that PCTK3 binds to several 14-3-3 isoforms.[3] Therefore, PKA phosphorylation at Ser^{66} and Ser^{109} of PCTK3 may mediate the interactions with 14-3-3 proteins and alter the subcellular localization of PCTK3.

Furthermore, PKA is involved in regulating of cell morphology through phosphorylation of cytoskeletal proteins including microtubules, intermediate filaments, and actin (46, 47). PKA also regulates actin filament turnover by modulating the activity of LIM domain kinase 1 (LIMK1) (48). A recent study reported that cyclin A2 plays a role in cytoskeletal reorganization via its association with RhoA, a member of the small GTPase family. Suppression of cyclin A2 by shRNA resulted in an increase in cell size and cortical localization of F-actin independent of the cell cycle, which led to increased cell migration and invasion (15). Similar to the cyclin A2-deficient phenotype,

[3] S. Matsuda, K. Kominato, and K. Yuasa, unpublished data.

PCTK3 knockdown HEK293T cells also exhibited an increase in cell size and F-actin accumulation at the edges of cell membranes and increased cofilin phosphorylation. These data suggest that PCTK3 activated through binding to cyclin A2, and phosphorylation by PKA is involved in regulating cell motility via reorganization of actin filaments. Although CDKs were originally identified as enzymes that controlled cell cycle events, some members of the CDK family are involved in other cellular processes (49). For example, CDK5 is not considered to play a significant role in cell cycle regulation; however, it has an important function in the control of neurogenesis, including neurite outgrowth, axon guidance, and cell migration. CDK5 activated by neuron-specific regulator p35 inhibits p21-activated kinase 1 (PAK1) activity through phosphorylation at Thr^{212} (PV<u>T</u>PTRD, where <u>T</u> is Thr^{212}) (50). PAK1, an effector of Rac, has a pivotal role in cell morphology and motility via the LIM domain kinase/cofilin pathway (51). Phosphorylation of PAK1 by p35/CDK5 is likely to be implicated in the dynamics of the actin cytoskeleton reorganization. PCTK3 may also phosphorylate substrate proteins, such as PAK1, and control actin dynamics.

In summary, we demonstrated that cyclin A2 is a specific activator of PCTK3 and that phosphorylation at Ser^{12} of PCTK3 significantly enhances its kinase activity in the absence of cyclin A2. These findings shed light on the activation mechanism of not only PCTK3 but also other uncharacterized members of the CDK family, such as PCTK2. Further investigations will be needed to clarify the physiological functions and pathological roles of PCTK3.

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PCTAIRE Kinase 3/Cyclin-dependent Kinase 18 Is Activated through Association with Cyclin A and/or Phosphorylation by Protein Kinase A

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