Protein kinase G signaling disrupts Rac1-dependent focal adhesion assembly in liver specific pericytes

Chittaranjan Routray,1 Chunsheng Liu,1 Usman Yaqoob,1 Daniel D. Billadeau,2 Kenneth D. Bloch,3 Kozo Kaibuchi,4 Vijay H. Shah,1 and Ningling Kang1

1GI Research Unit and Cancer Cell Biology Program, 2Department of Immunology, Mayo Clinic, Rochester, Minnesota; 3Anesthesia Center for Critical Care Research, Massachusetts General Hospital, Boston, Massachusetts; and 4Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Showa, Nagoya, Japan

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Routray C, Liu C, Yaqoob U, Billadeau DD, Bloch KD, Kaibuchi K, Shah VII, Kang N. Protein kinase G signaling disrupts Rac1-dependent focal adhesion assembly in liver specific pericytes. Am J Physiol Cell Physiol 301: C66–C74, 2011. First published March 30, 2011; doi:10.1152/ajpcell.00038.2011.—Nitric oxide (NO) is implicated in integrin-mediated cell adhesion to extracellular matrix, whether or how NO signaling regulates the assembly of focal adhesion complexes (FA) and migration of HSC is not known. With the help of complementary molecular and cell biological approaches, we demonstrate here that activation of PKG signaling in HSC inhibits vascular tubulogenesis, migration/chemotaxis, and assembly of mature FA plaques, as assessed by vascular tubulogenesis assays and immunofluorescence localization of FA markers such as vinculin and vasodilator-stimulated phosphoprotein (VASP). To determine whether PKG inhibits FA assembly by phosphorylation of VASP at Ser-157, Ser-239, and Thr-278, we mutated these putative phosphorylation sites to alanine (VASP3A, phosphoresistant mutant) or aspartic acid (VASP3D, phosphomimetic), respectively. Data generated from these two mutants suggest that the effect of PKG on FA is independent of these three phosphorylation sites. In contrast, activation of PKG inhibits the activity of small GTPase Rac1 and its activity is regulated by VASP (21, 24, 43, 44), we tested whether Rac1 activity is regulated by PKG signaling and its activity is regulated by VASP (21, 24, 43, 44), we tested whether Rac1 activity is regulated by PKG signaling and whether it plays a central role in PKG-induced FA disassembly in HSC. Our data demonstrated that activation of PKG in HSC inhibited the formation of lamellipodia and mature FA and that these effects were not mediated by phosphorylation of VASP.

In contrast, we found that activation PKG signaling in HSC inhibits Rac1-dependent focal adhesion assembly in liver specific pericytes. With the help of complementary molecular and cell biological approaches, we demonstrate here that activation of PKG signaling in HSC inhibits vascular tubulogenesis, migration/chemotaxis, and assembly of mature FA plaques, as assessed by vascular tubulogenesis assays and immunofluorescence localization of FA markers such as vinculin and vasodilator-stimulated phosphoprotein (VASP). To determine whether PKG inhibits FA assembly by phosphorylation of VASP at Ser-157, Ser-239, and Thr-278, we mutated these putative phosphorylation sites to alanine (VASP3A, phosphoresistant mutant) or aspartic acid (VASP3D, phosphomimetic), respectively. Data generated from these two mutants suggest that the effect of PKG on FA is independent of these three phosphorylation sites. In contrast, activation of PKG inhibits the activity of small GTPase Rac1 and its activity is regulated by VASP (21, 24, 43, 44), we tested whether Rac1 activity is regulated by PKG signaling and whether it plays a central role in PKG-induced FA disassembly in HSC. Our data demonstrated that activation of PKG in HSC inhibited the formation of lamellipodia and mature FA and that these effects were not mediated by phosphorylation of VASP.

In contrast, we found that activation PKG signaling in HSC inhibited FA disassembly by inhibiting Rac1.

NITRIC OXIDE (NO) generated by endothelial cells plays a major role in the control of vessel tone and vascular architecture (2, 10). In perivascular cells (pericytes), NO exerts its biological effects mainly by activating the cGMP-dependent protein kinase (PKG) via NO/cGMP/KG paracrine signaling. Similar to pericytes in other vascular beds, liver-specific pericytes hepatic stellate cells (HSC) are also regulated by the NO/cGMP/PKG paracrine signaling. For instance, NO/cGMP/PKG signaling inhibits the contractility, migration, and survival of HSC in vitro (27, 29, 38). Indeed, impaired NO production or attenuated response of HSC to NO stimulation in animal models of liver cirrhosis suggest that a defective NO/cGMP/PKG signaling cascade is implicated in the pathophysiology of liver fibrosis and ensuing portal hypertension (3, 12, 15, 17).

Focal adhesions (FA) are dynamic protein complexes that connect extracellular matrix to cells and convey these external stimuli into intracellular signals, which is fundamental for cell adhesion, migration, and survival (5, 14, 31, 45). Although it is known that PKG signaling in smooth muscle cells mediates FA disassembly induced by counter-adhesive extracellular matrix proteins such as thrombospondin and tenascin (34), and that PKG inhibits integrin-mediated cell adhesions (36), mechanistic evidence of how PKG influences FA in HSC is unknown.

FA consists of a large number of proteins including vinculin and vasodilator-stimulated phosphoprotein (VASP). VASP is a member of the Ena/VASP family of proteins and characterized as a substrate of PKG with a postulation of three major phosphorylation sites (Ser-157, Ser-239, and Thr-278). Moreover, VASP is required for optimal FA assembly in pericytes and endothelial cells (24, 48). Therefore, we performed experiments in the present study to test whether PKG signaling regulates FA assembly in HSC by phosphorylating VASP and inhibiting its function. Small GTPases such as RhoA and Rac1 are major regulators of cytoskeletal rearrangement, cell morphogenesis, and migration. PKG signaling is known to inhibit RhoA activity (28, 41, 42), the effect of PKG on Rac1 function in pericytes however is conflicting (35, 41). Based on prior data showing that Rac1 is required for FA assembly (4, 23, 24, 37) and its activity is regulated by VASP (21, 24, 43, 44), we tested whether Rac1 activity is regulated by PKG signaling and whether it plays a central role in PKG-induced FA disassembly in HSC. Our data demonstrated that activation of PKG in HSC inhibited the formation of lamellipodia and mature FA and that these effects were not mediated by phosphorylation of VASP. In contrast, we found that activation PKG signaling in HSC inhibited FA disassembly by inhibiting Rac1.

MATERIALS AND METHODS

Cell culture. LX2 cells, which are immortalized human-derived HSC, were used in this study (52). They have been well characterized and broadly utilized for their ease for plasmid transfection and adenovirus vectors-based gene transfer (7, 24). Cells were cultured under standard conditions in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin).

Generation of VASP phosphomutant constructs. Retroviral vector pMMP-VASPWT-YFP that encodes YFP-fused wild-type VASP was constructed as described (24). This vector was used as a template for site-directed mutagenesis to generate phosphoresistant mutant (pMMP-VASP3A-YFP) and phosphomimetic mutant (pMMP-
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VASP3D-YFP) (QuickChange Multi Site-Directed Mutagenesis Kit; Stratagene). All mutant constructs were sequenced before use. Transfection of 293T cells to generate retroviruses was carried out as previously described (11).

Adenoviral and retroviral transduction of LX2 cells. LX2 cells (1 × 10^6) were plated onto 60-mm culture dishes before adenoviral transduction. After cells were attached, 1 ml PBS containing 100 multiplicities of infection (moi) of adenovirus encoding target genes LacZ, GFP, PKG (wild type), or PKGca (constitutively active) was added to cells and incubated for 1 h at 37°C. To transduce cells with retrovirus, cells with 30–50% confluency were incubated with retroviral supernatant (1:4 dilution with DMEM-completed medium) for overnight at 37°C with Polybrene supplementation (8 μg/ml). The transduction efficiency of both methods was close to 100% by conventional fluorescence microscopy of green fluorescent protein (GFP) or LacZ staining.

Rac1 siRNA transfection. Validated control small interfering RNA (siRNA) (SI03650318) and Rac1 siRNA (318–338) (SI02655051) were purchased from Qiagen, and they were transfected into LX2 cells by using Oligofectamine Reagent (Invitrogen). Assays were performed at 72–96 h after transfection Rac1 knockdown by siRNA was confirmed by Western blot analysis (24).

Vascular tubulogenesis and Boyden chamber assays. Matrigel was used to coat the four-well chamber slides (100 μl/well, BD Bioscience and slides are from Lab-Tek Nalge Nunc International). Cells were then trypsinized and plated on the top of Matrigel (15,000 cells/well in 1 ml serum-free medium). Tubes formed on Matrigel were visualized and documented at different time points after seeding of cells. Tube length was calculated by using the Image Pro-Plus software (version 6.2, Media Cybernetics) (7, 24, 46). For cell migration studies, LX2 cells were transduced with either AdGFP (control), AdPKG, or PKGca. Cells were trypsinized and added in the upper wells of Boyden chamber (20,000 cells/well) 24 h later, and PDGF-BB (10 ng/ml) was added in the bottom wells as a chemoattractant for migration assays. 8-Br-cGMP (100 μM) was used to activate PKG signaling in cells. After 3 h of incubation at 37°C, the polycarbonate filter was removed, and migrated cells on the lower surface were fixed and stained with DNA dye DAPI. Cells passed through the filter were captured from random microscopic fields and quantification was done using Metamorph software (Molecular Devices).

Immunofluorescence and confocal microscopy. Cells that were fixed with 3% paraformaldehyde and permeabilized by 0.2% Triton X-100 were incubated with specific primary antibodies (anti-vinculin or anti-Rac1) for overnight at 4°C or 2 h at room temperature. Alexa Fluor 488 or 555 conjugated anti-mouse IgG (Molecular Probe) were used for secondary detection (1:250). Primary antibodies included anti-vinculin (Sigma, V9131), anti-Rac1 (BD transduction Laboratory, 610650), anti-IQGAP1 (Santa Cruz, H-109, 1:2,000), and anti-P-VASP (Ser239) (1:1,000, Cell Signaling, 3114).

Rac1 activity assay. Rac1 activity assay was performed using a Rac1 Activation Assay Kit (Millipore). Glutathione agarose beads conjugated with PKA P21 binding domain (GST-PBD) were used to pull down active Rac1 from fresh cell lysates. Bound Rac1 were then washed, eluted, and analyzed by Western blot analysis using anti-Rac1 antibody. Cell lysates incubated with 100 μM GTPγS or 100 μM GDP for 15 min at 30°C before were used as a positive or negative control (24).

GST pull-down assay. GST and truncated GST-fused IQGAP1 proteins were purified from Escherichia coli (BL21 DE3) by glutathione sepharose beads. GST pull-down assays were performed as described previously (6, 24).

Statistical analysis. Two-tailed Student’s t-test or two-way ANOVA was used to evaluate statistical significance (Graph Pad Prism 4). Value of P < 0.05 was considered statistically significant. All data was presented as means ± SE.

RESULTS

Activation of PKG signaling in HSC inhibits vascular tubulogenesis, migration/chemotaxis, and the formation of mature FA plaques. To test our hypothesis that NO/PKG paracrine signaling influences adhesion and migration of liver-specific pericytes HSC, we performed in vitro vascular tubulogenesis assays since the development of tubes in this assay is highly dependent on cell adhesion and migration on matrigel (32, 46). Immortalized human HSC cell line (LX2), which develops prominent mature FA plaques in cell culture (24), was used in our studies. These cells are defective in cGMP/PKG signaling (38), which provided us with an ideal cell model to study the role of cGMP/PKG in the regulation of cell adhesion and migration. We first transduced cells with adenoviruses encoding either LacZ, GFP (control), or wild-type PKG (9, 47) and treated cells with PKG agonist 8-Bromo-cGMP (100 μM) to activate PKG and then used them for vascular tubulogenesis assays. As shown in Fig. 1A, activation of PKG signaling significantly inhibited vascular tube formation of HSC on matrigel, suggesting that PKG signaling inhibits HSC adhesion and migration. Migration/chemotaxis assays further confirmed that activation of PKG inhibited HSC migration in vitro. As shown in Fig. 1B, combination of adPKG transduction and 8-Bromo-cGMP treatment significantly inhibited PDGF-in-
duced HSC migration as assessed by Boyden chamber assays. A similar inhibitory effect was also observed in cells transduced with AdPKGca (a constitutively active PKG), which did not require exogenous cGMP stimulation (Fig. 1B), thus obviating concerns that the aforementioned observations were due to nonspecific actions of the 8-Bromo-cGMP compound.

Phase-contrast microscopy of cells transduced with AdPKG and treated with 8-Bromo-cGMP revealed that activation of PKG signaling in HSC resulted in the disappearance of lamellipodia and concurrent appearance of multiple irregular membrane protrusions and elongations (Fig. 1C). These data validate the importance of PKG signaling in cellular locomotion in HSC. With these data in hand, we next prepared PKG-activated HSC for vinculin IF staining to test whether activation of PKG signaling inhibits the development of FA plaques. Vinculin IF and confocal microscopy demonstrated that in the absence PKG stimulation, the majority of cells contained mature FA plaques associated with prominent lamellipodia (arrows, Fig. 1D, top). In contrast, activation of PKG signaling disrupted FA assembly as well as the formation of lamellipodia (Fig. 1D, top). Quantitative analysis revealed that activation of PKG signaling in pericytes resulted in a threefold increase in cells that are devoid of mature FA (*P < 0.05, t-test; n = 3 independent experiments).

A similar inhibitory effect was also observed in cells transduced with AdPKGca (a constitutively active PKG), which did not require exogenous cGMP stimulation (Fig. 1B), thus obviating concerns that the aforementioned observations were due to nonspecific actions of the 8-Bromo-cGMP compound.

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As a complementary approach, we also analyzed changes in FA dynamics temporally using real-time fluorescence video microscopy with HSC that express YFP-tagged VASP, another known FA marker (Fig. 2A). The analysis for a period of 200 min immediately after addition of 8-Bromo-cGMP revealed that the average area of FA (Fig. 2B, left) and the number of FA (Fig. 2B, right) per cell were reduced by activation of PKG signaling. Identical results were also obtained in cells transduced with a constitutively active PKG construct that does not require exogenous cGMP (data not shown). As observed in other cell types such as smooth muscle cells and endothelial cells, PKG signaling is required for thrombospondin-1 and tenascin-C-mediated focal adhesion disassembly (34). By em-
ploying recombinant human thrombospondin-1 and tenascin-C, we found that these two counteradhesive extracellular matrix proteins also induced FA disassembly in HSC, and their effects were partially mediated by PKG signaling (data not shown).

PKG-induced FA disassembly is not mediated by phosphorylation of VASP. Although PKG signaling promotes diverse effects on actin membrane dynamics and cell adhesion that are dependent on culture conditions and cell type specificity (51), activation of PKG in our experimental model clearly simulated a FA phenotype reminiscent of perturbation of VASP, a known phosphorylation substrate of PKG (24). This observation led us to test the hypothesis that activation of PKG may inhibit FA assembly by phosphorylating VASP and therefore impairing VASP function. VASP contains three putative phosphorylation sites: Ser157, Ser239, and Thr278 (22). Indeed, Western blot analysis using an antibody that specifically recognizes one of phosphorylation sites of VASP Ser239, confirmed that VASP was highly phosphorylated in cells that were transduced with AdPKG and treated with 8-Bromo-cGMP compound (Fig. 3A).

To test whether these three phosphorylation sites on VASP mediate the effects of PKG on FA assembly, we generated YFP-tagged retroviral vectors that express a phospho-resistant mutant VASP3A-YFP in which these three sites were mutated to alanine and phosphomimetic VASP3D-YFP in which these three sites were mutated to aspartic acid (Fig. 3B, top). The full-length wild-type VASP (VASPWT-YFP) or YFP were used as controls. Expression of these constructs was confirmed by Western blot analysis (Fig. 3B, bottom). Cells expressing control or VASP phosphomutants were subjected to vinculin IF and, subsequently, FA plaques were analyzed in cells in the absence or presence of PKG activation. Surprisingly, both phosphomutants localized to FA and colocalized with vinculin (Fig. 3C). Furthermore, neither phosphomutant influenced FA in a manner different from YFP or VASP-WT YFP irrespective of PKG activation (Fig. 3D). These data indicate that phosphorylation of VASP on Ser-239, Ser-157, and Thr-278 is not required for the FA disassembly phenotype that is induced by PKG activation.

Activation of PKG signaling inhibits Rac1 activity. We next sought alternative mechanisms by which activation of PKG inhibits FA assembly in HSC. VASP binds and colocalizes with vinculin, and this interaction appears important for FA assembly (18, 39, 40). Therefore, we performed IP studies to determine whether activation of PKG signaling disrupts vinculin/VASP binding thereby preventing their targeting to FA. However, contrary to our initial prediction, IP results showing that equal amounts of vinculin were coprecipitated with VASP from control (AdLacZ-transduced cells) and PKG-activated cells (AdPKG-transduced cells) suggested that activation of PKG in HSC did not induce dissociation of vinculin/VASP complexes (Fig. 4A).
Rac1 is a member of the Rho family small GTPases, a known regulator for the development of lamellipodia and FA (4, 23–25, 37). Rac1 was recently identified as a binding partner of members of the Ena/VASP family proteins (21, 24), and its activity is regulated by VASP in both endothelial cells and pericytes (21, 24, 43, 44). As shown in Fig. 1C, activation of PKG signaling in LX2 cells inhibited the formation of lamellipodia, suggesting that Rac1 function was impaired in PKG-activated cells. Based on these data, we performed experiments to directly test whether activation of PKG in HSC inhibits Rac1 thereby inhibiting FA assembly. Indeed, Rac1 activity assays demonstrated that the amount of GTP-bound Rac1 was significantly reduced in cells transduced with AdPKG compared with cells transduced with AdLacZ (Fig. 4B, left). Consistent with this data, Rac1 IF and confocal microscopy revealed that in cells transduced with AdLacZ, a fraction of Rac1 localized to the plasma membrane, whereas in cells transduced with AdPKG, this plasma membrane localization of Rac1 was significantly diminished (Fig. 4B, right). Taken together, these data support the hypothesis that activation of PKG signaling inhibits Rac1 activity in LX2 cells.

Activation of PKG signaling in HSC inhibits the formation of a trimeric protein complex containing Rac1, IQGAP1, and VASP. Next we performed IP studies to test whether activation of PKG also inhibits Rac1 binding to IQGAP1, a putative effector protein of Rac1 and CDC42 that preferentially binds to GTP-bound small GTPases (26). Since the Rac1 antibody that was used for IF was suboptimal for IP, we performed IP using anti-IQGAP1. As shown in Fig. 4C, top, less Rac1 was coprecipitated with IQGAP1 from cells that were transduced with AdLacZ (control) or AdPKG and stimulated with 8-Bromo-cGMP (100 μM) were subjected to vinculin immunofluorescence for FA analysis. Quantitative data revealed that similar levels of FA disruption were observed in response to PKG activation irrespective of cells expressing VASP phosphomutants (*P < 0.05, ANOVA, n = 3 independent experiments).

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Thus we have identified VASP as another component of a trimeric protein complex Rac1/IQGAP1/VASP that may be implicated in the regulation of FA assembly. Next we performed in vitro GST-fusion protein pull-down assays to study how this trimeric protein complex is assembled. GST-fused truncated IQGAP1 proteins were purified from bacteria and their purities were verified by Ponceau S staining (Fig. 4D, bottom). Using these proteins as baits, we have identified that IQGAP1 functions as a scaffold that interacts with both VASP and Rac1. As shown in Fig. 4D, VASP was pulled down by both IQGAP1 (aa 746–1657) and IQGAP1 (aa 1503–1657). Since IQGAP1 (aa 746–1657) is a larger fragment that contains IQGAP1 (aa 1503–1657), we concluded that VASP interacts with COOH-terminus of IQGAP1 (aa 1503–1657). On the other hand, Rac1 interacts with an adjacent region of IQGAP1 (aa 746 and 1503), which is consistent with data reported previously (20, 26). In summary, our data suggest that activation of PKG in HSC induces dissociation of a trimeric protein complex Rac1/IQGAP1/VASP that may be required for maintaining Rac1 activity and Rac1 function.

Activation of PKG inhibits FA assembly by inhibiting Rac1. To test whether Rac1 indeed mediates PKG-induced FA disassembly, we next silenced Rac1 in LX2 cells using siRNA approach and stimulated the cells with AdPKG and 8-Bromo-cGMP to activate PKG signaling. Validated Rac1 siRNA purchased from QIAGEN has been shown to knockdown Rac1 efficiently in LX2 cells (24). As shown in Fig. 5A, Rac1 siRNA alone also induced FA disassembly. However, this effect of Rac1 siRNA was potentiated by only an additional 20% in response to PKG activation; the lack of synergistic or additive effect suggests that Rac1 is not the sole mediator of PKG-induced FA disassembly. Therefore, we next determined whether other components of the trimeric protein complex Rac1/IQGAP1/VASP are affected by PKG activation. As shown in Figs. 5B and 5C, PKG activation did not induce dissociation of vinculin/VASP complexes, but reduced Rac1 activity (Fig. 5B) and Rac1 localization on the plasma membrane (Fig. 5C). These results are consistent with the finding that PKG inhibits FA assembly by inhibiting Rac1, which in turn affects the localization of other components of the trimeric protein complex.
effects of PKG signaling upon Rac1 depletion suggested that perturbation of Rac1 function at least in part contribute to the effect of PKG signaling on FA disassembly.

To further test our hypothesis that Rac1 indeed mediates PKG-induced FA disassembly in LX2 cells, we expressed a constitutively active Rac1 mutant in cells to determine whether it would rescue cells from PKG-induced FA disruption. Rac1 cDNA with a point mutation Q61L was inserted into a retroviral vector (RacQL) and active Rac1 was generated in LX2 cells by RacQL retroviral transduction as we have previously observed (24). Cells transduced with AdLacZ or AdPKG were subjected to retroviral transduction as we have previously observed (24). Cells transduced with AdLacZ or AdPKG were subjected to retroviral transduction to express LacZ or RacQL, respectively, and the effect of RacQL on PKG inhibition of FA formation was examined. As shown in Fig. 5B, left, vinculin IF and confocal microscopy demonstrated that RacQL promoted the formation of lamellipodia and FA in cells upon PKG stimulation. Quantitative data revealed that RacQL significantly reduced the number of cells that were devoid of mature FA in both AdLacZ and AdPKG transduced cells (*P < 0.05; ANOVA; n = 3 independent experiments).

DISCUSSION

FA turnover is an essential component of cell motility, which in turn is fundamental for numerous cell-type specific functions (1, 5, 14, 31, 33). For example in vascular cells such as pericytes, motility is a dominant step in neovessel formation. Previous studies have demonstrated that NO/cGMP/PKG paracrine signaling inhibits the adhesion and migration of smooth
muscle cells, however, whether and how PKG signaling regulates FA assembly in HSC has not been investigated. Using a HSC cell line LX2 and adenoviral-based PKG gene transfer, we demonstrate here that activation of PKG signaling in HSC inhibits vascular tubulogenesis on Matrigel and morphologically inhibits the formation of lamellipodia and mature FA. In addition, we have generated the following relevant mechanistic findings: 1) PKG signaling induces FA disassembly independent of phosphorylation of VASP on Ser-157, Ser-239, and Thr-278; 2) activation of PKG inhibits Rac1 activity, the membrane localization of Rac1, and Rac1 interact with its effector protein, IQGAP1; and 3) constitutively active Rac1 mutant abrogates the effect of PKG on FA disassembly. Taken together, our data support that activation of PKG signaling disrupts FA assembly in HSC by inhibiting Rac1 function.

FA molecule VASP is a known substrate of PKG. In fact, VASP phosphorylation has been used as a diagnostic marker for activation of PKG signaling in cells. It is therefore reasonable to hypothesize that phosphorylation of VASP on Ser-157, Ser-239, and Thr-278 mediates the effects of PKG on FA disassembly in HSC. Indeed, in our cell model, enhanced VASP phosphorylation was readily detected by Western blot analysis. However, both YFP-tagged phosphoryreistant (VASP-3A) and phosphomimetic (VASP-3D) mutants localized to FA, where they colocalized with vinculin. Additionally, neither mutant influenced FA assembly in the presence or absence of PKG stimulation suggesting that phosphorylation of VASP by PKG does not mediate the FA disassembly phenotype induced by activation of PKG signaling. These findings are consistent with studies showing that a similar phosphomimetic mutant (EGFP-VASP3D) targeted to FA (19).

Numerous studies indicate that PKG signaling inhibits RhoA activity thereby inhibiting migration and contractility of smooth muscle cells (28, 41, 42). The effect of PKG on Rac1 function in pericytes however is conflicting (8, 35, 41, 49). Our data demonstrating that PKG activation inhibits Rac1 activity, thereby inhibiting the formation of lamellipodia and FA, provided us with an additional mechanism to explain how NO/PKG paracrine signaling regulates pericyte adhesion, migration, and survival. In addition, we identified VASP as a component of a trimeric protein complex of Rac1/IQGAP1/VASP that is implicated in the regulation of Rac1 activity and FA assembly. This protein complex is assembled by scaffold protein IQGAP1, which connects VASP and Rac1 by its distinct regions. These data provide a mechanism to supplement and extend a previously reported finding that Rac1 interacts with VASP (24, 44). Since VASP is required for maintaining Rac1 activity (24, 44), it is likely that Rac1/IQGAP1 act as downstream effector proteins of VASP to regulate FA assembly. However, it is not known how PKG signaling disrupts this trimeric protein complex and Rac1 function. IQGAP1 is known to interact with PKCε and to be phosphorylated by PKCe on S1443 (16). Furthermore, Rac1/IQGAP1 binding appears to be regulated by phosphorylation of IQGAP1 on S1443 (16). Therefore, in the future, it will be intriguing to study whether S1443 is phosphorylated by PKG and whether phosphorylation of IQGAP1 on S1443 mediates dissociation of IQGAP1/Rac1/VASP complexes.

HSC are a major cell type that contributes to the development of liver fibrosis and metastatic liver metastases owing to their role in the deposition of extracellular matrix proteins that are essential for fibrosis, tumor desmoplasia, and angiogenesis (13, 30, 50, 53). Thus inhibition of HSC activation can be regarded as a promising approach for prevention or treatment of these liver diseases. In addition, since NO/cGMP/PKG paracrine signaling plays a pivotal role in the regulation of pericyte function and vascular homeostasis, the downstream PKG signaling that mediates pericyte adhesion and migration may present additional therapeutic targets for treatment of vascular disorders as well.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
16. Grohmanova K, Schlaepfer D, Hess D, Gutierrez P, Beck M, Kroschewski R. Phosphorylation of IQGAP1 modulates its binding to Cdc42,


