Type II cGMP-dependent protein kinase inhibits RhoA activation in gastric cancer cells

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Abstract. Small GTPase RhoA is a key signaling component regulating cell migration and stress fiber formation. Previous studies have shown that RhoA activity is regulated by protein kinases, such as cAMP-dependent protein kinase (PKA) and type I cGMP-dependent protein kinase (PKGI), which phosphorylate the protein. This study was designed to investigate the effect of type II cGMP-dependent protein kinase (PKGII) on RhoA activity. Cells of the human gastric cancer line AGS were infected with adenoviral constructs bearing the *PKGII* cDNA in order to increase its endogenous expression, and were treated with 8-pCPT-cGMP to activate the PKGII enzyme. A transwell assay was performed to measure the migratory activity of the treated cells, and immunofluorescent microscopy was used to observe the formation of stress fibers. The phosphorylation of RhoA was detected by western blotting, and the activity of RhoA was measured by a pull-down assay. Co-immunoprecipitation (co-IP) was performed to detect binding of PKGII to RhoA. Glutathione S-transferase (GST)-fused fragments of RhoA and PKGII were expressed in *Escherichia coli* and used to investigate the domains required for the binding. The results showed that lysophosphatidic acid (LPA) treatment increased the migration and the formation of stress fibers in AGS cells and that this effect was RhoA-dependent. An increase in PKGII activity, not only inhibited LPA-induced migration and stress fiber formation, but also suppressed LPA-induced activation of RhoA. PKGII caused serine 188 (Ser188) phosphorylation of RhoA, but not the phosphorylation of the mutant RhoA Ser188A, and therefore had no inhibitory effect on the activity of the mutant protein. Co-IP results showed that there is direct binding of PKGII to RhoA. The GST pull-down assay showed that the fragment containing RhoA amino acid residues 1-44 and the N-terminal fragment of PKGII containing amino acid residues 1-176 are required for the binding between the two proteins. These results suggested that PKGII inhibits RhoA activity by binding to this small GTPase and causing phosphorylation at its Ser188 site.

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Introduction

Rho proteins are small GTPases of the Ras family that cycle between an active and an inactive GDP-bound form and regulate diverse cell functions such as cytoskeletal organization, smooth muscle contraction, muscle and neuronal differentiation, cell cycle progression and gene expression (1-4). RhoA is a key member of the Rho family and is activated downstream of multiple membrane receptors, such as thrombin and lysophosphatidic acid (LPA) receptors. RhoA activity is increased by the guanine nucleotide exchange factor, which promotes the release of bound GDP and subsequent binding of GTP, and is decreased by the GTPase-activating protein, which stimulates the hydrolysis of bound GTP (5). In addition, previous studies (6) have suggested that the phosphorylation-dephosphorylation cycle controls Rho protein activity. For example, the phosphorylation and inhibition of RhoA by the cAMP-dependent protein kinase (PKA) has been highlighted as one of the direct antagonizing mechanisms for RhoA activity. *In vitro* experiments have indicated that RhoA phosphorylation at serine 188 (Ser188) increases the ability of the Rho GDP-dissociation inhibitor to extract RhoA from the membrane (6-7). Another report further elucidated the significance of PKA in regulating, through phosphorylation, RhoA activity (8).

Type II cGMP-dependent protein kinase (PKGII) is a serine/threonine kinase. It is involved in several physiological functions including intestinal secretion, bone growth, learning and memory (9). Findings of previous studies have suggested that this kinase plays an important role in regulating additional biological activities such as cell proliferation, differentiation and apoptosis, especially in tumor cells (10-13). Previous results from our laboratory showed that PKGII inhibits the proliferation and migration of gastric cancer cell lines through inhibition of the EGF-induced activation of EGFR and of related signal transduction pathways (14-16). Since our previous results also showed that PKGII inhibits LPA-induced cell migration (17), whether this kinase exerts an inhibitory effect on RhoA activity should be elucidated. This study was designed to investigate the

potential inhibition, by PKGII, of LPA-induced RhoA activation and the underlying mechanism(s) involved.

Materials and methods

Cell lines and reagents. The human gastric cancer cell line AGS, African green monkey kidney fibroblast-like cell line COS-7 and human embryonic kidney (HEK) 293A cell line were provided by the Institute of Cell Biology (Shanghai, China). Adenoviral vectors encoding β-galactosidase (pAd-LacZ) and PKGII (pAd-PKGII), the recombinant pcDNA 3.1 vector bearing the genes encoding PKGII, wild-type RhoA and mutant RhoA Ser188A, and the expression vector pGEX-2T-RBD, encoding the cDNA of glutathione S-transferase (GST) coupled to the Rho-binding domain (RBD) of rhotekin were kind gifts from Dr Gerry Boss and Dr Renate Pilz (University of California, San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco-BRL (Grand Island, NY, USA). The antibody against PKGII was from Abgent Biotechnology (San Diego, CA, USA). Goat anti-β-actin, mouse anti-RhoA and rabbit anti-phosho (p)-RhoA (Ser188) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Mouse anti-FLAG was from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-p-LIMK (Thr505/508) was from Bioworld Technology Inc. (St. Louis Park, MN, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The cellular permeable cGMP analog 8-pCPT-cGMP and the ROCK inhibitor Y27632 were from Calbiochem (San Diego, CA, USA). LPA, TRITC-conjugated phalloidin and the plasmid p3xFLAG-Myc-CMV-24 were purchased from Sigma-Aldrich. The cell transfection reagent Lipofectamin™ 2000 and *Escherichia coli* BL21 (DE3) were from Invitrogen Life Technologies (Carlsbad, CA, USA). The plasmid pGEX-4T-1 and glutathione-sepharose beads were purchased from GE Healthcare Life Sciences (Piscataway, NJ, USA). Electrochemiluminescence (ECL) reagents were from EMD Millipore (Billerica, MA, USA). Glass bottom dishes were from Shengyou Biotechnology, Co., Ltd. (Hangzhou, China). All other reagents used were of analytical grade.

Preparation of adenoviral vectors. HEK 293A cells were transfected with pAd-LacZ and pAd-PKGII vectors and cultured for ≤10 days until cytopathic effects were observed. The cells and the culture medium were harvested and underwent three freezing-thawing cycles. The supernatants containing adenoviruses (Ad-LacZ and Ad-PKGII) were used to infect new HEK 293A cells to amplify adenoviruses. The amplified adenoviral preparations were titrated to determine the pfu/ml and kept at -80˚C until use.

Cell culture, transfection and infection. AGS cells were cultured in DMEM supplemented with 10% FBS and maintained at 37˚C in a humidified incubator with 95% air and 5% CO_2. The medium was changed every two days and the cells were sub-cultured until they reached confluence. For transfection of gastric cancer cells with plasmids, the cells were sub-cultured the day before the process and the transfection was performed according to the manufacturer's instructions. On the day before infection with Ad-LacZ and Ad-PKGII, the cells were cultured in fresh medium at 70-80% confluence.

Cloning and expression constructs. To generate FLAG-tagged wild-type RhoA and mutant RhoA Ser188A plasmids, cDNA fragments were amplified from the pcDNA 3.1/full-length wild-type RhoA and /RhoA mutant by PCR. The 5' primer for amplifying wild-type and mutant RhoA was 5'-CCC AAG CTT ATG GCT GCC ATC CGG AA-3'. The 3' primers were: for wild-type RhoA, 5'-CGC GGA TCC CAA GAC AAG GCA CCC AGA TT-3', and for mutant RhoA, 5'-CGC GGA TCC CAA GAC AAG GCA CCC AGC TT-3'. The PCR products were cleaved by *Hin*dIII and *Bam*HI and the target fragment was cloned into the expression vector p3xFLAG-Myc-CMV-24.

The cloning vectors for the bimolecular fluorescence complementation (BiFC) assay, pBiFC-VN155 and pBiFC-VC155, were kindly provided by Dr Chang-Deng Hu (Purdue University, West Lafayette, IN, USA). To construct pBiFC-RhoA-VC, a cDNA fragment encoding the full-length RhoA was amplified by PCR from pcDNA 3.1/ wild-type RhoA, using the following oligonucleotides: 5'-GGA AGA TCT ACA TGG CTG CCA TCC GGA A-3' (RhoA-VC-N), 5'-CGG GGT ACC CAA GAC AAG GCA CCC AGA TT-3' (RhoA-VC-C). The resulting PCR fragment was digested with *Bgl*II and *Kpn*I and then inserted into the pBiFC-VC155 vector. To construct pBiFC-PKGII-VN, a cDNA fragment encoding the full-length PKGII was amplified by PCR using the following oligonucleotides bearing *Bgl*II and *Kpn*I restriction sites: 5'-GGA AGA TCT CTA TGG GAA ATG GTT CAG TGA AAC-3' (PKGII-VN-N), 5'-CGG GGT ACC GAA GTC TTT ATC CCA GCC TGA T-3' (PKGII-VN-C). The amplified fragment was then cloned into the pBiFC-VN155 vector.

Full-length RhoA cDNA and cDNA fragments encoding RhoA amino acids 1-44 and 1-147 were amplified by PCR from the full-length pcDNA 3.1/wild-type RhoA vector. The sequence of the 5'-primer was: 5'-GCG GGA TCC ATG GCT GCC ATC CGG AAG-3'. The sequences of the 3'-primers were: 5'-CGC GTC GAC TCA CAA GAC AAG GCA CCC AGA TTT-3', 5'-CGC GTC GAC TCA GCC ACA TAG TTC TCA AAC AC-3' and 5'-CGC GTC GAC TCA CAT ATC TCT GCC TTC TTC AGG-3' for full-length, RhoA amino acids 1-44 and 1-147, respectively. The PCR products were cleaved by *Bam*HI-*Eco*RI and the target fragment was cloned into pGEX-4T-1. To generate PKGII cDNA fragments (encoding amino acid residues 1-176, 1-285, 286-762, 286-452, 453-711 and 712-762), cDNA fragments were amplified from the full-length pcDNA 3.1/PKGII by PCR. The following primers were used: 5' primer for PKGII (1-176) and PKGII (1-285), 5'-TCC CCC GGG TAT GGG AAA TGG TTC AGT G-3'; for PKGII (286-762) and PKGII (286-452), 5'-TCC CCC GGG TTT GCT GAA GAA TTT ACC TG-3'; for PKGII (453-711), 5'-TCC CCC GGG TCT TGA GAT TAT TGC AAC ACT GG-3' and for PKGII (712-762), 5'-TCC CCC GGG TAA TGG TTT TAA TTG GGA GGG ACT G-3'. The 3' primers were: for PKGII (1-176), 5'-CCG CTC GAG CTG CTG AGG ATC CAG TCT-3'; for PKGII (1-285), 5'-CCG CTC GAG GGA TAC ACT TCT GAG GAA GTT TCT G-3'; for PKGII (286-452), 5'-CCG CTC GAG GTT CTG GAA TGG GGA TGA TG-3'; for PKGII (453-711), 5'-CCG CTC GAG TAA CCA CCT

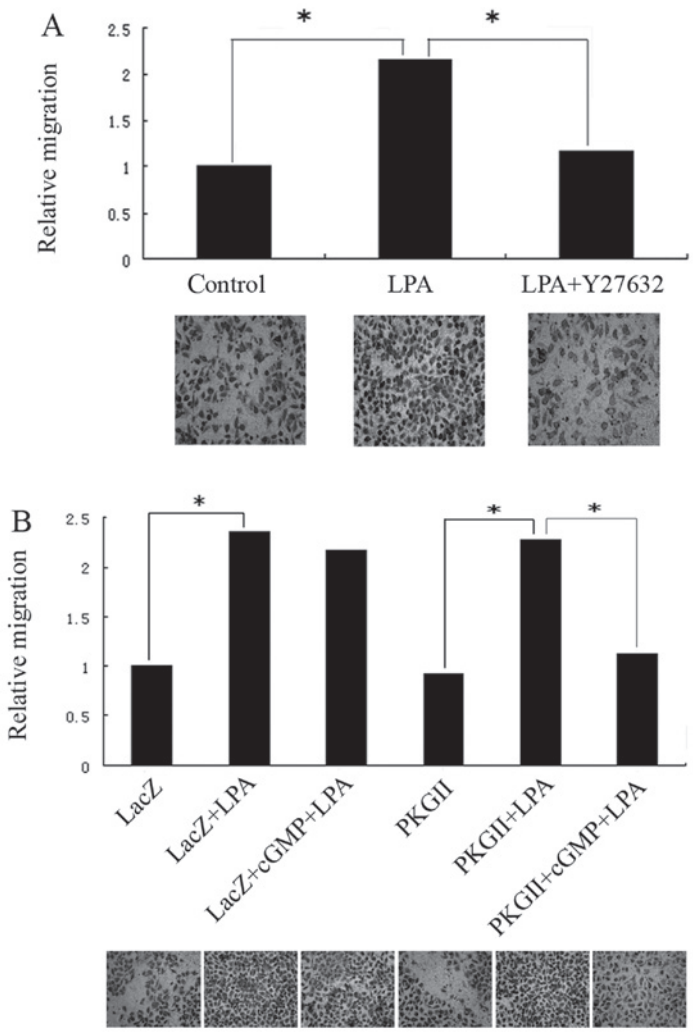

Figure 1. Type II cGMP-dependent protein kinase (PKGII) inhibits lysophosphatidic acid (LPA)-induced cell migration. The migratory activity of AGS cells was analyzed with a transwell assay and cells were examined under a light microscope (images below each graph, magnification, x200). (A) Serum-starved cells were treated with LPA (10 μM), or LPA and Y27632 (10 μM) for 1 h before LPA treatment. Control, cells not treated with LPA/Y27632. (B) The cells were infected with Ad-LacZ or Ad-PKGII for 48 h and were starved in serum overnight. In the Ad-LacZ + LPA and Ad-PKGII + LPA groups, LPA (10 μM) was added to the culture medium; in the Ad-LacZ + cGMP + LPA and Ad-PKGII + cGMP + LPA groups, cells were treated with 8-pCPT-cGMP (250 μM) for 1 h and then LPA (10 μM) was added to the culture medium. The migration time was 12 h. Data are shown as means ± SD from four independent experiments, each performed in duplicate (*P<0.01).

GTG TTT CTT AAT GTC-3' and for PKGII (712-762) and PKGII (286-762), 5'-CCG CTC GAG GAA GTC TTT ATC CCA GCC TGA TAG-3'. The PCR products were cleaved by *Sma*I-*Xho*I and the target fragment was cloned into pGEX-4T-1. The validity of all the constructs was confirmed by DNA sequencing.

Expression of GST-fusion proteins in E. coli. RhoA and PKGII fragments fused to GST were expressed in the *E. coli* competent cells BL21 (DE3), and transcription was induced with 0.2 or 1 mM isopropyl-β-D-thiogalactopyranoside. The expressed GST-fusion proteins were purified on glutathione-sepharose beads.

Phalloidin staining. Cells were plated on glass coverslips, serum-starved for 16 h and treated with 250 μM CPT-cGMP for 1 h prior to stimulation with 10 μM LPA for 5 min. Cells were fixed with freshly prepared 40 g/l paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, permeabilized with 0.3% Triton X-100 in PBS for 10 min and blocked with 3% bovine serum albumin in PBS. The cells were subsequently incubated with TRITC-labeled phalloidin (1:100) for 1 h at room temperature, and were visualized with fluorescence microscopy using a Leica DM LB2 microscope digital camera system (Leica Microsystems, Wetzlar, Germany).

Transwell migration assays. The migratory activity of AGS cells was detected using a transwell system comprising BD BioCoat™ Control Cell Culture inserts (8.0-μm PET membrane, 24-well) purchased from BD Biosciences (Franklin Lakes, NJ, USA). After trypsinization, $5x10^4$ cells were seeded into the upper chamber of a tissue culture incubator containing culture medium without FBS. Cell migration to the bottom side of the membrane was induced by medium containing 10% FBS in the lower chamber for 12 h at 37˚C. The cells remaining in the upper chamber were carefully removed with cotton swabs. Cells that had migrated to the bottom side of the membrane were fixed in 4% paraformaldehyde solution for

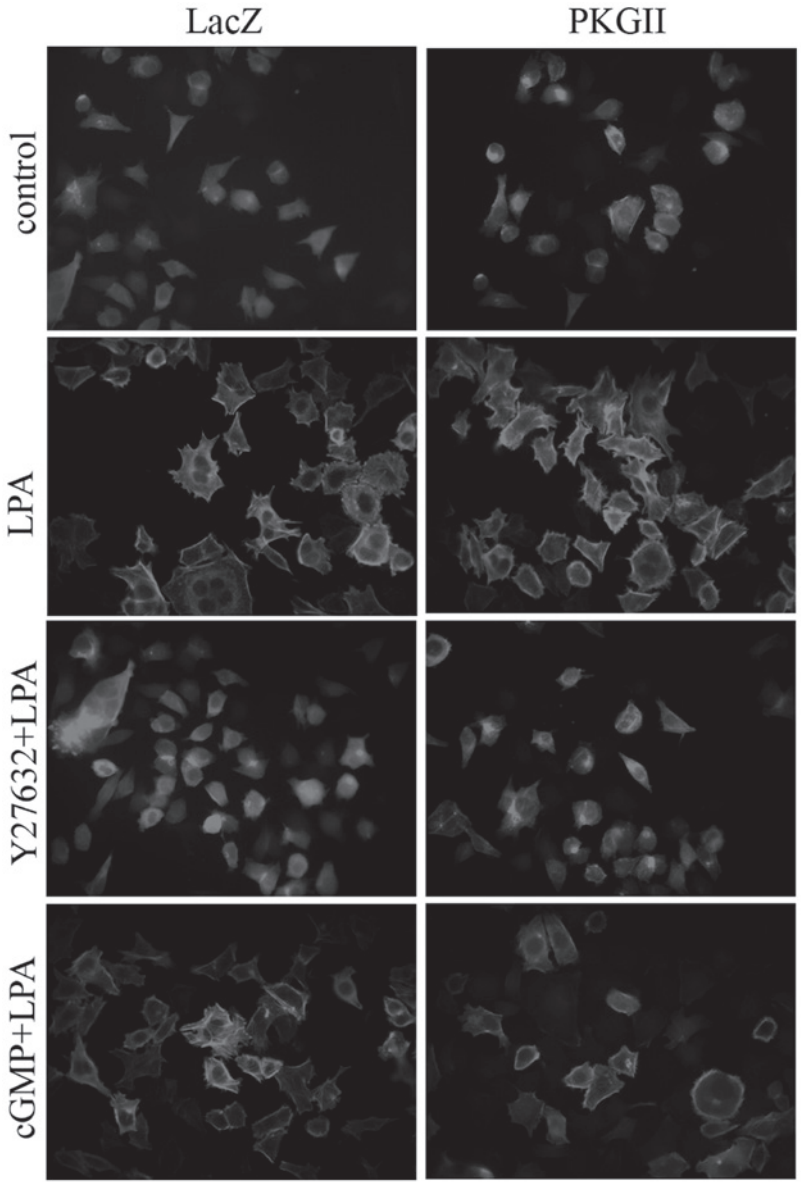

Figure 2. F-actin fluorescent staining with TRITC-conjugated phalloidin (phalloidin staining) in AGS cells treated with lysophosphatidic acid (LPA), or LPA and Y27632/8-pCPT-cGMP. The cells were infected with Ad-LacZ or Ad-PKGII for 48 h and were starved in serum overnight. In the Ad-LacZ + LPA and Ad-PKGII + LPA groups, LPA (1 μM) was added to the culture medium for 5 min; in the Ad-LacZ + cGMP + LPA and Ad-PKGII + cGMP + LPA groups, cells were treated with Y27632 (10 μM) or 8-pCPT-cGMP (250 μM) for 1 h and then LPA (1 μM). Control cells were not treated with LPA, Y27632 or 8-pCPT-cGMP. Magnification, x400. PKGII, type II cGMP-dependent protein kinase.

30 min, stained in Giemsa solution for 10 min and then rinsed in water. The stained cells were subjected to microscopic examination under a light microscope. Migrating cells were counted in five randomly selected fields per insert and the values were averaged. Each migration condition experiment was repeated three times.

Confocal laser scanning microscopy. Cos-7 cells (~2x10^5 cells/dish) were grown on 35-mm dishes with a 20-mm bottom well, and were transfected with BiFC pairs. The BiFC fluorescent signals were detected under a confocal microscope (Zeiss, Oberkochen, Germany).

Western blot analysis. Protein samples were electrophoresed on an SDS-PAGE (8-12%) gel, and membrane transfer was performed following the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The primary antibodies were incubated overnight at 4˚C in Tris-buffered saline with 2% Tween-20 (TBS-T) and the matching secondary antibodies were incubated for 1 h at room temperature in TBS-T, with three washes after each incubation. ECL reagents were used to allow visualizing the positive bands on the membrane. To perform densitometry analysis, digital images of the positive bands were obtained with the ChemiDoc XRS acquisition system and analyzed using the image analysis program Quantity One (Bio-Rad). The results were expressed as the ratio of target protein/loading control protein.

In vitro pull-down assay. Cells on 100-mm culture plates at ~90% confluence were washed three times with cold PBS and harvested in lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 25 mM NaF,

10 mM $MgCl_2$, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 10 mg/ml aprotinin and 10 mg/ml leupeptin) 48 h after transfection or infection. The supernatant obtained by centrifugation (13,800 x g, 10 min) was then mixed with GST-fusion protein beads and incubated for 2 h at 4˚C with rocking. After thorough washing with lysis buffer, the bound proteins were solubilized in 2X SDS buffer and analyzed by western blotting using antibodies against RhoA or PKGII. To assess the amount of protein used in each pull-down assay, 5% of the input lysate was loaded as a control. Beads loaded with GST alone were included as negative controls in all experiments. RhoA activity was detected by a similar method, and the supernatant was incubated with glutathione-sepharose beads and GST-RBD of rhotekin at 4˚C for 1 h.

Immunoprecipitation. The cells growing on 100-mm culture plates were washed twice with cold PBS and lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% Triton X-100, 1 mM EDTA, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 1 mM Na_3VO_4) 48 h after transfection and/or infection. The supernatant was obtained by centrifugation (13,800 x g, 10 min) and then mixed with target antibodies or matched immunoglobulin G as a negative control for 12 h at 4˚C with rocking. Fresh protein G conjugated to agarose was then added, followed by a 2-3 h incubation at 4˚C with rocking. Immunoprecipitates were centrifuged at 400 x g for 2 min at 4˚C. The supernatant was discarded and the pellet was washed four times with binding buffer (50 mM Tris-HCl, 250 mM NaCl, 0.05% Nonidet P40, 30 mM $MgCl_2$, pH 7.4) and then resuspended with the same volume of 2X SDS buffer. The precipitates were probed with antibodies against target proteins.

Statistical analysis. Data are expressed as means ± standard deviation (SD). Statistical significance was assessed with analysis of variance (ANOVA) analyses using the SPSS statistical software (IBM, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

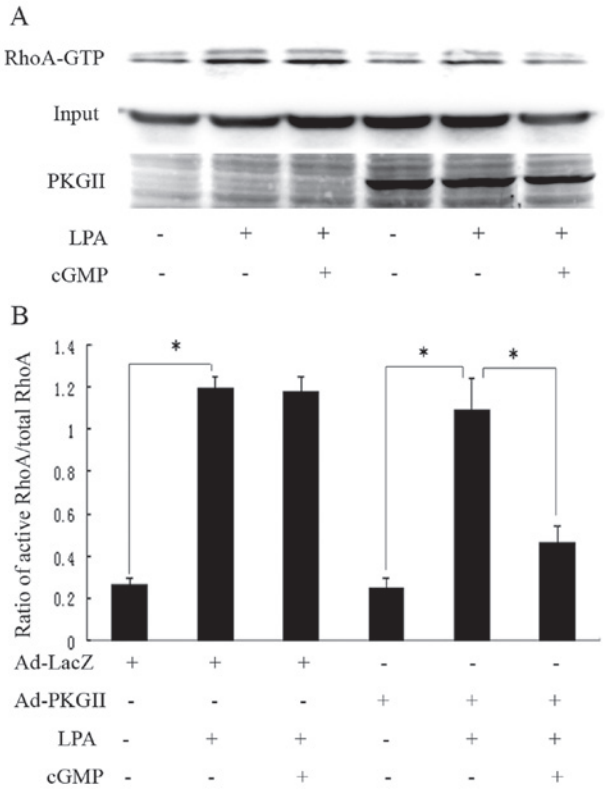

Figure 3. Type II cGMP-dependent protein kinase (PKGII) inhibits lysophosphatidic acid (LPA)-induced RhoA activation. The cells were infected with Ad-LacZ or Ad-PKGII for 48 h and were starved in serum overnight. In the Ad-LacZ + LPA and Ad-PKGII + LPA groups, LPA (1 μM) was added to the culture medium for 5 min; in the Ad-LacZ + cGMP + LPA and Ad-PKGII + cGMP + LPA groups, the cells were treated with 8-pCPT-cGMP (250 μM) for 1 h and then LPA (1 μM) was added to the culture medium for 5 min. The pull-down assay was used to detect the activated RhoA. For the input, 5% of cell lysate and equal amounts of RhoA were incubated with GST-RBD beads as described in 'Materials and methods'. The pull-down product containing active RhoA was analyzed by western blotting with an anti-RhoA antibody. Densitometry analysis was performed to quantify the positive bands and the raw volume ratio of active RhoA to RhoA input (pull-down/input) was calculated. (A) Representative blot and (B) Results of the densitometry analysis. Data are shown as means ± SD from three independent experiments (*$P<0.05$).

Results

PKGII inhibits the LPA-induced migration and cytoskeletal changes in AGS cells. LPA is a potent mediator in the modulation of the cell migration process. A previous study suggested that the G_{12}/G_{13}-RhoA signaling pathway contributes to efficient LPA-stimulated cell migration (18). To detect the role of RhoA in LPA-induced migration of gastric cancer cells, AGS cells were treated with 10 μM Y27632 (ROCK inhibitor) for 1 h before LPA stimulation. A transwell assay was used to measure the migratory activity of the cells. The results showed that LPA stimulation resulted in ~2-fold increase in cell migration compared to that of unstimulated cells (Fig. 1A). Treatment with Y27632 inhibited the LPA-stimulated cell migration, indicating that LPA-induced cell migration is controlled by the activation of the RhoA/ROCK pathway (Fig. 1A). To investigate the effect of PKGII on LPA-induced migration, AGS cells were infected with Ad-LacZ or Ad-PKGII and then treated with 250 μM 8-pCPT-cGMP for 1 h prior to treatment with LPA. Treatment with 8-pCPT-cGMP markedly and significantly inhibited LPA-induced cell migration in the cells infected by Ad-PKGII but not in those infected by Ad-LacZ, indicating that PKGII activity is important, not only for impeding the LPA-induced migration, but also for inhibition of the LPA-induced activation of RhoA (Fig. 1B). To confirm that PKGII functions through cross-talk with the RhoA/ROCK pathway, we examined the effect of PKGII on LPA-induced stress fiber formation, which is well-known to be dependent on RhoA activation. The results showed that pre-infection with Ad-PKGII and treatment with 8-pCPT-cGMP reduces the formation of stress fibers caused by LPA stimulation (Fig. 2).

PKGII inhibits RhoA activation by phosphorylating RhoA at Ser188. We used a pull-down assay to assess whether RhoA activation is affected by PKGII. Treatment of Ad-PKGII-infected cells with 8-pCPT-cGMP marginally decreased the basal RhoA activity, but inhibited LPA-induced RhoA activation by >50% (Fig. 3). LIMK is a signaling component downstream of RhoA, participating in regulating the formation of stress fibers. We detected Thr505/508 phosphorylation/

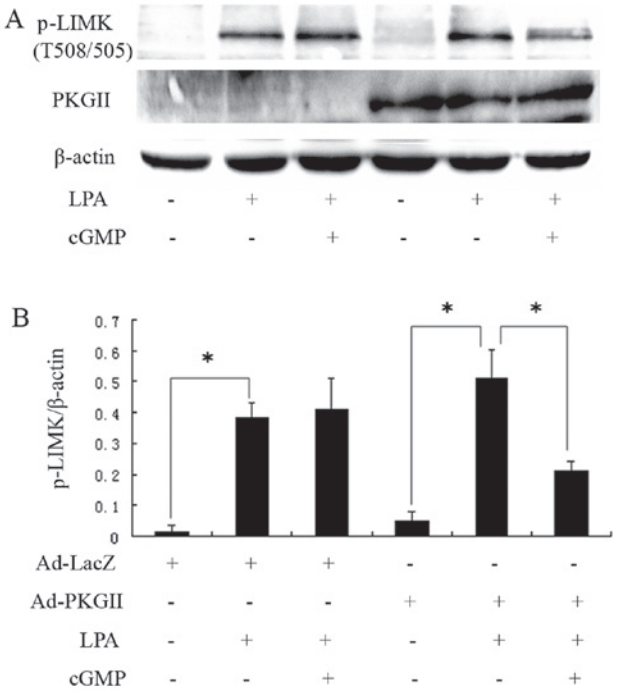

Figure 4. Type II cGMP-dependent protein kinase (PKGII) prevents lysophosphatidic acid (LPA)-induced Thr505/508 phosphorylation of LIMK. AGS cells were treated as described in Fig. 3. Western blotting was performed to detect Thr505/508 phosphorylation of LIMK and densitometry analysis was performed to quantify the positive bands. β-actin was used as a loading control. (A) Representative blot and (B) results of the densitometry analysis. Data are shown as means ± SD from three independent experiments (*P<0.05).

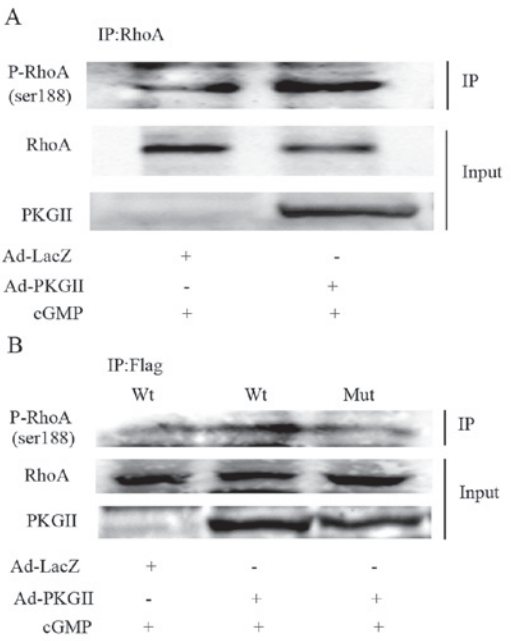

Figure 5. Type II cGMP-dependent protein kinase (PKGII) induces serine 188 (Ser188) phosphorylation of RhoA. (A) AGS cells were infected with Ad-LacZ or Ad-PKGII for 48 h and were starved in serum overnight. 8-pCPT-cGMP (250 μM) was added to the culture medium for 1 h. Immunoprecipitation (IP) of RhoA was achieved with an antibody targeting RhoA, and the phosphorylation of precipitated RhoA was analyzed by western blotting with antibody against phospho (p)-RhoA (Ser188). (B) AGS cells were transfected with FLAG-tagged plasmids expressing wild-type (Wt) RhoA or Ser188A mutant (Mut). Six hours after transfection, the cells were infected by Ad-LacZ or Ad-PKGII for 48 h. The cells were harvested and lysed. IP with antibody against FLAG was performed to precipitate wild-type or mutant RhoA and the phosphorylation of precipitated RhoA was analyzed by western blotting with antibody against p-RhoA (Ser188). Representative blot of three independent experiments.

activation of LIMK and found that PKGII reduced the levels of phosphorylated LIMK which was likely to have been induced by LPA (Fig. 4). These results indicated that inhibition of LPA-induced migration and stress fiber formation by PKGII is associated with inhibition of LPA-induced RhoA activity and its downstream signaling.

It was previously shown that Ser188 is the phosphorylation site through which protein kinases exert their inhibitory effect on RhoA activity (8). We used western blotting with an antibody against RhoA phosphorylated at Ser188 to detect the PKGII-induced phosphorylation of RhoA in AGS cells. The cells were treated as described above. The results showed that the level of phosphorylated RhoA Ser188 was increased in cells infected by Ad-PKGII and treated with 8-pCPT-cGMP (Fig. 5A). In the cells transfected with the plasmid expressing wild-type RhoA, the PKGII activity was sufficient to phosphorylate exogenous RhoA at Ser188. By contrast, in the cells transfected with the plasmid expressing mutant RhoA Ser188A, the phosphorylation of exogenous RhoA at Ser188 did not increase after treatment with 8-pCPT-cGMP (Fig. 5B). These results indicated that PKGII phosphorylates RhoA at Ser188.

PKGII interacts directly with RhoA. The prerequisite for a kinase to phosphorylate its substrate protein is its ability to directly bind the substrate. To determine whether RhoA is the substrate of PKGII, we used a co-immunoprecipitation (co-IP) approach to detect the potentially direct binding of PKGII to RhoA. A

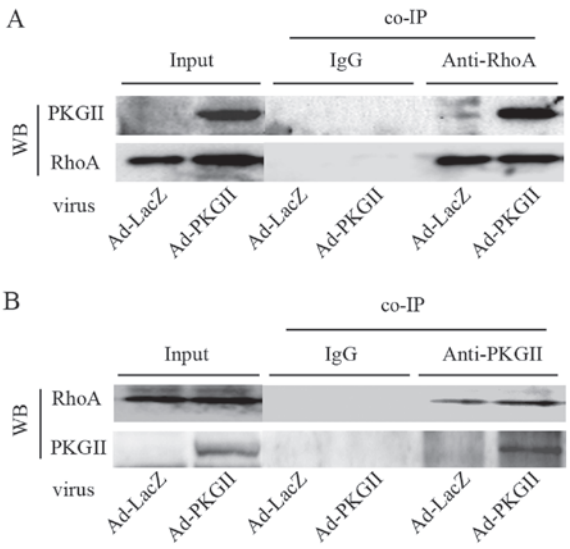

Figure 6. Type II cGMP-dependent protein kinase (PKGII) binds to RhoA. AGS cells were grown in 100-mm plates and infected with Ad-LacZ or Ad-PKGII for 48 h, the cells were lysed and the lysate was subjected to co-immunoprecipitation (co-IP). (A) The lysate was co-immunoprecipitated with anti-RhoA antibody or isotype-matched IgG and the precipitates were probed in a western blotting (WB) experiment with anti-PKGII and anti-RhoA antibodies. As a protein input control, 5% of cell lysates was used. (B) The opposite experiment was also performed, i.e., the lysate was immunoprecipitated with anti-PKGII antibody and probed with anti-RhoA and anti-PKGII antibodies.

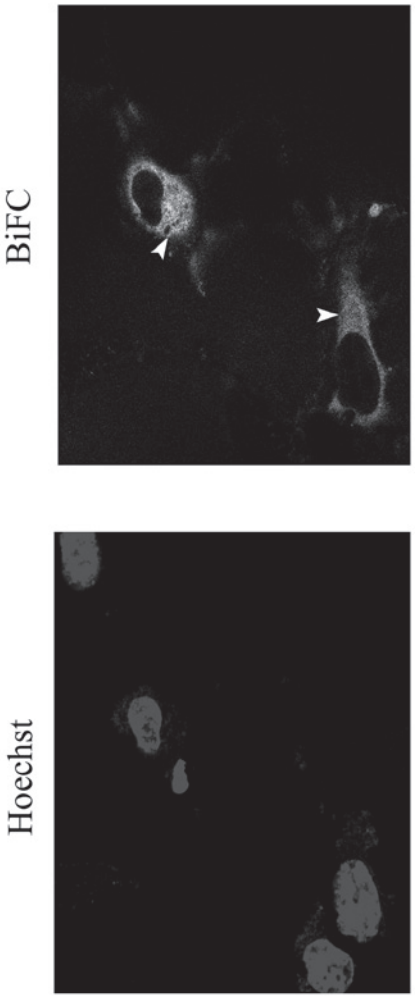

Figure 7. BiFC analysis of the interaction between type II cGMP-dependent protein kinase (PKGII) and RhoA *in vitro*. Cos-7 cells were transfected with PKGII-VN155 and RhoA-VC155. Twenty hours after transfection, the cellular DNA was stained with Hoechst 33342 and cells were observed under a confocal microscope. The arrows indicate the binding signal between PKG II-VN and RhoA-VC (green fluorescent protein).

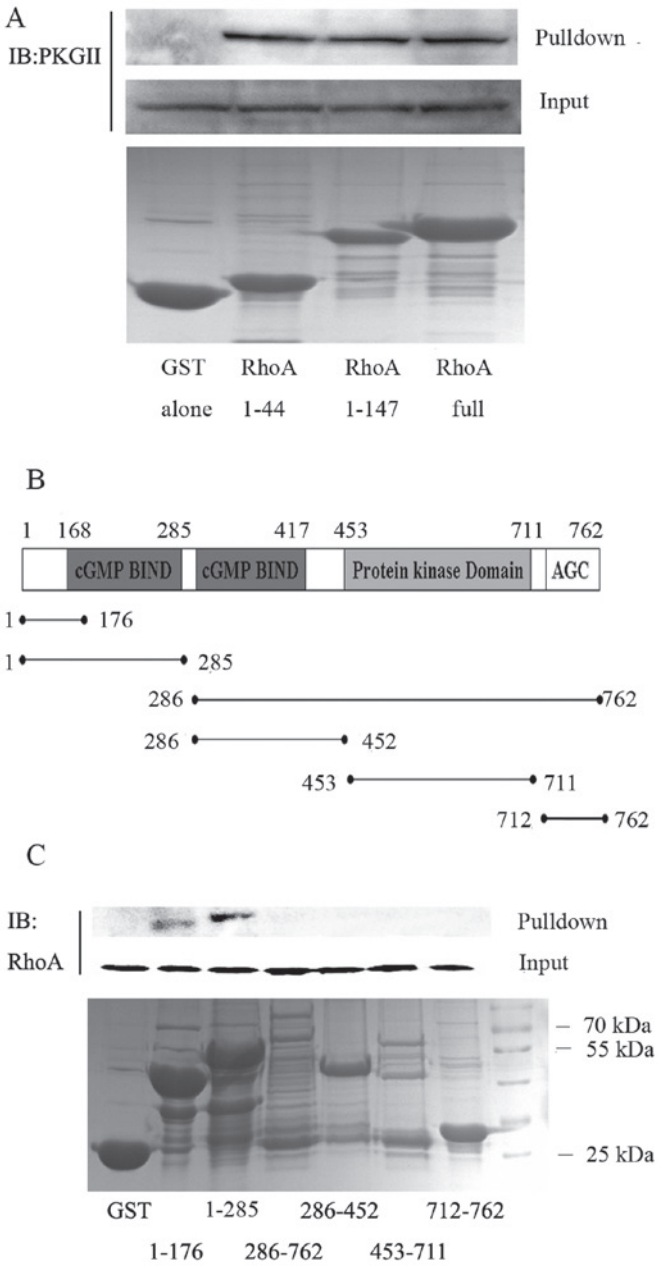

Figure 8. Identification of the type II cGMP-dependent protein kinase (PKGII) binding domains of RhoA and the RhoA binding domains of PKGII. (A) AGS cells were infected with Ad-PKGII for 48 h. Cell lysate was prepared and incubated with glutathione S-transferase (GST)-fused RhoA fragments and GST-fused full-length RhoA protein for 2 h at 4˚C. Interaction of PKGII with GST fusion proteins was determined by immunoblotting (IB) with anti-PKGII antibody. (B) Schematic presentation of PKGII deletion mutants used in the experiments. (C) Proteins constituted of PKGII fragments fused to GST were immobilized with GST beads and incubated for 2 h at 4˚C with cell lysate containing the RhoA protein. Interaction of RhoA with GST fusion proteins was determined by IB using the anti-RhoA antibody.

binding complex between PKGII and RhoA was detected in AGS cells (Fig. 6). To confirm this result, we performed a BiFC assay, based on the formation of a fluorescent complex by two non-fluorescent fragments of the Venus protein, VN155 (VN) and VC155 (VC), brought together by the association of proteins fused with each Venus fragment (19). We found that diffuse cytoplasmic signals with a few aggregates were present in cells expressing PKGII-VN and RhoA-VC (Fig. 7). This result indicated that PKGII can bind RhoA protein *in vitro*.

To define the specific domains of RhoA required for binding to PKGII, different RhoA fragments were fused with the GST protein according to Kato *et al* (20): the RhoA switch I domain (residues 1-44), the RhoA switch I and switch II domains (residues 1-147), and the full-length RhoA (residues 1-192). The fragments were expressed in *E. coli*, purified and added to cell extracts to test PKGII precipitation. The results showed that all three GST-RhoA constructs, but not GST alone, precipitated PKGII, suggesting that amino acid residues 1-44 of RhoA, constituting the switch I domain, are sufficient for the interaction with PKGII (Fig. 8A). We also investigated which domains of PKGII mediate the PKGII-RhoA interaction. GST-fusion proteins containing specific domains of PKGII (Fig. 8B) were generated and incubated with cell lysate containing abundant RhoA protein. Co-IP results showed that RhoA is detected in pull-downs containing the PKGII N-terminal portion, either using the fragments containing amino acid residues 1-176 or residues 1-285 (Fig. 8C). This indicated that the fragment with amino acid residues 1-176 of PKGII is important for the interaction with RhoA.

Discussion

PKGs are serine/threonine kinases that are widely distributed in eukaryotes. Two genes (*prkg1* and *prkg2*) code for PKGs, the type I cGMP-dependent protein kinase (PKGI) and PKGII. In mammals, PKGI is widely expressed in vascular smooth muscle cells, platelets, lung, certain endothelial cells, fibroblasts, heart and the cerebellum (21). PKGI has been identified as a tumor suppressor protein based on its profound anti-tumor effect (22,23). Unlike PKGI, PKGII is a tissue-specific enzyme that is expressed mainly in the brush border of intestinal mucosa and in specific regions of the brain (24,25). PKGII is involved in regulating electrolyte and water secretion, renin and aldosterone secretion and in the adjustment of the biological clock (9,25). In recent years, new insights on the roles of PKGII on cell proliferation, migration and apoptosis were reported. For example, Swartling *et al* (11) found that PKGII has anti-proliferative effects on human glioma cells. Wang *et al* (12) reported a pro-differentiation effect of PKGII in colon cancer cells. Results from our laboratory have shown that PKGII inhibits proliferation and MAPK-mediated signal transduction in human gastric cancer cells (14,15). Furthermore, we demonstrated that PKGII inhibits migration of gastric cancer cells by blocking EGFR activation and consequently, PLCγ1- and MAPK/ERK-mediated signal transduction pathways (16). Overall, these studies indicate that PKGII plays important roles in regulating a number of tumor cell biological activities, and that this kinase, similarly to PKGI, is a potential tumor suppressor protein.

The small G protein RhoA, a member of the Rho subfamily of the Ras superfamily, is important for actin/myosin-based cortical contractility, migration, invasion, transformation, proliferation and survival of tumor cells (1-4). In the GTP-bound active state, RhoA translocates to the plasma membrane, where it interacts with effectors to transduce downstream signals. It has been confirmed that RhoA activates ROCK1 and then LIMK proteins to regulate the formation of stress fibers and induce the migration of cancer cells (26-28). Numerous signaling molecules may cross-talk with RhoA. Among them, protein kinases exert their effect through phosphorylation-dephosphorylation of the protein. In most cases, phosphorylation occurs at a serine residue located at the C-terminal domain of RhoA protein, and modifies its cellular localization. The phosphorylation and inhibition of RhoA by PKA is well established (6,7). It was shown that PKGI can also phosphorylate RhoA at Ser188 and thereby, inactivate RhoA signaling (29,30). However, no study thus far has provided evidence that RhoA is the direct substrate of PKGII.

In the present study, we confirmed the inhibitory effect of PKGII on RhoA and elucidated the underlying mechanism *in vitro*. Previous studies have shown that the G_{12}/G_{13}-RhoA pathway is important for LPA-induced cell migratory activity, and the majority of studies available on stress fiber signaling pathways have identified RhoA as the major regulator of stress fiber formation under most physiological conditions (18,31). To confirm this in gastric cancer cells, we used Y27632, an inhibitor of ROCK, to identify the role of the RhoA/ROCK pathway in the process. The results showed that Y27632 partially blocked LPA-induced migration and stress fiber formation in AGS cells, indicating that the RhoA/ROCK pathway is involved in these processes. To determine whether RhoA is the key target of PKGII, we examined the inhibitory effect of PKGII on RhoA activation. The results showed that PKGII inhibits the LPA-induced activation of RhoA. Since the inhibition of RhoA is associated with its phosphorylation status, we measured the phosphorylation level of RhoA in cells with high activity of PKGII. The results confirmed that PKGII phosphorylates RhoA at Ser188, and that this phosphorylation partially contributes to the decrease in RhoA activation.

To elucidate whether RhoA is the direct substrate of PKGII, we investigated and confirmed the binding between RhoA and PKGII by using co-IP and BiFC assays. Kato *et al* (20) found that PKGI binds directly to RhoA via a direct interaction between the amino-terminus of RhoA (residues 1-44), containing the switch I domain and the amino-terminus of PKGI (residues 1-59), which includes a leucine zipper heptad repeat motif. PKGII has a domain structure similar to PKGI, consisting of an N-terminal regulatory domain, which contains a dimerization and an auto-inhibitory region, two cGMP-binding domains and a C-terminal catalytic domain. However, the position of the high- and low-affinity cGMP-binding domains in PKGII is reversed in comparison to PKGI (32). Moreover, the two PKG iso-enzymes exhibit a different affinity towards various membrane permeable cGMP-analogs, which allows their differentiation (24). To identify the domains of PKGII and RhoA that are involved in their interaction, we expressed fusion proteins with GST and RhoA or PKGII fragments and assessed their binding ability by GST pull-down assays. These experiments demonstrated that the N-terminal of PKGII and the switch I domain of RhoA are required for PKGII-RhoA binding. Taken together, these data support that PKGII directly binds to RhoA.

Since RhoA is involved in the biological activity of tumor cells, new and more effective methods blocking the RhoA/ROCK signal transduction pathway are expected to be useful in cancer therapy. Therefore, our finding that PKGII can inhibit RhoA and downstream signal transduction molecules is of high significance. It provides the evidence that PKGII is a potential tumor inhibitor and may also provide information on the development of therapeutic strategies against cancer.

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