cGMP-dependent Protein Kinase Phosphorylates p21-activated Kinase (Pak) 1, Inhibiting Pak/Nck Binding and Stimulating Pak/Vasodilator-stimulated Phosphoprotein Association

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Endothelial cells are normally non-motile and quiescent; however, endothelial cells will become permeable and invade and proliferate to form new blood vessels (angiogenesis) in response to wounding, cancer, diabetic retinopathy, age-related macular degeneration, or rheumatoid arthritis. p21-activated kinase (Pak), an effector for the Rho GTPases Rac and Cdc42, is required for angiogenesis and regulates endothelial cell permeability and motility. Although Pak is primarily activated by Rac and Cdc42, there are additional proteins that regulate Pak activity and localization, including three AGC protein kinase family members, Akt-1, PDK-1, and cAMP-dependent protein kinase. We describe phosphorylation and regulation of Pak localization by a fourth AGC kinase family member, cGMP-dependent protein kinase (PKG). Using in vitro mapping, a phosphospecific antibody, co-transfection assays, and untransfected bovine aortic endothelial cells we determined that PKG phosphorylates Pak at serine 21. Phosphorylation was accompanied by changes in proteins associated with Pak. The adaptor protein Nck was released, whereas a novel complex with vasodilator-stimulated phosphorylated was stimulated. Furthermore Ser-21 phosphorylation of Pak appears to be important for regulation of cell morphology. In both human umbilical vein endothelial cells and HeLa cells, activation of PKG in the presence of Pak stimulated tail retraction and cell polarization. However, in cells expressing S21A mutant Pak1, PKG activation or treatment with a peptide that blocks Nck/Pak binding caused aberrant cell morphology, blocked cell retraction, and mislocalized Pak, producing uropod (tail-like) structures. These data suggest that PKG regulates Pak and that the interaction plays a role in tail retraction.

The Rho family of small GTPases regulates cell proliferation, apoptosis, motility, and morphology (4–6). Rac and Cdc42 are two of the best characterized Rho GTPase family members, and one of their shared effectors is the serine/threonine p21-activated kinase (Pak)2 (7). Signals as varied as insulin, estrogen, glial growth factor, interleukin–3, or angiogenic factors activate and regulate Pak (8–13). In endothelial cells, Pak is stimulated by vascular endothelial growth factor, basic fibroblast growth factor, and angiopoietins 1 and 2 to regulate angiogenesis, motility, and cell survival (8, 13, 14). Microinjection of mutant forms of Pak such as dominant negative mutants or an inhibitory peptide called the Pak PID (Pak inhibitory domain, amino acids 83–149) reduces motility at multiple stages. These models suggest that Pak is required for motility by coordinating the formation of new adhesions at the front of the cell while detaching the rear of the cell by promoting tail retraction (15).

Pak contains a number of proline-rich regions that serve as docking sites for proteins with SH3 domains including Ptx, Grb2, or Nck. In most cases, these proteins regulate Pak largely by controlling its subcellular localization. Nck is a 51-kDa protein that binds the N-terminal proline-rich region of Pak, is associated with Pak in endothelial cells, and is recruited to focal adhesions in vascular endothelial growth factor-treated cells (8, 16, 17). When Nck/Pak binding is disrupted with peptides that interfere with the binding, endothelial cell migration, network formation in three-dimensional assays, and chick chorioallantoic membrane angiogenesis are all inhibited (18). Furthermore when Nck expression is blocked by antisense DNA, the assembly of focal adhesions is blocked (17). These data indicate that Nck, through its regulation of Pak, is important in regulating endothelial cell migration and morphology.

The most well characterized mechanism for regulation of Nck/Pak binding is by phosphorylation of serine 21 on Pak (19). When Ser-21 is phosphorylated, which can come about either by autophosphorylation or by other kinases such as the AGC kinase family member Akt-1, Nck/Pak binding is disrupted (19, 20). The AGC family of kinases include protein kinase A (cAMP-dependant protein kinase), protein kinase C, and cGMP-dependant protein kinase (PKG). PKG is a serine/threonine kinase activated by the short lived second messenger cGMP. Activation is rapid and transient.

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§ This abbreviations used are: Pak, p21-activated kinase; SH3, Src homology 3; PKG, cGMP-dependent protein kinase; VASP, vasodilator-stimulated phosphorylated protein; HUVEC, human umbilical vein endothelial cell; DMEM, Dulbecco’s modified Eagle’s medium; 8-Br-cAMP, 8-bromo-cAMP, 8-Br-cAMP, 8-(2-chloro-adenosine 3′,5′-monophosphate; 8-pCPT-cGMP, 8-(2-chloro-adenosine 3′-5′-monophosphate; N-NTA, nickel-nitrilotriacetic acid; BAEC, bovine aortic endothelial cell; EBM-2, endothelial basal medium-2; WT, wild-type; KD, kinase-dead; GFP, green fluorescent protein; PBS, phosphate-buffered saline; EVH1, Ena-VASP homology 1; MLC, myosin light chain.
because cGMP is rapidly degraded by intracellular phosphodiesterases thereby enabling PKG to serve as a rapid yet transient transducer of extracellular signals. In endothelia, NO or phosphodiesterase inhibitors rapidly elevate cGMP levels and stimulate PKG. This causes changes in endothelial cell permeability and motility (21).

Given the importance of both PKG and Pak in regulating endothelial cells, we wished to determine any cross-regulation between the kinases. In this study, we demonstrated that PKG phosphorylates Pak at Ser-21, disrupts Nck/Pak binding, alters Pak localization, and causes remodeling of focal adhesions. Additionally we demonstrated a novel phosphorylation-dependent association between Pak and the PKG substrate, Ena/Mena family member, and actin cytoskeleton-regulatory protein: vasodilator-stimulated phosphoprotein (VASP). Through regulation of these processes, PKG phosphorylation of Pak regulates HeLa cell and human umbilical vein endothelial cell (HUVEC) morphology and cellular remodeling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Invitrogen. FuGENE 6 transfection reagent, Complete protease inhibitor mixture tablets, and fibronectin were from Roche Applied Science. cAMP-agarose, 8-Br-cAMP, 8-pCPT-cGMP, EDTA, forskolin, HEPES, 3-isobutyl-1-methylxanthine, Nonidet P-40 detergent, Nunc chamber slides, phenylmethylsulfonyl fluoride, and sodium orthovanadate (Na3VO4) were from Sigma. Mouse monoclonal anti-Nck antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal N-20 anti-Pak1 antibody and protein A/G PLUS-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-Myc1–9E10 was from University of Pennsylvania Cell Center (Philadelphia, PA). Mouse monoclonal anti-VASP was from A. G. Scientific Inc. (San Diego, CA). Rabbit polyclonal anti-cyclis GMP-dependent protein kinase was from Calbiochem. Nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from Qiagen. Alexa Fluor 594-tagged phalloidin and highly cross-absorbed Alexa Fluor 594 goat anti-mouse immunoglobulin G (heavy plus light chains) were from Molecular Probes (Eugene, OR). HUVECs (passages 2–6), bovine aortic endothelial cells (BAECs) (passages 2–11488), and visualized by autoradiography.

**Kinase Assays**—Kinase assays were conducted by incubating proteins, as indicated in the figure legends, with 20 μM ATP in kinase buffer (10 mM MgCl2, 40 mM HEPES, pH 7.4) in a reaction volume of 25 μl. Reactions were initiated by adding 20 μM ATP in some cases with 5 μCi of [γ-32P]ATP, and the reactions were incubated at 30 °C for 5 min. The reactions were terminated with 25 μl of 2× SDS sample buffer. Samples were then boiled, resolved by SDS-PAGE, and visualized by autoradiography.

**Cell Culture and Transfection**—COS-7 or 293T cell lines were maintained in DMEM containing 10% heat-inactivated fetal bovine serum and supplemented with 2 mM glutamine, 100 units of penicillin G/ml, and 100 μg of streptomycin/ml at 37 °C in 5% CO2. For transfection of COS-7 or 293T cell lines, cells were split, grown for a day to 60–80% confluence, and transfected with FuGENE as recommended. For each well of a 6-well (35-mm dish) plate, cells were transfected with 0.25 μg of WT Pak1 plasmid along with 0.75 μg of PKG 1β plasmid. pCMV6 vector was used to control for total transfected DNA. Cells were allowed to express proteins for 24 h after DNA was added. Cells were washed in 37 °C DMEM three times and serum-starved in DMEM 18–24 h. To harvest, cells were washed twice with ice-cold PBS, washed with 0.8 ml of ice-cold lysis buffer (40 mM HEPES, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 1 mM EDTA, 25 mM NaF, 1 mM Na3VO4) containing the recommended concentration of Complete protease inhibitor, and the mixture was agitated in the wells on ice for 5 min. The lysis mixture was transferred into centrifuge tubes and centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. The supernatant was collected as cell lysate and used immediately.

BAECs were split and grown for 1 day in endothelial growth medium-2 in 6-well plates. Twenty-four hours after replating, the cells were washed twice with 37 °C EBM-2 and treated with water or 500 μM...
PKG Phosphorylates Pak

PKG Phosphorylates Pak in Vitro—The AGC kinase family members PDK-1 (25), cAMP-dependent protein kinase (26), and Akt (20, 27) phosphorylate and regulate Pak. We hypothesized that PKG, another AGC kinase family member, also phosphorylates Pak1. To test this hypothesis, we incubated recombinant Pak1 and PKG, both purified from baculovirus, in an in vitro [$\gamma^32P$]ATP kinase assay, separated the reactions on an SDS-polyacrylamide gel, and analyzed protein phosphorylation by autoradiography (Fig. 1A). Because Pak1 phosphorylates at activated, we tested Pak1 K299R, a KD mutant of Pak1, to eliminate the variable of Pak kinase activity on Pak itself. We noted that PKG phosphorylated Pak1.

To determine the region of Pak that was being phosphorylated we tested a number of Pak1 fragments for phosphorylation by PKG (Fig. 1B). Overlapping fragments of Pak1 covering the entire protein were tested. The best substrate for PKG was the fragment covering the N-terminal portion of Pak, amino acids 1–74. PKG did not phosphorylate a fragment encompassing amino acids 67–149 and only weakly phosphorylated fragments from the central region of Pak1 encompassing amino acids 147–231 and the C-terminal catalytic region, amino acids 231–544 (constructed in a KD mutant to prevent autophosphorylation) (Fig. 1B). Because phosphorylation of these fragments was weak, they were not pursued. We also noted that phosphorylation of the N-terminal fragment (amino acids 1–74) was considerably better than that observed with Akt1 (data not shown).

Because the most highly phosphorylated fragment of Pak1 was the N-terminal portion, we wished to identify the residue phosphorylated by PKG within fragment 1–74. Because Akt phosphorylates serine 21 on Pak1 we ran an in vitro [$\gamma^32P$]ATP kinase assay with PKG and fragment 1–74 of Pak containing a mutation from serine to alanine at amino acid 21. The S21A mutant fragment was phosphorylated much less readily by PKG than the original fragment (Fig. 1C). To confirm the data with fragments, we incubated purified full-length KD Pak1 and S21A-Pak1 with PKG in an in vitro [$\gamma^32P$]ATP kinase assay and then analyzed the reactions on an SDS-polyacrylamide gel by autoradiography (Fig. 1D). Again we observed that PKG phosphorylated Pak1; however, the phosphorylation was not seen in the S21A mutant Pak.

We also confirmed PKG phosphorylation of Pak at serine 21 with a phosphospecific antibody. To do this, we incubated recombinant bacu-
lovirus wild-type Pak1, a kinase-dead Pak1 (K299R), and a S21A/K299R mutant Pak with PKG in a non-isotopic ATP kinase assay, separated the proteins on an SDS-polyacrylamide gel, and detected phosphate incorporation by Western blot (Fig. 1E). Again we observed a signal at serine 21 when PKG was incubated with Pak1, whereas no signal was detected with the S21A mutant Pak1.

As a control for the phosphospecific antibody, we probed the same samples with a phosphospecific antibody that recognizes threonine 423, a major phosphorylation site on Pak (Fig. 1E). Although there was a slight Thr-423 phosphorylation of KD Pak treated with PKG, the S21A-Pak did not have a signal, suggesting that the observed signal may be due to cross-reactivity of the Thr-423 antibody with serine 21. In this experiment, the wild-type Pak1 was highly phosphorylated at Thr-423, and activated PKG did not affect the signal. The signal in the wild-type protein is probably due to partial activation of Pak in baculovirus. Note that PKG was also recognized by this antibody, which cross-reacts with a number of related kinases. From these experiments we conclude that PKG, like Akt, phosphorylates Pak at serine 21 in vitro and does not phosphorylate Thr-423.

To determine whether PKG phosphorylated Pak1 in cells we searched for a Pak kinase activity in BAECs. BAECs provide a good model to study the endogenous effects of PKG because they express PKG, unlike other endothelial cell lines. For the assay, we treated BAECs with cGMP for 15 or 60 min. We then lysed the cells, ran the lysate on an SDS-polyacrylamide gel, and detected serine 21 phosphorylation by Western blot (Fig. 1F). Only in cells treated with cGMP were we able to detect serine 21 phosphorylation. Thus, in untransfected BAECs, cGMP stimulated phosphorylation of Pak at serine 21.

**PKG Does Not Immediately Affect Pak Activation** —To test whether PKG stimulates phosphorylation of Pak in vivo, we conducted a time course dosing of 500 μM 8-pCPT-cGMP in COS-7 (Fig. 2A and B) and 293T (Fig. 2C) cells transfected with WT Pak1β and/or WT Pak1 plasmids. We observed similar results in both cell types, but because the signal was more robust in 293T cells, most experiments were performed in these cells.
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Treated cells were lysed, a portion of the lysate was incubated with Myc antibody to immunoprecipitate Pak, and the sample was then Western blotted. Additionally we probed cell lysate Western blots for PKG and Myc to confirm PKG and Pak expression (Fig. 2A and data not shown).

Using the Ser-21-phosphospecific antibody, we observed that PKG stimulated serine 21 phosphorylation on Pak1. We used the known PKG substrate VASP as a positive control. Phosphorylation of VASP at serine 239 increased through 4 h of treatment with 500 μM 8-pCPT-cGMP as measured with a VASP Ser-239-phosphospecific antibody. However, in positive controls from cells co-transfected with Pak and Q61L activated Rac, Rac stimulated phosphorylation of Ser-21 on Pak1 but had no effect on VASP phosphorylation status. The Ser-21 phosphosignal, as with the Ser-239 phospho-VASP signal, peaked at 1 h of treatment with cGMP and decreased by the 24-h time point (Fig. 2, A and C).

To determine whether PKG might also activate Pak, we analyzed Pak activity as well as phosphorylation at Thr-423 as a measure of Pak kinase activity. We conducted a time course of treatment with 500 μM 8-pCPT-cGMP in COS-7 cells transfected with DNA plasmids for mammalian cell expression of WT Pak1 and/or WT PKG1 (Fig. 2B). A positive control lane of Pak plus an activated Rac showed increased Pak activity in a Pak immunokinase assay and positive staining in a Pak1 Thr-423-phosphospecific blot. However, there was no effect of PKG on Pak activation until after 18–24 h of treatment with 8-pCPT-cGMP. We conclude from these experiments that PKG can phosphorylate Pak1 directly on serine 21, and this phosphorylation follows the kinetics of PKG activation as measured by VASP phosphorylation at serine 239; however, there is no immediate effect of PKG activation on Pak activity.

**PKG Activation Reduces Nck Binding to Pak**—Nck binds Pak1 at a proline-rich SH3 binding site near serine 21, and serine 21 phosphorylation has been demonstrated to prevent association of Pak1 with the adaptor protein Nck (16, 19). Additionally our laboratory has shown that Akt phosphorylates Pak1 on serine 21 and reduces Nck/Pak binding (20). To test the effect of PKG on Nck/Pak binding, we transfected 293T cells with wild-type Pak1 and/or WT PKG1 and then tested whether a 2-h cGMP treatment would reduce Nck/Pak binding (Fig. 3A). Nck binding was determined by performing Western blots for Pak1 after Nck immunoprecipitation. Consistent Pak expression was confirmed by Western blots for total Pak1 (data not shown). We observed reduced Nck/Pak binding after cGMP treatment. We did not see a disruption in Nck/Pak binding in 293T cells transfected only with Pak1 and treated with vehicle or cGMP. We were able to rule out the possibility that the effects on Nck/Pak binding were caused indirectly after autophosphorylation of Pak1 because we did not see reduced Nck binding when an activated form of Rac was co-transfected with kinase-dead Pak1 as well as wild-type Pak (Fig. 3B). We confirmed that the kinase-dead Pak1 was inactive with a Pak1 phospho-Thr-423 Western blot (data not shown). Additionally non-cGMP-treated cells transfected with PKG and Pak had levels of Nck/Pak binding and Ser-21 phosphorylation similar to Pak negative controls. These results indicate that PKG phosphorylates Pak1 at serine 21 in a cGMP-dependent manner, and this phosphorylation releases the Nck adaptor protein from Pak1 independently of Pak kinase activity.

**PKG Stimulates Association of VASP with Pak**—Pak1 contains five proline-rich regions that bind proteins with SH3 motifs. Binding partners can direct localization of Pak1 and help Pak1 direct cell polarity (7). VASP can also bind proline-rich proteins through an Ena-VASP homology 1 (EVH1) proline binding domain. Additionally cells from VASP(−/−) mice have prolonged Rac and Pak activation after platelet-derived...
growth factor treatment suggesting that VASP might directly regulate Rac and Pak (28). To determine whether Pak associates with VASP we immunoprecipitated transfectected 293T cells with a VASP antibody and then probed Western blots for Pak. The transfected Pak coprecipitated with VASP (Fig. 3C). Because both Pak and VASP are substrates for PKG, we also tested the effects of PKG activation on VASP association with Pak (Fig. 3C). Activation of PKG stimulated association of Pak1 with VASP. Within 20 min of PKG activation, serine 21 phosphorylation of Pak1 rose, and VASP/Pak1 association rose, whereas Nck/Pak association decreased (Fig. 3C). These results indicate that cGMP activation of PKG stimulates association of VASP with Pak while inhibiting the association of Nck with Pak.

**Rac Also Stimulates VASP/Pak Association**—Because serine 21 phosphorylation of Pak by PKG correlated with increased VASP/Pak association (Fig. 3C) we wished to determine whether Rac might also induce VASP/Pak association. Rac also stimulated serine 21 phosphorylation as well as association of Pak with VASP (Fig. 2, A and C, and supplemental Fig. 1A). However, Rac did not stimulate the complex between VASP and the S21A-Pak despite elevated levels of basal Pak activity and a strong activation by Rac (supplemental Fig. 1B).

**PKG-mediated Relocalization of Pak and Cell Polarization Are Blocked by S21A-Pak**—To view the dynamics of Pak following PKG stimulation, we transfected HeLa cells with wild-type or mutant GFP-Pak1 and then observed Pak localization after transfection and activation of PKG using time lapse fluorescence microscopy (Fig. 4). The cells were treated with 8-pCPT-cGMP and monitored by taking phase and fluorescence micrographs every 5 min for 45–60 min. We noted significant differences in both the localization of Pak and cell polarization following PKG activation (Fig. 4). Initially GFP-Pak1 was concentrated at the edges of cells in the punctate patterns of focal adhesions. Following PKG stimulation, GFP-Pak1 localized away from what would later become “rear” portions of the cell and consolidated at newly forming lamellipodia of the polarizing cells (Fig. 4). There was no change in Pak localization or cell morphology in untreated cells or cells that were not transfected with PKG (Fig. 4). The time lapse observations were consistent with the activated PKG-induced WT GFP-Pak1 localization and vinculin staining we observed in fixed HeLa cells (data not shown).

To determine whether the change in Pak localization and cell morphology was mediated by PKG phosphorylation of Pak, we tested the mutant S21A-GFP-Pak1. Although the S21A-GFP-Pak1-transfected cells exhibited thinner, more diffuse, less punctate base-line localization compared with wild-type Pak, the localization largely resembled wild-type controls. However, following PKG activation, there was a signifi-
activated PKG relocalizes WT GFP-Pak1 and polarizes HeLa cells, but this effect is blocked by S21A-GFP-Pak1. HeLa cells were co-transfected with WT GFP-tagged Pak1, WT GFP-tagged Pak1 and PKG, S21A-GFP-tagged Pak1, or S21A-GFP-tagged Pak1 plus PKG and observed with fluorescence and phase microscopy before and after treatment with 8-pcP7-cGMP. Samples were observed for 10–15 min with phase and fluorescence microscopy to establish base-line cell morphology and Pak localization. Samples were treated with 500 μM 8-pcP7-cGMP, and phase and fluorescence micrographs were taken of the cells every 5 min for 45 min. The cells were observed under a Nikon TE2000-U fluorescence microscope, and micrographs were taken with a Hamamatsu camera operated with Image Plus software. A21 Pak, S21A-Pak.

**TABLE 1**

Activated PKG causes GFP-Pak1-expressing HeLa cells to polarize, but this effect is blocked by S21A-GFP-Pak1

| HeLa cell plasmid expression | Polarized cells | Cells with uropods |
|-----------------------------|----------------|-------------------|
|                             | %              | %                 |
| Pak                         | 9.1            | 0                 |
| Pak + PKG                   | 29             | 5.5               |
| S21A-Pak                    | 9.5            | 19                |
| PKG + S21A-Pak              | 6.9            | 45                |

to Pak (15, 18). To test the interactions between Pak and PKG we transfected HUVECs with PKG, Myc-tagged Pak1 (S21A or WT), and a GFP vector to identify transfected cells. Cells were then treated with cGMP, fixed, and stained with phalloidin. As we observed in HeLa cells, activated PKG stimulated cell polarization. The S21A-Pak1 alone caused a mild aberrant fringe- or tail-like cell morphology; however, S21A-Pak1-expressing cells had a more severe aberrant uropod morphology upon treatment with cGMP (Fig. 5).

The HUVEC results were confirmed using immunofluorescence and time lapse phase microscopy. In cells transfected with GFP vector, PKG, Pak, or Pak + PKG there was no apparent difference in cell morphology following cGMP treatment (Fig. 5 and Table 2). The morphology of these cells was polarization characterized by random formation of lamellipodia, extension of the cell, consolidation, and contraction of the cell toward the extension followed by tail retraction. In cells transfected with Myc-S21A-Pak1 the process began similarly: cells first formed lamellipodia, which extended and consolidated, and the cell attempted to retract the rear. However, cells transfected with Myc-S21A-Pak1 had reduced tail retraction and exhibited a fringelike morphology following random cell movement. The most severe uropod morphology was in cells transfected with S21A-Pak following PKG activation with cells developing long rod-shaped tail-like extensions (Fig. 5, supplemental Fig. 1, and Table 2).

**FIGURE 4.** Activated PKG relocalizes WT GFP-Pak1 and polarizes HeLa cells, but this effect is blocked by S21A-GFP-Pak1. HeLa cells were co-transfected with WT GFP-tagged Pak1, WT GFP-tagged Pak1 and PKG, S21A-GFP-tagged Pak1, or S21A-GFP-tagged Pak1 plus PKG and observed with fluorescence and phase microscopy before and after treatment with 8-pCPT-cGMP. Samples were observed for 10–15 min with phase and fluorescence microscopy to establish base-line cell morphology and Pak localization. Samples were treated with 500 μM 8-pCPT-cGMP, and phase and fluorescence micrographs were taken of the cells every 5 min for 45 min. The cells were observed under a Nikon TE2000-U fluorescence microscope, and micrographs were taken with a Hamamatsu camera operated with Image Plus software. A21 Pak, S21A-Pak.

**FIGURE 5.** HUVECs expressing S21A-Pak fail to retract their tails following PKG activation or treatment with a Tat-Pak peptide. HUVECs were transfected with GFP vector, PKG + GFP, GFP + Pak, GFP + Pak1 + PKG, GFP + S21A-Pak1, or GFP + S21A-Pak1 + PKG. Cells were starved and not treated or starved and treated with 500 μM 8-pCPT-cGMP for 2 h. Cells were fixed after treatment and stained with Alexa Fluor 594-conjugated phalloidin to visualize F-actin. No significant difference was observed in the morphology of most cells (vector, PKG, Pak1, PKG + Pak, and S21A-Pak1 alone). However, aberrant tail morphology was apparent in untreated HUVECs transfected with S21A-Pak1 + PKG, whereas a more severe rod-shaped morphology was apparent in cells transfected with S21A-Pak1 + PKG and treated for 2 h with cGMP or following treatment with the Tat-Pak peptide in HUVECs transfected with S21A alone or S21A-Pak + PKG. GFP-positive cells are identified by arrowheads. The cells were observed under a Nikon TE2000-U fluorescence microscope, and micrographs were taken with a Hamamatsu camera operated with Image Plus software. A21 Pak, S21A-Pak.
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TABLE 2
Activated PKG causes HUVECs to polarize, but this effect is blocked by S21A-GFP-Pak1 or S21 peptide

| HUVEC plasmid expression and treatment condition | Polarized cells | Cells with uropods |
|------------------------------------------------|-----------------|-------------------|
| GFP untreated                                   | 10              | 0                 |
| GFP + cGMP                                      | 10              | 0                 |
| GFP + S21 pep.                                  | 11              | 18                |
| Pak untreated                                   | 11              | 2.6               |
| Pak + cGMP                                      | 14              | 5.4               |
| Pak + S21 pep.                                  | 8.3             | 29                |
| PKG untreated                                   | 12              | 0                 |
| PKG + cGMP                                      | 44              | 0                 |
| PKG + S21 pep.                                  | 6.3             | 25                |
| PKG + Pak untreated                             | 8.8             | 0                 |
| PKG + Pak + cGMP                                | 28              | 2.3               |
| PKG + Pak + S21 pep.                            | 0               | 63                |
| S21A-Pak untreated                              | 5.3             | 32                |
| S21A-Pak + cGMP                                 | 6.8             | 36                |
| S21A-Pak + S21 pep.                             | 3.9             | 49                |
| PKG + S21A-Pak untreated                        | 4.9             | 29                |
| PKG + S21A-Pak + cGMP                           | 1.8             | 45                |
| PKG + S21A-Pak + S21 pep.                       | 2.9             | 35                |

Samples were treated with 500 μM 8-pCPT-cGMP or S21 peptide (pep.), fixed, and stained with phalloidin. Fluorescence micrographs were taken of the cells, and the GFP-positive cells were scored for morphology. The percentage of cells that were polarized or had uropods (tails) after treatment is provided. Between 25–60 cells per treatment were analyzed over the course of four to eight individual experiments.

peptide is fused to the Nck binding site on Pak and inhibits Nck binding to Pak (18). We observed that the Tat-Pak fusion peptide stimulated the appearance of a mild uropod morphology in control, PKG-, WT Pak-, and WT Pak plus PKG-transfected cells (Fig. 5 and Table 2). However, in cells transfected with A21-Pak1 alone or A21-Pak1 plus PKG the Tat-Pak peptide caused the cells to adopt a more severe, long, rod-shaped uropod morphology similar to the effects of activated PKG in A21 cells (Fig. 5). Together these observations indicate that PKG acts at the level of Nck and Pak to regulate polarization and tail retraction.

DISCUSSION

PKG regulates endothelial cell motility, contractility, and permeability. Similarly PKG and its target, VASP, also play critical roles in endothelial migration. Yet previously no mechanism has been established as to how the two pathways interact with each other. Herein we demonstrate that in vitro and in cells the AGC kinase family member, PKG, phosphorylated Pak at serine 21 to disrupt binding to Nck and thereby stimulate cell polarization. We also show that PKG stimulated a previously undescribed association between Pak and the PKG substrate VASP, and this association inversely correlated with Nck/Pak binding in response to PKG activation (Fig. 3C). PKG activation stimulated polarization, whereas a Nck-Tat peptide or disruption of Pak phosphorylation by PKG activation (data not shown and Fig. 4), and VASP translocates from focal adhesions upon phosphorylation by PKG (31). And finally, over-expression of VASP in fibroblasts results in decreased cell motility, whereas cells lacking VASP are significantly more motile (32). Yet expression of a kinase-dead or activated form of Pak in combination with activated PKG did not lead to formation of uropods (data not shown). Consequently these data indicate that a major consequence of disruption of VASP/Pak association is improper motility and cytoskeletal regulation due to both improper Pak activity and localization, thereby disrupting the tightly regulated process of cell polarization.

Interestingly our observations correlate well with observations of a mutant form of PAKα in Dictyostelium. In Dictyostelium the Pak homologue, PAKα, regulates cell polarity and chemotaxis partly through control of myosin II assembly. In these cells, Akt phosphorylates PAKα in response to chemotactants (33). Furthermore expression of the analogous Akt negative mutant form of PAKα caused highly elongated cell posteriors, again suggesting that tail retraction was blocked.

Yet the molecular mechanisms by which Pak, VASP, PKG, and Nck regulate polarization and how S21A-Pak blocks this polarization and stimulates uropod formation are unclear. We suspect that the VASP/Pak interaction is mediated by VASP binding a Pak polyproline motif because Pak contains five polyproline domains, and VASP often associates with polyproline-rich domains through its EVH1 domain (34). This VASP/Pak association may also be part of a larger complex that includes Nck because in platelets Nck and VASP co-associate with the adaptor protein SLP-76 (35). This model would involve a complex of Pak, VASP, and Nck regulated by exogenous phosphorylation at serine 21 because, although activation of Pak by Rac is sufficient to stimulate VASP/Pak association and stimulate Pak serine 21 autophosphorylation, it is not sufficient to disrupt Nck/Pak binding (Fig. 3 and supplemental Fig. 1). Furthermore our data are consistent with this model because disruption of PKG phosphorylation of Pak by expression of S21A-Pak disrupted cytoskeletal regulation indicating misregulation of an effector downstream of both Pak and PKG. However, the possible common target downstream of both Pak and PKG is not known.

One potentially attractive target for Pak and PKG regulation of the cytoskeleton is myosin light chain phosphorylation. It has been proposed that the effects of Pak on cell contraction are caused by modulation of myosin light chain phosphorylation (36). Myosin light chain can be phosphorylated at serine 19 by myosin light chain (MLC) kinase, Rok, or Pak (7). Furthermore in smooth muscle, it has been observed that PKG reduces MLC phosphorylation through Rho and Rok inhibition. Yet the RhoA inactivation by PKG in smooth muscle cells is not sufficient to explain all of the PKG-dependent decrease in MLC phosphorylation, indicating there are additional PKG target(s) responsible for decreased MLC phosphorylation (37). Our data suggest that Pak may be that additional target and that uropod formation occurs due to improper MLC phosphorylation because of aberrant Pak activity and localization.

In summary, we have shown that PKG phosphorylated Pak in a cGMP-dependent manner in vitro and in transfected and untransfected endothelial cells. PKG phosphorylation of Pak decreased Nck/Pak binding and stimulated a novel VASP/Pak interaction. In HeLa cells and HUVECs, PKG activation stimulated cell polarization, and the
endothelial cell polarization stimulated by PKG activation could be blocked through expression of an S21A mutant Pak, and this effect could be mimicked by treatment with a Nck/Pak blocking peptide. We hypothesize that PKG controls cell morphology through Pak by regulation of VASP/Pak association, MLC phosphorylation, and polarization of focal adhesions.

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