

# Ahnak Protein Activates Protein Kinase C (PKC) through Dissociation of the PKC-Protein Phosphatase 2A Complex\*

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We have previously reported that central repeated units (CRUs) of Ahnak act as a scaffolding protein networking phospholipase C $\gamma$  and protein kinase C (PKC). Here, we demonstrate that an Ahnak derivative consisting of four central repeated units binds and activates PKC- $\alpha$  in a phosphatidylserine/1,2-dioleoyl-*sn*-glycerol-independent manner. Moreover, NIH3T3 cells expressing the 4 CRUs of Ahnak showed enhanced c-Raf, MEK, and Erk phosphorylation in response to phorbol 12-myristate 13-acetate (PMA) compared with parental cells. To evaluate the effect of loss-of-function of Ahnak in cell signaling, we investigated PKC activation and Raf phosphorylation in embryonic fibroblast cells (MEFs) of the Ahnak knock-out (Ahnak<sup>-/-</sup>) mouse. Membrane translocation of PKC- $\alpha$  and phosphorylation of Raf in response to PMA or platelet-derived growth factor were decreased in Ahnak null MEF cells compared with wild type MEFs. Several lines of evidence suggest that PKC- $\alpha$  activity is regulated through association with protein phosphatase 2A (PP2A). A co-immunoprecipitation assay indicated that the association of PKC- $\alpha$  with PP2A was disrupted in NIH3T3 cells expressing 4 CRUs of Ahnak in response to PMA. Consistently, Ahnak null MEF cells stimulated by PMA showed enhanced PKC-PP2A complex formation, and add-back expression of Ahnak into Ahnak null MEF cells abolished the PKC-PP2A complex formation in response to PMA. These data indicate that Ahnak potentiates PKC activation through inhibiting the interaction of PKC with PP2A.

Ahnak is a large protein with a size of 700 kDa that was initially identified in human neuroblastomas and skin epithelial cells (1–3). Ahnak can be divided into three regions based on the protein structure; they are the amino-terminal 251 amino

acids, the large central region of about 4300 amino acids made up of conserved domains repeated 36 times, and the carboxyl-terminal 1002 amino acids. Several lines of recent evidence indicate that the carboxyl-terminal region of Ahnak plays an important role in the formation of cytoskeletal structure, calcium homeostasis, and muscle regeneration (4–6). The complex of Ahnak and the annexin 2/S100A10 regulates the formation of cortical actin cytoskeleton and cell membrane cytoarchitecture (7, 8). Ahnak also has been reported to play a role in modulation of L-type channel in response to  $\beta$ -adrenergic stimulation in blood vessels (9–11). In low calcium concentrations, Ahnak proteins are mainly localized in nucleus, but an increase in intracellular calcium level leads to translocation of the protein to the plasma membrane. Phosphorylation of serine 5535 in the carboxyl-terminal of Ahnak protein by nuclear protein kinase B was shown to be essential for its export from nucleus (12). The typical central repeated unit of Ahnak is 128 amino acids in length and contains a hepta-sequence motif, (D/E) $\phi$  $\Omega$  $\phi$ K(A/G)P, where  $\phi$  and  $\Omega$  represent hydrophobic and hydrophilic amino acid residues, respectively. The 128-amino acid-long domain represents structurally a polyionic rod with hydrophobic amino acids facing inward and hydrophilic amino acids facing outward. The overall structure is a typical  $\beta$ -strand that is predicted to form a propeller structure. Therefore, a single Ahnak molecule would contain a large number of tandem propeller structures. It is suggested that the central repeat unit likely supports the structural integrity and scaffolding activity of Ahnak (13). It has been reported that central repeat unit of Ahnak binds to and activates phospholipase C $\gamma$ 1 in the presence of arachidonic acid (14). We have previously reported that 4 CRUs<sup>4</sup> of Ahnak interact with phospholipase C $\gamma$  and protein kinase C- $\alpha$  (PKC- $\alpha$ ), leading to an activation of inositol metabolism including inositol 1,4,5-trisphosphate production and intracellular calcium mobilization (15). The result demonstrated that central repeated units of Ahnak plays the role of a molecular linker for calcium homeostasis in response to agonists.

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<sup>4</sup> The abbreviations used are: CRUs, central repeated units; MEF, mouse embryonic fibroblast; PP2A, protein phosphatase 2A; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; JNK, c-Jun NH<sub>2</sub>-terminal kinase; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase; PBS, phosphate-buffered saline; HA, hemagglutinin; TRITC, tetramethylrhodamine isothiocyanate; PS, phosphatidylserine; GST, glutathione S-transferase.

It is well established that PKC family members play important roles in the regulation of differentiation and proliferation of many cell types in response to diverse stimuli including hormones, neurotransmitters, and growth factors (16). The PKC isozymes can be classified into three groups according to their regulatory properties, which are in turn governed by the presence of specific domains in the proteins. The conventional PKCs (cPKCs) include PKC- $\alpha$ , - $\beta$ I, - $\beta$ II and - $\gamma$ , and these isoforms can be activated by Ca<sup>2+</sup> and/or by diacylglycerol and phorbol esters. The novel PKCs (nPKCs) including PKC- $\delta$ , - $\epsilon$ , - $\theta$ , and - $\eta$ , can also be activated by diacylglycerol and phorbol esters but are Ca<sup>2+</sup>-independent (16–18). The atypical PKCs, which include PKC- $\xi$  and PKC- $\iota$ , are unresponsive to Ca<sup>2+</sup> and diacylglycerol/phorbol esters. Consistent with these structural differences, individual PKC isozymes mediate unique cellular functions through the interaction with distinct PKC-binding proteins. PKC-binding proteins including receptor for activated protein kinase C (RACK), MARCKS, and PICK1 can influence subcellular localization, mechanism of activation, and specificity of substrate of PKCs (11, 19, 20).

Protein phosphatase 2A (PP2A), a trimeric protein complex composed of catalytic C subunit, structural A subunit, and regulatory B subunit, is an ubiquitously expressed cytoplasmic serine/threonine phosphatase (21). PP2A activity is regulated by mechanisms that include subunit phosphorylation, methylation of catalytic subunit, and specific targeting to subcellular localization. Evidences suggest that PP2A interacts with PKC and regulates its activity (22–24). In this report, we describe the effect of 4 CRUs of Ahnak on PKC activity through regulation of the interaction of PKC with PP2A. We also demonstrate that 4 CRUs of Ahnak protein play a critical role in the activation of Raf/MEK/Erk/Elk-1 cascade as a downstream effector of PKC.

## EXPERIMENTAL PROCEDURES

**Materials**—[ $\gamma$ -<sup>32</sup>P]ATP (10mCi/ml) was purchased from Amersham Biosciences. GF109203X and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem. Platelet-derived growth factor-BB (PDGF-BB) was purchased from Upstate Biotechnology. Phosphatidylserine and 1,2-dioleoyl-*sn*-glycerol was purchased from Sigma. Effectogen and Lipofectamine were purchased from Qiagen and Invitrogen, respectively. Antibodies against phospho-Erk1/2 (Thr-202/Tyr-204), phospho-MEK1/2 (Ser-217/221), phospho-JNK (Thr-183/Tyr-185), phospho-p38 MAPK (Thr-180/Tyr-182), p38 MAPK, phospho-PKC (Ser-160), and JNK were obtained from Cell Signaling Technology. Antibodies against phospho-Raf-1 (Ser-338), PKC- $\alpha$ , and PP2A and purified recombinant PKC- $\alpha$  protein were purchased from Upstate Biotechnology. Antibodies to PKC- $\delta$  and phospho-PKC (Ser-660) and integrin  $\beta$ 1 were purchased from BD Transduction Laboratories. Antibody to HA was purchased from Roche Molecular Biochemicals, and a luciferase assay kit was purchased from Promega, Madison, WI.

**Cell Cultures**—NIH3T3 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Mouse embryonic fibroblasts (MEF) cells were cultured in DMEM supplemented with 10% fetal bovine serum.

**Construction of Plasmids**—Various kinds of pcDNA3(HA)-Ahnak fragment including N (amino acid residues 1–257), 4

CRUs (amino acid residues 3860–4412), C1 (amino acid residues 4640–5386), and C2 (amino acid residues 5258–5643) domains as previously described (11) were subcloned into pGEX4T1. All constructs were checked by restriction mapping and nucleotide sequencing.

**Generation and Characterization of Ahnak Knock-out Mice**—Genomic DNA was extracted from 129/Svj mouse J1 embryonic stem cells and was used as template DNA for PCR. A targeting vector was constructed to delete a 502-bp genomic fragment containing a segment of amino-terminal region of *ahnak* gene using 3' 5.1-kb long arm fragment and 5' 1.9-kb short arm fragment ligated into the pPNT vector. To construct 3' 5.1-kb long arm fragment, a NotI-linked forward primer (5'-gcgccgccagctacatgtggctaccatg-3') and a XhoI-linked reverse primer (5'-ctcagtggaactcggatccctcttt-3') were used. To construct the 5' short arm, a XbaI-linked forward primer (5'-tctagactctcatcaggttcaaggg-3') and a reverse primer (5'-ccctctatgttggcagac-3') were used.

The positive selection gene was the neomycin phosphotransferase gene (*neo*) resistant to the neomycin analog, G418. The negative selection gene was the herpes simplex virus thymidine kinase (*HSVtk*), which conferred sensitivity to the guanosine analog, ganciclovir (GANC). The targeting vector was linearized with NotI and electroporated into 129/Svj mouse J1 embryonic stem cells. Clones resistant to G418 and GANC were selected, and homologous recombination was confirmed by Southern blotting. Among 252 clones screened, 8 clones with successfully modified *ahnak* gene were verified. To produce chimeric mice, the embryonic stem clones containing the targeted mutation were injected into C57BL/6 blastocysts, and these were subsequently transferred to recipient females on day 3 of pseudo-pregnancy. The resulting male chimeric mice were bred to C57BL/6 females to obtain heterozygous Ahnak mice. Germ-line transmission of the mutant allele was verified by Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Interbreeding of the heterozygous mice was performed to generate homozygous Ahnak-deficient mice.

For Southern blot analysis, a 460-bp probe located just outside the 5' short arm of the knock-out vector was prepared using two primers (the forward primer, 5'-gcccaagtcagatgctac-3'; the reverse primer, 5'-gagacatcaagtcagccttg-3'). Mouse genomic DNA was isolated from mouse tail, digested with BamHI, and hybridized with the probe. A 8.6-kb Ahnak wild-type DNA fragment and a 9.9-kb Ahnak mutant DNA fragment were detected.

**Preparation MEFs**—MEFs were prepared from day 13.5 embryos and were maintained in DMEM containing 10% fetal bovine serum. Tissues from embryo were incubated with 3 ml of trypsin-EDTA (Invitrogen 25300–096) with gentle shaking at 37 °C for 30 min. Trypsinization was terminated by the addition of 200  $\mu$ l of fetal bovine serum (FBS) and fresh medium (DMEM, 10% FBS). Cells from the tissue were allowed to attach the culture dish. MEF cells were cultured at 37 °C in atmosphere of 5% CO<sub>2</sub> in DMEM (JBI, Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (JBI) and 1% (v/v) antibiotic-antimycotic solution (Invitrogen).

**Transfection**—NIH3T3 cells were plated at a density of 1.5  $\times$  10<sup>5</sup> cells/well in 6-well plates. The cells were transfected with

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0.8  $\mu\text{g}$  of pcDNA3-HA and pcDNA3-HA-4 CRUs using Effec-tin (Qiagen) according to the manufacturer's protocol and maintained in the complete medium for 24 h. The cells were serum-starved for 12 h and then stimulated with 100  $\mu\text{M}$  or 10 ng/ml PMA for the indicated times. MEF cells were plated at a density of  $1 \times 10^5$  cells/well in 6-well plates. The cells were transfected with 2  $\mu\text{g}$  of pcDNA3-HA and pcDNA3-HA-4 CRUs using Lipofectamine reagent according to the manufacturer's protocol and maintained in the complete medium for 24 h. The cells were serum-starved for 24 h and then stimulated with 100 ng/ml PMA or PDGF for the indicated times.

**GST Pulldown Assays**—Cultures of *Escherichia coli* BL21 containing pGEX4T1 or various pGEX4T1-Ahnak derivatives (see above) were induced with 0.4 mM isopropyl- $\beta$ -D-thioga-lactopyranoside for 3 h at 30 °C. The harvested bacteria were suspended in phosphate-buffered saline (PBS) containing 1% Triton X-100 and protease inhibitors (0.1  $\mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride, 1  $\mu\text{g}/\text{ml}$  aprotinin, and 1  $\mu\text{g}/\text{ml}$  leupeptin) and lysed by sonication. After centrifugation at  $15,000 \times g$  for 20 min, the supernatant was incubated with glutathione-agarose beads for 3 h at 4 °C. The beads were subsequently incubated with NIH3T3 cell lysates overexpressing HA-PKC- $\alpha$ . The samples were washed three times with PBS containing 1% Triton X-100 and subjected to immunoblotting assay with antibody against HA.

**Cell Fractionation Experiment**—After washing twice with PBS, hypo-osmotic solution (20 mM Tris-HCl, pH 7.5, 2 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA) containing protease inhibitors (1  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin) was added to the culture plates. Cells were subsequently scraped off the plates and homogenized on ice. The homogenate was centrifuged at  $13,000 \times g$  for 15 min at 4 °C to prepare the cytosolic fraction. The membrane fraction was collected by solubilizing the remaining pellet in radioimmune precipitation assay buffer (RIPA) buffer containing protease inhibitors, and RIPA lysates were centrifuged at  $100,000 \times g$  for 15 min at 4 °C. Equal amounts of the cytosol or the membrane fractions were resolved by 8% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were immunoblotted with the indicated primary antibodies followed by species-specific horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by chemiluminescence.

**Immunoprecipitation and Immunoblotting**—Lysates ( $1\text{--}2 \times 10^6$  cells) were mixed with antibodies (0.5–1  $\mu\text{g}$ ) for 4 h or overnight and then incubated with 40  $\mu\text{l}$  of protein G-Sepharose for 2 h at 4 °C. Immune complexes were washed 5 times with lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 0.1  $\mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 1  $\mu\text{g}/\text{ml}$  aprotinin, 1  $\mu\text{g}/\text{ml}$  leupeptin, and 10% glycerol). After boiling in 2 $\times$  SDS-PAGE sample buffer, the samples were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were immunoblotted with the indicated primary antibodies followed by horseradish peroxidase-conjugated goat secondary antibodies. Bands were visualized by chemiluminescence.

**Immunofluorescence**—MEF cells were grown on coverslips. The cells were serum-starved for 24 h, stimulated with 100 ng/ml PDGF for 10 min, washed with cold PBS, fixed with 3.5%

paraformaldehyde in PBS for 10 min at room temperature, washed with PBS, and then permeabilized in 0.5% Triton X-100. Nonspecific sites were blocked by treating the cells with PBS containing 0.5% bovine serum albumin for 4 h at 4 °C. The cells were incubated with primary antibodies against PKC- $\alpha$  in PBS for overnight at 4 °C, washed with PBS, incubated with the secondary antibody (TRITC-conjugated goat anti-mouse-IgG; KPL) for 40 min at room temperature in the dark, and then mounted on glass slides using a drop of Aqua-Poly/mount. Images were recorded using a confocal laser scanning microscope (Carl Zeiss 510).

**PKC Activity Assay**—PKC- $\alpha$  (5 ng) was incubated in 20 ml of 50 mM HEPES, pH 7.3, containing 3  $\mu\text{M}$  magnesium acetate, 750  $\mu\text{M}$   $\text{CaCl}_2$ , 200  $\mu\text{M}$  ATP (0.2  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]ATP), 1  $\mu\text{g}$  of histone I, and purified Ahnak proteins with or without phospholipid micelles (0.8  $\mu\text{g}$  of phosphatidylserine (PS)) and 0.08  $\mu\text{g}$  of DG) at 30 °C for 30 min. Reactions were stopped by the addition of 5 $\times$  SDS-PAGE sample buffer. Products were subjected to 12% SDS/PAGE and autoradiographed by BAS-2500 (Fuji Co.) (25).

**Luciferase Reporter Assays**—NIH3T3 cells were transfected with indicated combinations of pFA2-Elk-1, pFR-luciferase reporter, 4 CRUs of Ahnak, and control plasmids (Stratagene Co.). Cells were maintained in the complete medium for 24 h. Cells were incubated in the presence or absence of 10 ng/ml PMA for 7 h in serum-free media and then harvested for measurement of luciferase and  $\beta$ -galactosidase activities according to the manufacturer's protocol (Promega). Transfection efficiencies were normalized by measuring  $\beta$ -galactosidase activities (26).

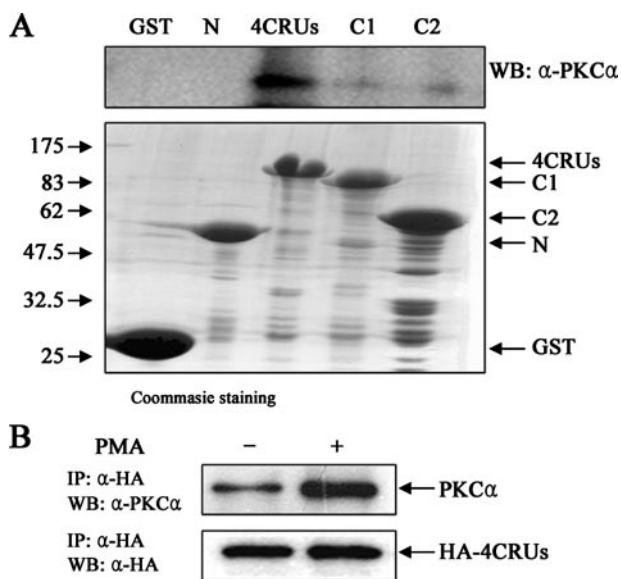
## RESULTS

**Central Repeated Units of Ahnak Interact and Activate PKC- $\alpha$** —To identify the domains of Ahnak responsible for interaction with PKC- $\alpha$ , we made four GST-conjugated Ahnak derivatives; GST-N (amino acids 1–257), GST-4 central repeated units (4 CRUs, amino acids 3859–4412), GST-C1 (amino acids 4640–5386), and GST-C2 (amino acids 5258–5643). PKC- $\alpha$ , the major isoform in NIH3T3 cells, interacts strongly with 4 CRUs of Ahnak *in vitro* (Fig. 1A). A weak interaction was also detected between PKC- $\alpha$  and C1 or C2 regions of Ahnak. The amino-terminal fragment of Ahnak did not bind to PKC- $\alpha$  (Fig. 1A).

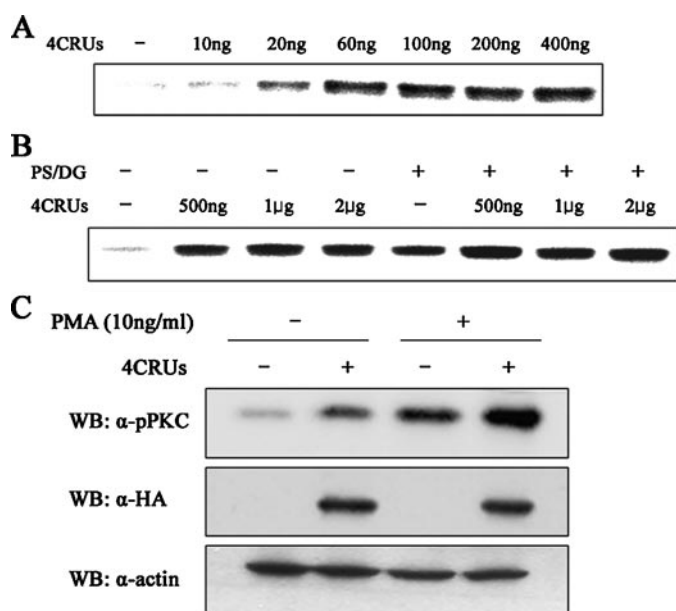
To investigate whether 4 CRUs of Ahnak associates with PKC- $\alpha$  isozyme in cells, we transfected NIH3T3 cells with plasmids harboring HA-tagged 4 CRUs. Cells were incubated in the absence or presence of PMA, and then cell lysates were subjected to immunoprecipitation with antibodies to HA. Immunoblot analysis of the resulting precipitates with antibodies to PKC- $\alpha$  revealed that 4 CRUs of Ahnak interacted with PKC- $\alpha$  (Fig. 1B). Moreover, it was shown that interaction of PKC- $\alpha$  with 4 CRUs of Ahnak was increased by PMA stimulation (Fig. 1B).

To explore the direct effect of 4 CRUs of Ahnak on PKC activity in the complex, we performed PKC activity assay using histone I phosphorylation in the presence or absence of purified 4 CRUs of Ahnak. Incubation of purified PKC- $\alpha$  with 4 CRUs of Ahnak showed an increase in the activity of PKC- $\alpha$  in a dose-dependent manner (Fig. 2A). PS/DG is a well known PKC acti-





**FIGURE 1. Interaction of 4 CRUs of Ahnak with PKC- $\alpha$ .** *A*, GST and GST-Ahnak fragment fusion proteins were purified on glutathione-Sepharose beads and incubated with NIH3T3 cell lysates. After washing with PBS containing 1% Triton X-100 and 5% Nonidet P-40, they were analyzed on SDS/PAGE. The interaction between Ahnak and PKC was determined by Western blot (WB) using anti-PKC- $\alpha$  (upper panel). The lower panel shows Coomassie staining of GST and GST-conjugated Ahnak fragments. *B*, NIH3T3 cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. After serum starvation for 16 h, cells were incubated in the absence or presence of PMA for 30 min. Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to HA, and the resulting precipitates were subjected to immunoblot analysis with antibody to PKC- $\alpha$ .



**FIGURE 2. Enhanced PKC activity by 4 CRUs of Ahnak.** *A* and *B*, 5 ng PKC- $\alpha$  was incubated (30 min 30 °C) in 20  $\mu$ l of 50 mM HEPES, pH 7.3, containing 3 mM magnesium acetate, 750  $\mu$ M CaCl<sub>2</sub>, 200  $\mu$ M ATP (0.2  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP), 1  $\mu$ g of histone, and purified proteins Ahnak without or with phospholipids micelles (0.8  $\mu$ g of PS and 0.08  $\mu$ g of DG) (*B*). Reactions were stopped and subjected to SDS/PAGE and autoradiographed by BAS-2500. *C*, NIH3T3 cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. After serum starvation for 12 h, cells were incubated in the absence or presence of PMA for 30 min. Cell lysates were then subjected to immunoblot analysis (WB) with antibodies to phospho-specific PKC- $\alpha$  and HA.

vator that imparts its effect through the interaction to C1 domain of PKC. Oversaturation of PKC- $\alpha$  activity by the addition of excess 4 CRUs of Ahnak did not show further activation in the presence of PS/DG, indicating that 4 CRUs of Ahnak bind to C1 domain of PKC- $\alpha$  (Fig. 2*B*). We next investigated the effect of 4 CRUs of Ahnak on the phosphorylation of PKC- $\alpha$  in cells. Expression of 4 CRUs of Ahnak in NIH3T3 cells resulted in increased PKC- $\alpha$  phosphorylation in response to PMA compared with parental cells (Fig. 2*C*).

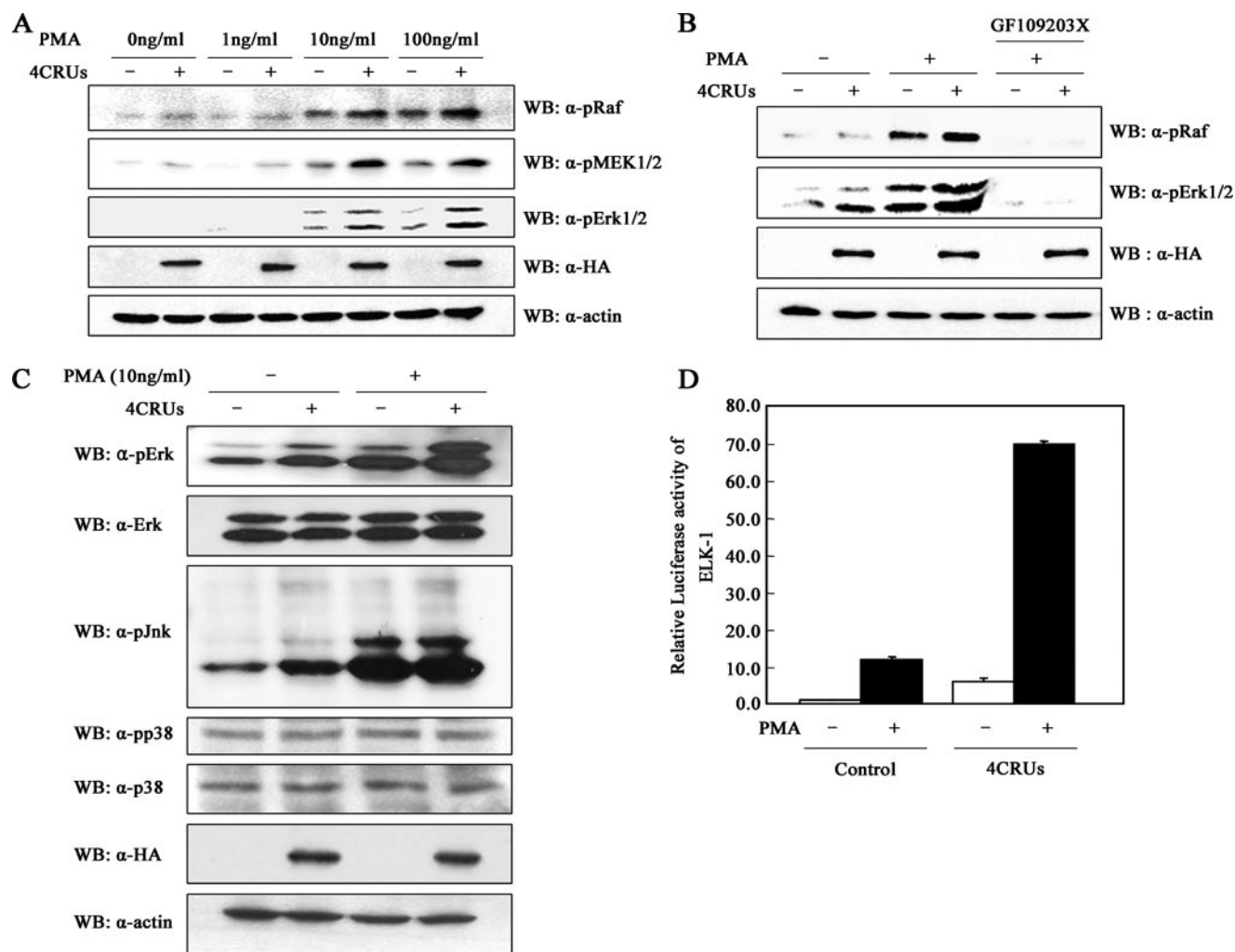
**4 CRUs of Ahnak Activate the c-Raf/MEK1/2/Erk Pathway**—It has been well established that PKC plays an important role in the regulation of various cellular functions such as cell division, survival, and proliferation through the extracellular signal-regulated kinase/mitogen-activated protein kinase (Erk/MAPK) cascade. It is also known that PKC- $\alpha$  functions as a potent activator of Raf and thereby functions as that of the Erk pathway as well (27, 28). Therefore, we examined whether 4 CRUs of Ahnak activate the c-Raf/MEK/Erk signaling cascade in response to PMA. We performed immunoblot assays with antibodies against the phosphorylated forms of c-Raf, MEK, and Erk in NIH3T3 cells expressing 4 CRUs of Ahnak in the absence or presence of PMA. Expression of 4 CRUs of Ahnak in NIH3T3 cells resulted in enhanced c-Raf, MEK1/2, and Erk phosphorylation in a PMA concentration-dependent manner compared with control cells (Fig. 3*A*). Pretreatment of the cells with GF109203X, a potent PKC inhibitor, completely blocked the effect of 4 CRUs of Ahnak on phosphorylation of Raf in response to PMA (Fig. 3*B*). These results suggest that 4 CRUs of Ahnak potentiated PKC-dependent phosphorylation of c-Raf and downstream signaling cascade.

We investigated the effect of 4 CRUs of Ahnak on other MAPK family members. NIH3T3 cells expressing 4 CRUs of Ahnak were incubated in the absence or presence of PMA, and then cell lysates were subjected to immunoblot analysis with antibodies to the phosphorylated form of JNK or p38 MAPK. 4 CRUs of Ahnak consistently potentiated Erk phosphorylation in response to PMA but not JNK or p38 MAPK phosphorylation (Fig. 3*C*). These results indicate that Ahnak-dependent cell signaling is specific for c-Raf/MEK/Erk pathway.

Elk-1 is a well known transcription factor that is phosphorylated and activated by Erk (29). We, thus, studied whether 4 CRUs of Ahnak can potentiate the Elk activation in response to PMA. We transiently transfected the Elk-1 dependent luciferase reporter with vector pcDNA3(HA) or pcDNA3(HA)-4 CRUs in NIH3T3 cells and exposed to 10 ng/ml PMA for 7 h. PMA activated Elk-1 transcriptional activity by 12-fold in control cells, whereas the basal and PMA-induced transcriptional activity of Elk-1 were increased in NIH3T3 cells expressing 4 CRUs of Ahnak by 6- and 70-fold, respectively (Fig. 3*D*). The result suggests that Ahnak enhances PKC-mediated Elk-1 activation pathway in cells.

**Failure of PKC Activation in Ahnak Null MEF Cells**—To verify the function of Ahnak in cell signaling, we made Ahnak gene knock-out mouse by homologous recombination (Fig. 4*A*) and established MEF cell culture from mouse with the homozygous Ahnak null genotype (Ahnak<sup>-/-</sup>). Genotypic analysis of genomic DNA and Western blot analysis with antibody against Ahnak confirmed that the Ahnak gene and its product were not

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**FIGURE 3. Ahnak mediates the activation of c-Raf/MEK/Erk pathway in NIH3T3 cells by PMA.** *A*, NIH3T3 cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. After serum starvation for 12 h, cells were incubated in the absence or presence of varying concentrations of PMA. Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated active forms of c-Raf, MEK1/2, or Erk1/2. The membrane was subsequently re-probed and immunoblotted (WB) with antibodies against actin or HA. *B*, NIH3T3 cells expressing 4 CRUs of Ahnak were pretreated with GF109203X (5  $\mu$ M) for 10 min before treatment of PMA (10 ng/ml). Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated active forms of c-Raf and ERK1/2. *C*, concerted action of 4 CRUs of Ahnak and PMA is specific for Erk phosphorylation but not JNK or p38 MAPK phosphorylation. NIH3T3 cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. After serum starvation for 12 h, cells were incubated in the absence or presence of PMA for 30 min. Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated active forms of ERK1/2, JNK, and p38 MAPK. *D*, 4 CRUs of Ahnak activate Elk1-luc activity in response to PMA. NIH3T3 cells were co-transfected with pFA2-Elk-1, pFR-luciferase reporter, and either pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. Cells were maintained in the complete medium for 24 h. Cells were incubated in the absence or presence of 10 ng/ml PMA for 7 h in serum-free media. Then, cells were harvested for measurement of luciferase and  $\beta$ -galactosidase activities. Data are the means  $\pm$  S.E. of values from three independent experiments.

present (Fig. 4B). We investigated the effect of loss of Ahnak gene on PKC and Raf phosphorylation. Stimulation of Ahnak<sup>-/-</sup> MEF cells with PMA failed to lead to PKC and Raf phosphorylation, in contrast to wild type MEF cells (Fig. 5A). To verify biological relevance of the Ahnak function in PKC activation, we next investigated PKC activation in terms of membrane translocation in response to PDGF. MEF cells were starved for 24 h and then treated with PDGF for the indicated periods of time. The distribution of PKC- $\alpha$  in the membrane and cytoplasmic fraction was subsequently determined. Stimulation of wild type MEF cells with PDGF resulted in decreased localization of PKC- $\alpha$  from cytoplasmic fraction and increased localization of PKC- $\alpha$  in the membrane fraction. In clear contrast, the membrane translocation of PKC- $\alpha$  did not occur in Ahnak null MEF cells in response to PDGF (Fig. 5B). To further verify the function of 4

CRUs of Ahnak, we performed an add-back experiment of 4 CRUs into the Ahnak null MEF cells. Add-back expression of 4 CRUs resulted in restoration of PKC- $\alpha$  membrane translocation in response to PDGF (Fig. 5B). Moreover, laser-based confocal microscope further confirmed that PKC- $\alpha$  is translocated to the plasma membrane in wild type MEF cells (Fig. 5C, arrow) but not in Ahnak null MEF cells (Fig. 5C). Next we asked whether PDGF regulates Raf phosphorylation in Ahnak null MEF cells. Raf phosphorylation by PDGF in Ahnak null MEF cells was significantly decreased compared with that in wild type MEF cells (Fig. 5D). Furthermore, add-back expression of 4 CRUs into Ahnak null MEF cells also showed a recovery of PDGF-induced Raf phosphorylation (Fig. 5D).

**4 CRUs of Ahnak Inhibit Interaction of PKC- $\alpha$  with PP2A**—Several lines of evidence suggest that PMA induces a transient

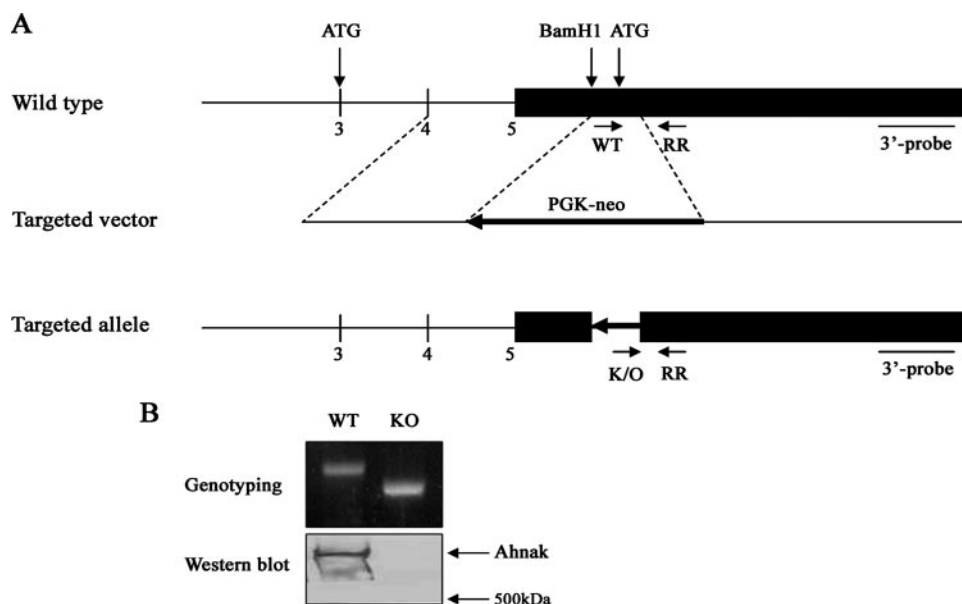


FIGURE 4. **Scheme for Ahnak knock-out mice.** *A*, structure of the Ahnak genomic locus and the targeting construct. The targeting vector was used to delete ~502 bp of genomic DNA, including the presumed promoter region of Ahnak and the ATG start codon (PGK, 3-phosphoglycerate kinase; neo, neomycin resistance gene; WT, wild-type sense primer; KO, knock-out sense primer; RR, right arm reverse antisense primers). *B*, genotyping by PCR. Genomic DNA was extracted from tail of wild-type and knock-out mice. PCR was performed using separate sense primers for wild type and knock-out mice and the common antisense primer (upper panel). Cell lysates from MEF cells were then subjected to immunoblot analysis with KIS (anti-Ahnak, Lab Frontier, Seoul, Korea) antibody (lower panel).

translocation of PKC- $\alpha$  and PP2A as a complex to regulate protein phosphorylation in various cells. To elucidate the mechanism of Ahnak-mediated PKC- $\alpha$  phosphorylation, we investigated the effect of 4 CRUs of Ahnak on the association of PKC- $\alpha$  with PP2A. NIH3T3 cell lysates were subjected to immunoprecipitation with antibodies to PP2A. Immunoblot analysis of the resulting precipitates with antibodies to PP2A or PKC- $\alpha$  revealed that endogenous PKC- $\alpha$  interacts with PP2A whether or not there was PMA stimulation in NIH3T3 cells. However, the expression of 4 CRUs of Ahnak in NIH3T3 cells resulted in diminished interaction of PKC- $\alpha$  with PP2A in response to PMA (Fig. 6A). It is likely that the interaction of phosphorylated with 4 CRUs of Ahnak and with PP2A is mutually exclusive such that the presence of Ahnak leads to disruption of phosphorylated PKC- $\alpha$  and PP2A complex. To further verify the function of Ahnak in the regulation of PKC-PP2A complex, we analyzed the complex dissociation by PMA in Ahnak null MEF cells. Stimulation of Ahnak null MEF cells with PMA did not result in the disruption of PKC-PP2A complex (Fig. 6B). Moreover, add-back expression of 4 CRUs into Ahnak null MEF cells abolished the PKC-PP2A complex in response to PMA. Taken together, these results indicate that Ahnak is involved in the regulation of PKC- $\alpha$  phosphorylation through dissociation of PP2A from PKC- $\alpha$ .

## DISCUSSION

It is well established that PKC isozymes are activated by phospholipids, diacylglycerol, and calcium resulting from receptor-mediated cell signaling (17, 18). Thus, activated kinases are targeted to specific cellular location through PKC isozyme-specific binding partners to function in cell growth,

differentiation, and survival (30). Specifically, our previous results indicated that 4 CRUs of Ahnak interact with PKC- $\alpha$ , which is the primary isozyme in NIH3T3, and the complex of the repeated units of Ahnak with PKC is translocated to plasma membrane (Fig. 5C). We found that repeated units of Ahnak protein bind to cPKCs, PKC- $\alpha$  (Fig. 1B). Furthermore, nPKCs such as PKC- $\delta$  and atypical PKCs such as PKC- $\zeta$  also appear to interact with 4 CRUs of Ahnak (data not shown). The cPKC and nPKC contain a C1 domain that has conserved cysteine and histidine residues that are responsible for the coordination of two Zn<sup>2+</sup> ions. The atypical PKCs also contain a single zinc-finger motif. We also noticed that PS/DG, which interacts with C1 domain of PKCs, is no longer effective in enhancing the PKC activity in the presence of a saturating concentration of Ahnak (Fig. 2). Taken together, these data suggest that the

repeated units of Ahnak protein likely interact with PKC isozyme through the zinc binding motif in C1 domain.

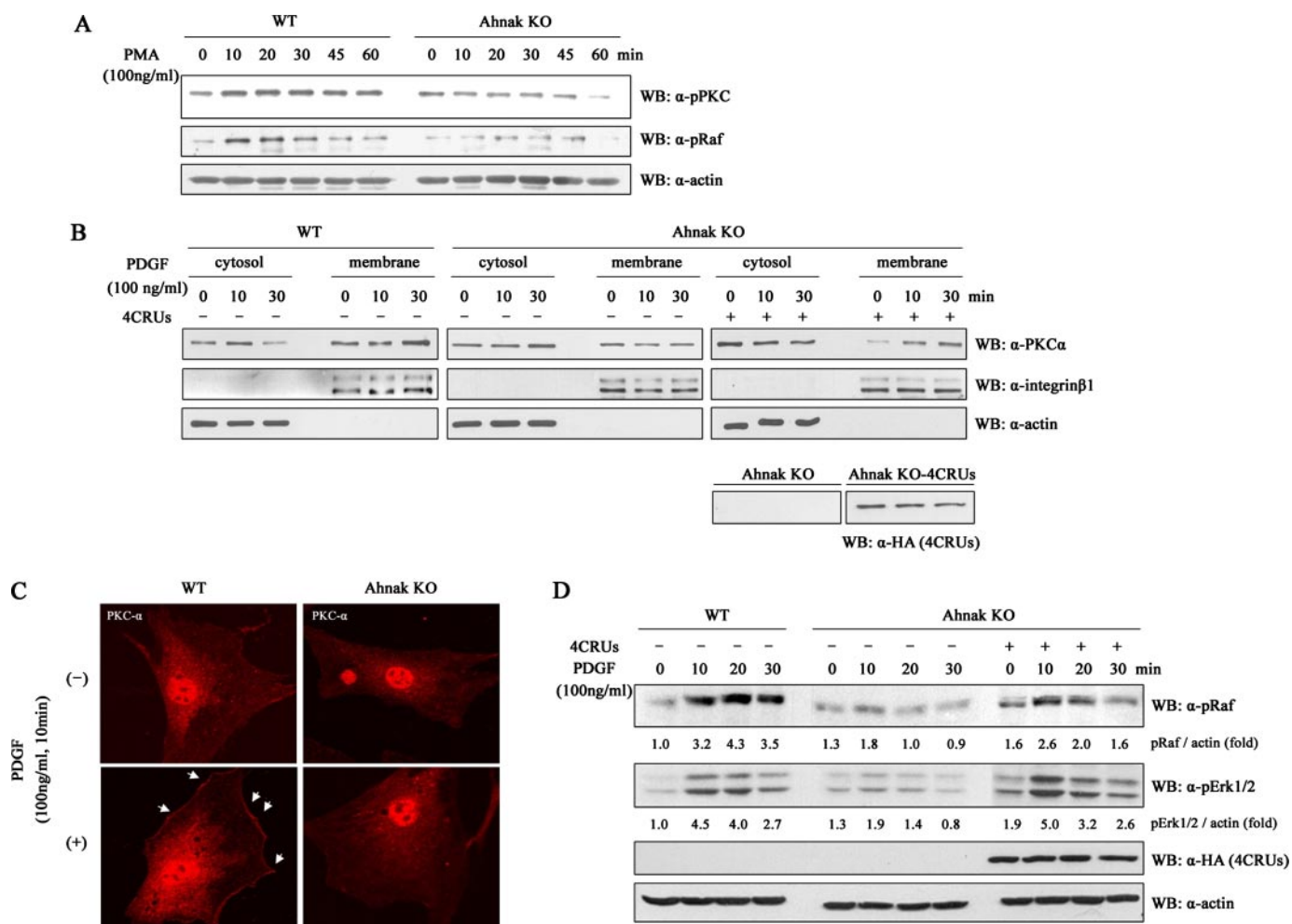
Although 4 CRUs of Ahnak interact with many PKC isozymes, we have observed that the interaction between Ahnak and PKC isozymes is not universal. Specifically, PKC- $\gamma$ , a cPKC, and PKC- $\epsilon$ , a nPKC, failed to interact with 4 CRUs of Ahnak (data not shown). Stahelin *et al.* (31) demonstrated that PKC- $\alpha$  and PKC- $\beta$ II among cPKCs and PKC- $\delta$ , a nPKC, strongly prefer PS, whereas PKC- $\gamma$  and PKC- $\epsilon$  show a low selectivity for PS. Binding of PS to C1 domain in PKC- $\alpha$ , PKC- $\beta$ II, or PKC- $\delta$  results in relief of intramolecular tethering in C1 domains (31–37). It is the high conformational flexibility of C1 domain in PKC- $\gamma$  and PKC- $\epsilon$  that is believed to be responsible for the lack of PS selectivity. Combining our results and the finding of Stahelin *et al.* (31, 36) finding leads to the hypothesis that the flexibility of C1 domain in PKC isozymes is critical for the interaction of 4 CRUs of Ahnak with PKC isozymes.

As one of the most important downstream pathways affected by PKC activation, we analyzed c-Raf/MEK/Erk pathway in terms of the regulatory effect of Ahnak. Ectopic expression of 4 CRUs of Ahnak to NIH3T3 cells resulted in potentiation of c-Raf, MEK, and Erk phosphorylation in response to PMA compared with parental cells (Fig. 3). Furthermore, pretreatment of GF109203X, a well known potent PKC inhibitor, to cells expressing 4 CRUs of Ahnak resulted in complete inhibition of c-Raf/MEK/Erk phosphorylation, indicating that the activation of Erk pathway is PKC-dependent (Fig. 3C).

PP2A is mammalian cytoplasmic serine/threonine phosphatase. Several lines of evidence indicate that PP2A plays an important role in negative regulation of Erk pathway (38, 39). Interestingly, small t antigen in simian virus 40 (SV40) associ-



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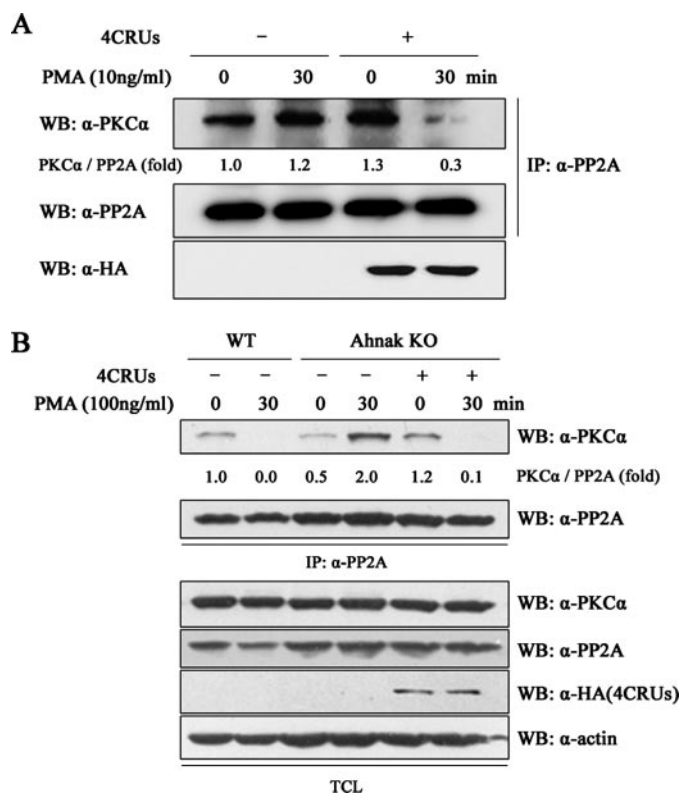


**FIGURE 5. Verification of the role of Ahnak using Ahnak knock-out cell lines.** *A*, stimulation of Ahnak null MEF cells with PMA failed to induce PKC and Raf phosphorylation. Wild type and Ahnak null MEF cells were serum-starved for 24 h and then stimulated with 100 ng/ml concentrations of PMA for the indicated times. Cell lysates were then subjected to immunoblot analysis (WB) with antibodies to phospho-specific PKC- $\alpha$ , phospho-c-Raf, and actin. *WT*, wild-type sense primer; *KO*, knock-out sense primer. *B*, membrane translocation of PKC- $\alpha$  was not induced in Ahnak null MEF cells in response to PDGF. Wild type and Ahnak null MEF cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs. Wild type, Ahnak null MEF cells, and Ahnak null MEF cells expressing 4 CRUs of Ahnak were serum-starved for 24 h and then stimulated with 100 ng/ml concentrations of PDGF for the indicated times. Cells were lysed and fractionated, and lysates from each fraction were resolved by SDS-PAGE and subjected to immunoblot analysis with antibody to PKC- $\alpha$ . Integrin  $\beta$ 1 and actin were used as a loading control of each fraction. *C*, PKC- $\alpha$  is translocated to plasma membrane in wild type MEF cells in response to PDGF. Wild type and Ahnak null MEF cells were unstimulated (*upper panel*) or stimulated with 100 ng/ml PMA for 10 min (*lower panel*) and fixed and stained with PKC- $\alpha$ . TRITC-conjugated goat anti-mouse IgG was used as the secondary antibody for staining PKC- $\alpha$ . *D*, Raf phosphorylation by PDGF in wild type, Ahnak null MEF cells, or null MEF cells expressing 4 CRUs of Ahnak. Wild type and Ahnak null MEF cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs. Wild type, Ahnak null MEF cells, and Ahnak null MEF cells expressing 4 CRUs of Ahnak were serum-starved for 24 h and then stimulated with 100 ng/ml concentration of PDGF for the indicated periods of time. Cell lysates were subjected to immunoblot analysis with antibodies to phospho-c-Raf, phospho-Erk1/2, and actin. The ratio of phosphorylated form of PKC (*pPKC*) to actin (loading control) is indicated.

ates with the regulatory subunit of PP2A and inhibits PP2A activity, resulting in mitogenic effect during SV40 transformation (40). Moreover, small t antigen interferes the interaction of PP2A with Shc, leading to enhanced epidermal growth factor-stimulated Shc phosphorylation with increased MAPK pathway (41). It has been reported that PP2A has the capacity to down-regulate PKC- $\alpha$  activity (22, 24). Therefore, we hypothesized that the interaction between PKC- $\alpha$  and PP2A might be involved in Ahnak-mediated PKC phosphorylation and activation of its downstream molecules. This hypothesis led us to determine the interaction between PP2A and PKC- $\alpha$  in the absence or presence of Ahnak. We have observed that PKC- $\alpha$  interacts with PP2A constitutively in NIH3T3 cells, and the interaction of PKC- $\alpha$  with PP2A is disrupted by the concerted action of 4 CRUs of Ahnak and PMA (Fig. 6A). PKC-PP2A

complex formation was enhanced in Ahnak null MEF cells, and ectopic expression of Ahnak in the MEF cells led to disruption of PKC-PP2A complex. The results indicate that Ahnak stimulates disruption of PKC-PP2A complex, and the released PKC from the complex leads to activation of c-Raf/MEK/Erk cascade. Taken together, it is likely that Ahnak acts as a novel activator of PKC through interfering with the PKC-PP2A complex.

Several lines of evidence indicate that reciprocal action of kinase and phosphatase regulates  $Ca^{2+}$  channel in cardiac and neuronal cells (24, 42, 43). Yang *et al.* (42) demonstrated that PKC interacts with the  $\alpha_{1c}$  subunit of L-type calcium channel and phosphorylates Ser-1928 residue in the subunit leading to positive regulation of calcium channel activity. It was also shown that PP2A and PP2B abolish calcium influx through



**FIGURE 6. Dissociation of PKC-PP2A complex by Ahnak.** *A*, NIH3T3 cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs of Ahnak. After serum starvation for 12h, cells were incubated in the absence or presence of PMA for 30 min. Cell lysates were then subjected to immunoprecipitation (IP) with antibodies to PP2A, and the resulting precipitates were subjected to immunoblot analysis (WB) with antibodies to PKC- $\alpha$  and PP2A. The ratio of PKC- $\alpha$  to actin (loading control) is indicated. *B*, wild type and Ahnak null MEF cells were transfected with pcDNA3-HA or pcDNA3-HA-4 CRUs and maintained in the complete medium for 24 h. The cells were serum-starved for 24 h and then incubated in the absence or presence of PMA for 30 min. Cell lysates were then subjected to immunoprecipitation with antibodies to PP2A, and the resulting precipitates were subjected to immunoblot analysis with antibodies to PKC- $\alpha$  and PP2A. Ratio of PKC- $\alpha$  to actin (loading control) is indicated. TCL means total cell lysate.

L-type calcium channel phosphorylated by PKC, and inhibition of PP2A and PP2B by pharmacological inhibitor increased calcium influx in arterial myocytes (43). Calcium influx is totally abolished in PKC- $\alpha^{-/-}$  cells, indicating that regulation of calcium influx by PKC appears to be a typical feature of arterial myocytes (43). The result indicates that gating modality of L-type calcium channel is regulated by relative activities of kinase and phosphatase associated with the calcium channel complex. Interaction of the COOH-terminal region of Ahnak and the  $\beta$  subunit of L-type calcium channel inhibits channel activity, and phosphorylation of Ahnak and the  $\beta$  subunit of L-type calcium channel by protein kinase PKA results in relief from Ahnak suppression (3, 9). Our results show that Ahnak interacts and activates PKC activity (Figs. 1 and 2). Interestingly, Ahnak stimulates disruption of PKC-PP2A complex after stimulation of PKC (Fig. 6). Our results suggest a possibility that Ahnak protein serves as a molecular scaffold networking between L-type calcium channel and kinase/phosphatase. Further molecular mechanistic studies on scaffolding function of Ahnak are needed to determine the interconnections between calcium channel and protein phosphorylation.

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