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PKCδ Influences p190 Phosphorylation and Activity: Events Independent of PKCδ-Mediated Regulation of Endothelial Cell Stress Fiber and Focal Adhesion Formation and Barrier Function

Akua K. Fordjour and **Elizabeth O. Harrington**
Vascular Research Laboratory, Providence Veterans Affairs Medical Center, Department of Medicine, Alpert Medical School of Brown University, Providence, RI 02908

Abstract

Background—We have shown that protein kinase Cδ (PKCδ) inhibition results in increased endothelial cell (EC) permeability and decreased RhoA activity; which correlated with diminished stress fibers (SF) and focal adhesions (FA). We have also shown co-precipitation of p190RhoGAP (p190) with PKCδ. Here, we investigated if PKCδ regulates p190 and whether PKCδ-mediated changes in SF and FA or permeability were dependent upon p190.

Methods—Protein-protein interaction and activity analyses were performed using co-precipitation assays. Analysis of p190 phosphorylation was performed using *in vitro* kinase assays. SF and FA were analyzed by immunofluorescence analyses. EC monolayer permeability was measured using electrical cell impedance sensor (ECIS) technique.

Results—Inhibition of PKCδ increased p190 activity, while PKCδ overexpression diminished p190 activity. PKCδ bound to and phosphorylated both p190FF and p190GTPase domains. p190 protein overexpression diminished SF and FA formation and RhoA activity. Disruption of SF and FA or increased permeability induced upon PKCδ inhibition, were not attenuated in EC in which the p190 isoforms were suppressed individually or concurrently.

Conclusion and General Significance—Our findings suggest that while PKCδ can regulate p190 activity, possibly at the FF and/ or GTPase domains, the effect of PKCδ inhibition on SF and FA and barrier dysfunction occurs through a pathway independent of p190.

Introduction

The pulmonary vasculature is a crucial barrier regulating the flux of fluid and molecules between the blood vessels and the interstitium and alveolar space of the lung. Disruption of this fluid flux balance can lead to pulmonary edema formation and acute respiratory failure, as occurs in settings of acute lung injury (ALI). Endothelial barrier integrity occurs in part through the maintenance of cell-cell and cell-extracellular matrix protein complexes and associated actin microfilaments and microtubules. While much work has been done to identify signaling molecules important in agonist-induced endothelial permeability [1-3], the

Corresponding Author: Elizabeth O. Harrington, PhD Providence VA Medical Center 151, Research Services 830 Chalkstone Avenue Providence, RI 02908 Phone: 401−273−7100, ext 3651 Fax: 401−457−3305 E-mail: Elizabeth_Harrington@brown.edu.

mechanisms regulating endothelial barrier function under basal, unstimulated states are not as well understood.

Protein kinase C (PKC) is a family of serine/ threonine kinases important in signaling pathways affecting a multitude of cellular functions, including endothelial cell proliferation, adhesion, migration, and tube formation. In addition, PKC is important in regulating endothelial barrier function under basal, unstimulated conditions and in response to edematogenic agents [4-9]. We have shown pulmonary edema formation *in vivo* upon inhibition of PKCδ, but not in response to inhibition of other PKC isoforms [10]. Additionally, PKCδ overexpression enhanced endothelial barrier function through increased RhoA GTPase activation and focal adhesion formation [11]. Conversely, we have shown that chemical or molecular inhibition of PKCδ resulted in diminished RhoA GTPase activity, stress fiber and focal adhesion disruption, and endothelial barrier dysfunction *in vitro* [12]. The signaling mechanism by which PKCδ regulates endothelial basal barrier function and stress fiber and focal adhesion formation through RhoA GTPase is not known.

RhoA GTPase has been shown to play an intimate role in regulating endothelial monolayer permeability under basal, unstimulated states and in response to a number of agonists, including thrombin, histamine, TNFα, and activated neutrophils [13-18]. RhoA GTPase activation is a key mediator in actomyosin filament contraction and in the formation of focal adhesions in endothelial cells [15,19]. RhoA GTPase cycles between a GTP-bound, active state and a GDP-bound, inactive state. GTPase activating proteins (GAP) enhance the intrinsic Rho GTPase activity through direct binding, thus promoting the hydrolysis of GTP to GDP. Of the GAP proteins shown to modulate RhoA GTPase activity, p190RhoGAP is the best characterized. Two isoforms for p190RhoGAP have been identified, p190RhoGAP (referred to as p190) and p190-B RhoGAP (referred to as p190-B), which are ubiquitously expressed and the primary GAP proteins known to regulate RhoA GTPase activity [20-22] and promote stress fiber formation [20,22]. Additionally, RhoA inhibition through cadherin engagement to extracellular matrix protein was shown to occur through a p190-mediated pathway [23]. Interestingly, adhesion to extracellular matrix proteins promoted the recruitment of p190 to the focal adhesion protein complexes formed [24]. A recent study suggested focal adhesion kinase (FAK) regulates RhoA activity through phosphorylation and activation of p190, thus regulating endothelial barrier restoration following thrombin exposure [23]. Additionally, depletion of p190 protein attenuated the ability of angiopoietin-1 to protect against LPS-induced increase in endothelial monolayer permeability *in vitro* and lung edema formation [25]. Hence, there is evidence supporting a role for p190 in regulating endothelial barrier function.

In a previous study, we demonstrated co-precipitation of PKCδ with p190 and 120RasGAP, but not with other PKC isoforms (α, η, or ε) tested [12]. Thus, we hypothesized that PKCδ maintains endothelial barrier integrity through p190-mediated signaling pathway regulating stress fiber and focal adhesion formation and RhoA GTPase activation. We show herein that PKCδ activity inversely affected the activity of p190. In addition, PKCδ bound to and phosphorylated both the phenylalanine-rich (FF) and GTPase domains of p190. We further show that p190 overexpression in endothelial cells resulted in diminished stress fiber formation and focal adhesion formation and decreased RhoA activity; effects that were similar to those seen upon PKCδ chemical or molecular inhibition. Suppression of neither p190 nor p190-B protein protected against disruption of stress fibers and focal adhesions, or increased permeability induced by PKCδ inhibition. Our findings suggest that while p190 and PKCδ are both involved in signaling mechanisms which regulate the actin cytoskeleton and focal adhesion formation and barrier dysfunction in endothelial cells, the effects of PKCδ inhibition occurs through a signaling pathway which is independent of p190.

Materials and Methods

Cell lines and Reagents

Pulmonary artery endothelial cells (PAEC) were propagated in Eagle minimal essential medium (MEM; GIBCO, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA) and used between passages 4 and 8. Lung microvascular endothelial cells (LMVEC) were propagated in MCDB-131 media (Vec Technologies, Rensselaer, NY) and used between passages 4 and 10. Cultures of both endothelial cell types were purchased from Vec Technologies.

pGEX plasmid vectors expressing p190 constructs encompassing the FF domain (amino acids 266 through 544) (pGST-p190 FF) and GTPase domain (amino acids 1 through 266) (pGST-p190 GTPase) were gifts from Dr. J Settleman, Harvard Medical School, Charlestown, MA [26]. Constructs encoding GFP conjugated dominant-negative p190, pGFP-p190^{R1283A} (pGFP-p190RA), or wild-type p190, pGFP-p190, and pGST-Rho(Q63L) were gifts from Dr. K. Burridge, University of North Carolina, Chapel Hill, NC [27]. pGST-C21 construct was a generous gift from Dr. John G. Collard, The Netherlands Cancer Institute [28].

The Ad5.CMV GFP adenovirus (referred to as Ad GFP) was purchased from Q-BIOgene (Carlsbad, CA). Adenoviral particles expressing PKCδ wild-type protein (referred to as Ad wt PKCδ) was a gift from Dr. M. Ohba (Showa University) [29].

DMSO, rottlerin, diolein, myelin basic protein, and human recombinant PKCδ and vinculin-specific antibodies were purchased from Sigma Chemicals (St. Louis, MO). Glutathione Sepharose 4B and phosphatidylserine were purchased from GE Healthcare (Piscataway, NJ) and Avanti (Alabaster, AL), respectively. [γ-^{32} P]-ATP was purchased from PerkinElmer (Waltham, MA). Immunobilon membranes were obtained from Pierce Chemical (Rockford, IL). Antibodies directed against PKCδ and p190 and p190-B were purchased from BD Biosciences (San Jose, CA) and Epitomics (Burlingame, CA), respectively. RhoA and actin directed antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red and Alexa conjugated phalloidin were obtained from Molecular Probes (Eugene, OR).

p190 and p190-B siRNA targeted against rat p190 mRNA were modified from previously designed siRNA sequences targeting mouse p190 mRNA [30]. Customized p190 specific siRNA with a sense-strand sequence of $^{5'}$-CCC TGG AGG ACT GTG TGG AAT ATA AdTdT-$^{3'}$ and antisense strand sequence of $^{5'}$-TTA TAT TCC ACA CAG TCC TCC AGG GdTdT-$^{3'}$, p190-B specific siRNA with sense strand $^{5'}$-GTA CGT ATC CTC GTA AAT TdTdT-$^{3'}$ and antisense strand $^{5'}$-AAT TTA CGA GGA TAC GTA CdTdT-$^{3'}$, and control (non-silencing) siRNA with sense strand $^{5'}$-UUC UCC GAA CGU GUC ACG UdTdT-$^{3'}$ and antisense strand $^{5'}$-UUC UCC GAA CGU GUC ACG UdTdT-$^{3'}$ were purchased from Qiagen (Valencia, CA).

Recombinant Protein Purification

E. coli BL21 transformed with constructs encoding glutathione S-transferase (GST), GST-C21 (rhotekin), GST-Rho(Q63L), GST-p190 FF or GST-p190 GTPase were grown at 37°C in Luria broth medium until the culture density reached 0.7 OD_{600nm}. Recombinant protein expression was induced with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3h and bacteria were harvested by centrifugation at 4000 × g for 10min. Bacteria pellets were lysed by sonication in lysis buffer (0.1% aprotinin, 0.1% leupeptin, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.5% sarkosyl, and 1% Triton X-100). Recombinant proteins were purified using glutathione-conjugated Sepharose 4B beads. Proteins bound to the beads were resolved by SDS-PAGE and the gel was stained with Coomassie brilliant blue. The concentration of

recombinant proteins was determined via extrapolation using a standard curve containing various concentrations of bovine serum albumin.

Affinity Analyses

To determine if PKCδ interaction with the p190 domains, EC grown to confluence were washed in 1X PBS and lysed in FISH buffer (10% glycerol, 100mM NaCl, 5mM Tris.Cl, pH 7.4, 1% NP-40, 2mM $MgCl_2$, 10μg/ml aprotinin, 10μg/ml leupeptin, and 1mM PMSF supplemented with serine /threonine phosphatase inhibitors I and tyrosine phosphatase inhibitor II cocktail. Six hundred micrograms of cell lysates were incubated with 50μg glutathione-Sepharose beads conjugated GST, GST-p190 GTPase, or GST-p190 FF for 3h at 4°C. Protein conjugated glutathione beads were washed with lysis buffer and suspended in 1X Laemli buffer. Proteins were resolved via SDS-PAGE and transferred to membranes. Membranes were immunoblotted for indicated protein.

p190 activity was determined as previously described [31,32]. RhoA and p190 activities were determined in confluent EC washed with either cold phosphate-buffered saline or subjected to indicated treatments and washed with HBS wash buffer (20mM HEPES, pH 7.5, 150mM NaCl), respectively. Cell pellets were resuspended in FISH buffer (10% glycerol, 50mM Tris, pH 7.4, 100mM NaCl, 1% NP 40, and 2mM $MgCl_2$) for RhoA activity or lysis buffer (20mM HEPES, pH 7.5, 150mM NaCl, 5mM $MgCl_2$, 1mM dithiothreitol, 1% Triton X-100, 10μg/ml aprotinin, 10μg/ml leupeptin and 1mM PMSF) for the p190 activity assay. Six hundred micrograms of cell lysates were incubated with 80μg glutathione-Sepharose beads conjugated to GST-C21 or GST-RhoA(Q63L) for 1h at 4°C for the RhoA or p190 activity assays, respectively. Protein conjugated glutathione beads were washed with lysis buffer and suspended in 1X Laemli buffer. Proteins were resolved via SDS-PAGE and transferred to membranes. Membranes were immunoblotted for indicated protein.

In vitro Kinase Assay

The purified GST fused proteins-bound to glutathione beads (50μg) were washed twice with 500μl 25mM Tris-Cl, pH 7.4, 5mM $MgCl_2$, 0,.5mM EDTA and 1mM DTT. The washed beads were resuspended in 30μl PKC reaction buffer (25mM Tris-Cl, pH7.4, 5mM $MgCl_2$, 0,.5mM EDTA, 1mM DTT, 10μM ATP, 2μg/ml diolein, 20mg/ml phosphatidylserine, and 0.2mCi/ml [γ-^{32}P] ATP (3000 Ci/mmole)) with or without recombinant, enzymatically active PKCδ (20ng) for 30min at 30°C, shaking. As posititve and negative controls, recombinant PKCδ was incubated with 10μg myelin basic protein or no substrate, respectively. The reactions were terminated with 10μl 4X Laemli buffer. Proteins were subsequently resolved by SDS-PAGE and transferred to PVDF membranes. Phosphorylation of proteins was determined by autoradiography. Subsequently, the PVDF membranes were stained with Ponseau to visualize the GST and GST-fused proteins (data not shown).

Transfection and Adenoviral Infection

PAEC were transfected with GFP-conjugated wild-type p190, GFP-conjugated dominant negative p190, or GFP cDNA using Lipofectamine 2000 reagent, according to the manufacturer's protocol. Overexpression of transiently transfected cDNAs was determined by fluorescence microscopy.

For analysis of RhoA activity, GFP-positive cells were purified from untransfected cells using fluorescent activated cell sorter (FACs) analysis to obtain pure populations of endothelial cells overexpressing each construct. Briefly, PAEC were removed with trypsin from the cell culture plate and resuspended in DMEM media. Following centrifugation, the cells were resuspended at 5×10^7cells/ml in DMEM and placed on ice until sorting. Flow cytometry was performed

on a FACStar *plus* flow cytometer at an excitation of 488-nm wavelength and 630DF22 emission.

LMVEC were transfected with 240nM siRNA targeting rat p190 or p190-B mRNA or with equivalent amounts of control siRNA using Amaxa nucleofector solution (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's protocol. Depletion of the p190 proteins in endothelial cell lysates were confirmed by immunoblot analysis of the protein 72−96h after transfection. Protein suppression was shown to be maintained over this time period (data not shown).

Endothelial monolayers containing equivalent numbers of cells were infected with adenoviral particles at optimal particle units and experiments were performed 24−48h post-infection as previously described [11]. Transient overexpression of the protein of interest was confirmed in each experiment by immunofluorescence and immunoblot analysis.

Immunoblot Analysis

Equivalent amounts of cell lysates were resolved by SDS-PAGE and immunoblotted for indicated proteins as previously described [11].

Endothelial monolayer permeability assay

Equivalent numbers of endothelial cells were seeded on collagen-coated gold electrode (8W10E) culture plates and treated as described. Changes in electrical resistance were determined as previously described [10,12].

Immunofluorescence Analyses

Cells were grown to confluence on collagen-coated glass coverslips and treated as described. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were immunofluorescently stained for vinculin or stress fibers as previously described [10, 12]. Images were viewed at 1000x magnification using a Nikon Eclipse E400 fluorescence microscope interfaced with a SPOT Diagnostics Instruments digital camera.

The ImageJ “particle analysis” feature (National Institutes of Health (NIH)) was used for quantitation of the number of focal adhesions per cell. In each view, we determined the number of focal adhesions by measuring regions of PAEC immunofluorescently stained for vinculin containing structures for transfected cells. The total number of cells in each view was determined by counting the number of DAPI-stained nuclei. The number of focal adhesions per cell was calculated as the ratio of the total number of vinculin immunofluorescence stained structures relative to the total number of cells. Data is representative of three experiments. In each experiment, multiple (6−9) images were acquired for each sample.

Statistical Analyses

For three or more groups, differences among the means were tested for significance in all experiments, using ANOVA with Fisher's least significance difference test. For two groups, differences among the means were tested for significance using Students’ unpaired t-test. Significance was reached when $p<0.05$. All data are presented as mean ± standard error. n is indicated for each set of data.

Results

PKCδ influences the activity of p190RhoGAP (p190)

p190 activity has been shown to be influenced by protein-protein interactions and phosphorylation [33]. Because we had previously demonstrated co-immunoprecipitation of p190 with PKCδ [12], we assessed whether altered PKCδ activity would affect the activity of p190. Using primary cultures of microvascular endothelial cells, derived from rat lungs, we exposed confluent endothelial cells to the PKCδ chemical inhibitor, rottlerin, or vehicle for 30 minutes and assessed p190 activity using affinity precipitation assays of RhoA-bound p190. We noted a significantly increased level of active p190 in rottlerin exposed endothelial cells relative to endothelial cells exposed to vehicle (Figure 1a). Similarly, overexpression of cDNA encoding wild type PKCδ in endothelial cells resulted in a significant decrease in p190 activity (Figure 1b). Together, these results suggest that PKCδ may regulate the activity of p190.

PKCδ interacts with and phosphorylates both the GTPase and FF domains of p190

Because the phenylalanine-rich (FF) domains are important for protein-protein interactions [26,34] and the N-terminal GTPase domain influences the GAP activity of p190 [27], we next investigated whether PKCδ bound p190 at either domain. Using prokaryotic expression vectors, we bacterially expressed and purified recombinant proteins encoding either the p190 FF (amino acids 266 through 544) or p190 GTPase (amino acids 1 through 266) domain fused to glutathione-S-transferase (GST), referred to as GST-p190 FF or GST-p190 GTPase, respectively (Figures 2a and 2b). As a negative control for non-specific binding, we purified unconjugated GST protein. Lysates of microvascular endothelial cells isolated from rat lungs were incubated with the purified GST, GST-p190 FF or GST-p190 GTPase protein and precipitation of PKCδ was assessed by immunoblot analysis. We noted that PKCδ bound to both the GST-p190 FF and GST-p190 GTPase proteins (Figure 2c); protein interactions which were unaltered with increasing ionic concentrations (Figure 2d). Using *in vitro* kinase assays, we next assessed whether either p190 domain could serve as a substrate for PKCδ. Enzymatically active, recombinant PKCδ was incubated either with GST alone or recombinant GST fused p190 proteins. In parallel, the GST or recombinant GST fused p190 proteins were incubated in the PKC reaction buffer without PKCδ. As shown in Figure 2e, while no phosphorylation of the p190 FF or GTPase domains was noted in the absence of PKCδ, both domains of p190 were phosphorylated in settings in which PKCδ was present.

Endothelial cells overexpressing p190 display fewer stress fibers and focal adhesions and attenuated RhoA activity

Earlier studies have demonstrated that increased p190 expression or activity resulted in decreased fibroblast stress fiber formation; however, whether or not this effect by p190 is unique to fibroblasts is unknown [22,27]. Thus, we assessed the effects of altered p190 protein expression on stress fiber and focal adhesion formation in endothelial cells. Because primary cultures of microvascular endothelial cells isolated from rat lung were unable to overexpress the p190 cDNA sufficiently, we transiently transfected eukaryotic vectors encoding GFP, GFP-conjugated wild type p190, or GFP-conjugated dominant negative p190 ($p190^{R1283A}$) cDNA in primary cultures of endothelial cells derived from bovine pulmonary arteries. Following overexpression, the endothelial cells were immunofluorescently stained with markers for stress fibers or focal adhesions (Figure 3). In comparison to endothelial cells overexpressing GFP (Figures. 3a-3d), endothelial cells overexpressing wild type p190 demonstrated diminution in the amount of stress fibers and focal adhesions (Figures 3e-3h). Endothelial cells overexpressing dominant negative p190 demonstrated no significant changes in stress fiber or focal adhesion formation (Figures 3i-3l). The attenuation of focal adhesion formation in endothelial cells overexpressing wild type p190 was significant relative to overexpression of GFP or dominant negative p190 (Figure 3m). Since RhoA GTPase is a key regulator of the

actin stress fiber formation and focal adhesion structures [35], we next determined the effect of overexpression of wild type p190 on the activity of RhoA in these endothelial cells using affinity precipitation assays of active, GTP-bound RhoA. We noted a significant reduction in RhoA activity in endothelial cells overexpressing wild type p190, relative to endothelial cells overexpressing either dominant negative p190 or GFP alone (Figure 4a). Cell lysates run in parallel confirmed overexpression of GFP or GFP conjugated to dominant negative or wild type p190 (Figure 4b). Our data suggests that while increased p190 activity promotes inactivation of RhoA and disassembly of stress fibers and focal adhesion structures, decreased p190 activity does not significantly affect RhoA or its downstream mediated cytoskeletal structures.

Rottlerin-induced endothelial actin stress fiber and focal adhesion disruption or increased permeability is not attenuated by p190 isoform suppression

We have shown that PKCδ chemical or molecular inhibition leads to disruption of stress fibers and focal adhesion formation and increased permeability in endothelial cell monolayers; effects which were shown to correlate with diminished RhoA activity [12]. Thus, we investigated if PKCδ modulated endothelial cell stress fiber and focal adhesion formation and regulated endothelial monolayer permeability through a p190-dependent pathway. Primary cultures of microvascular endothelial cells isolated from rat lungs were transiently transfected with siRNA directed against either p190 or p190-B individually, or concurrently. As a negative control, endothelial cells were transfected with non-silencing siRNA in parallel. Protein suppression of the targeted p190 isoforms was confirmed by immunoblot analysis for all experiments (Figure 5a-5c). Transfected endothelial cells were then exposed to vehicle or rottlerin for 30 minutes and immunofluorescently stained with markers for stress fiber or focal adhesions. Similar to our previous studies, PKCδ inhibition with rottlerin promoted the disruption of stress fibers (Figures 5d-5g) and focal adhesions (Figures 6a-6d) in endothelial cells transfected with non-silencing siRNA. Interestingly, depletion of neither p190, p190-B (data not shown), nor both p190 and p190-B proteins abrogated stress fiber (Figures 5h-5k, 5l-5o) and focal adhesion (Figures 6e-6h, 6i-6l) disruption in rottlerin-exposed endothelial cells. In similar experiments, we assayed the effects of the p190 isoform protein suppression on barrier dysfunction induced upon PKCδ inhibition using the electrical cell impedance system. In comparison to vehicle exposed endothelial cells, rottlerin promoted a significant increase in monolayer permeability in endothelial cells transfected with non-silencing siRNA (Figures 7a-c), as we have previously shown [10]. Suppression of either p190 isoform individually (Figures 7a and 7b) or both p190 isoforms simultaneously (Figure 7c) had no significant effect on rottlerin-induced endothelial cell barrier dysfunction. Similarly, p190 protein depletion had no significant effect on thrombin-induced endothelial barrier dysfunction (data not shown). Thus, taken together, these results suggest that rottlerin-induced endothelial cell stress fiber and focal adhesion disruption or endothelial barrier dysfunction are not mediated through p190.

Discussion

In this study, we showed that the activity of PKCδ inversely affected p190 activity. Additionally, we demonstrated that the FF and GTPase domains of p190 interacted with and served as substrates for PKCδ. We further showed that overexpression of p190 resulted in diminished stress fibers, a significant decrease in focal adhesions, and decreased RhoA activity; effects that were similar to those seen upon PKCδ inhibition. We also demonstrated that disruption of endothelial cell stress fibers and focal adhesions and increased monolayer permeability induced by inhibition of PKCδ was not attenuated by p190 and/ or p190-B protein suppression. Thus, our study provides scientific support that p190 activity may be regulated by PKCδ, possibly through protein-protein interactions and/ or altered phosphorylation state. Our findings further suggest that while p190 is important to RhoA-mediated formation of actin

microfilaments and associated cell-extracellular matrix protein complexes, p190 is not involved in the signaling mechanism by which PKCδ inhibition promotes endothelial barrier dysfunction via stress fiber and focal adhesion disruption (Figure 8).

Previous studies have suggested that the activity of RhoGAP proteins is regulated by various mechanisms; including phosphorylation state, interactions with other proteins and phospholipids, and subcellular localization [33]. Phosphorylation of p190 has been demonstrated to occur at serine and threonine residues, as well as tyrosine residues by a multitude of kinases, including PKC, glycogen synthase kinase-3β (GSK-3β), Abl-related gene (Arg) tyrosine kinase, c-Src, Rho kinase (ROK), and focal adhesion kinase (FAK) [23, 36-41]; posttranslational modifications which have correlated with modulation of p190 activity, subcellular localization, and interaction with other proteins. For example, p190 tyrosine phosphorylation has been shown to be associated with growth factor induced p190 translocation to distinct actin cytoskeletal structures in c-Src overexpressing fibroblasts [42]. Also, FAK-mediated p190 tyrosine phosphorylation and activation, in response to thrombin, was shown to correlate with attenuated RhoA activation [23], while dephosphorylation via protein tyrosine phosphatases resulted in diminished p190 activity and enhanced RhoA activity [43,44]. Additional studies have shown that the phosphorylation state of p190 dictated its binding to protein partners, including Rnd, p120RasGAP, and p120 catenin, which in turn affected the activities of various monomeric GTPases and cell-cell and cell-extracellular matrix interactions [31,45-47]. More recently, several serine/ threonine residues within p190 have been identified which are phosphorylated by MAPK, GSK-3β, and ROK [40,41]. p190 phosphorylation by GSK-3β resulted in diminished activity with concomitant alteration in cell polarization during migration [40]. Similarly, ROK-mediated p190 phosphorylation at S^{1150} resulted in sustained RhoA GTPase activation in vascular smooth muscle cells [41].

While p190 phosphorylation by PKC has been shown in *in vitro* kinase assays using crude PKC extract and in mouse embryonic fibroblasts in response to the general PKC-activating phorbol esters [39], evidence is lacking as to whether PKC affected p190 activity *in vivo*, the sites at which p190 was phosphorylated, and which PKC isoforms were involved *in vitro*. In the current study, we demonstrate that alterations in PKCδ isoform activity translates to altered p190 activity. We further show that PKCδ was able to phosphorylate and bind distinct domains of p190. Thus, we speculate that p190 activity in endothelial cells is regulated by PKCδ primarily through direct phosphorylation. It is likely, however, that a combination of kinases and phosphatases create a phosphorylation profile of distinct tyrosine, threonine, and serine residues within p190, hence dictating the state of activation, protein or lipid binding, and subcellular location and the subsequent signal cascade output.

While much work has been done to elucidate the role of PKC in cell function, few studies have identified substrates for select isoforms. In addition to our previous work [12] and current study demonstrating PKCδ interactions with p190 in endothelial cell lysates, other PKC isoforms have been shown to be associated with p190. Similar to PKCδ, the PKCζ isoform was shown to co-immunoprecipitate with p190 in hippocampus lysates [48], however whether p190 can be phosphorylated by PKCζ is unknown. Additionally, PKCε was noted to colocalize with p190 in fibroblasts and neuronal cells, but the two proteins did not co-immunoprecipitate [49]. Another study suggested PKCα-mediated phosphorylation of p190 and subsequent membrane recruitment in a syndecan-dependent signaling pathway following integrin ligation of fibroblasts [50]. Our study is the first to demonstrate that p190 activity is influenced by the activity of select PKC isoforms, namely PKCδ. Since the endothelial cells used in this study lack PKCζ and interactions between PKCα or PKCε with p190 were not noted in endothelial cell lysates via co-precipitation [12], it is possible that δ is the primary PKC isoform affecting p190 activity in pulmonary endothelial cells. Future studies will assess the influence of each

PKC isoform expressed in pulmonary endothelial cells on p190 phosphorylation, activity, and subcellular localization.

The protein sequence of p190 consists of several conserved domains, including the commonly found RhoGAP and proline-rich, SH3 binding domains, as well as the unique GTPase and phenylalanine-rich (FF) domains. While the functionally active GTPase domain is typically found in heterotrimeric and monomeric GTPases, the GTPase domain within p190 lacks any intrinsic activity [51]. In addition, no other RhoGAP proteins include a GTPase domain, suggestive of a discrete function for this domain in p190 [52]. GTP binding to the p190 GTPase domain has been shown to promote conformational stability and enhancement of RhoGAP activity [27,53]. Furthermore, phosphorylation of tyrosine residues within the GTPase domain via c-Src have been shown to attenuate binding of this domain to the GTP nucleotide [53]. The conserved FF domains are encoded primarily in proteins which regulate transcription and RNA splicing, in addition to the p190 isoforms. Jiang and colleagues showed that p190 FF domain binding with the transcription factor TFII-I caused its sequestration within the cytosol [26]. Upon growth factor mediated tyrosine phosphorylation of the FF domain, TFII-I was released from p190, whereby it translocated to the nucleus and gene expression ensued [26] .

Little data is available regarding the effects of serine/ threonine phosphorylation of these two unique domains. However, the ExPASy server, NetPhosK 1.0 proteomic program (sponsored by the Center for Biological Sequence Analysis, Technical University of Denmark) identified multiple potential PKC-specific phosphorylation motifs localized throughout p190 and within both the GTPase and FF domains. While we showed PKCδ phosphorylated the p190 GTPase and FF domains *in vitro*, future experiments will assess if this occurs *in vivo* and monitor the functional effect of these posttranslational modifications on p190 activity and on endothelial cell function. In addition, we cannot discount the possibility that PKCδ interacts with and/ or phosphorylates other regions of p190 not tested, including the GAP domain.

Work from multiple laboratories has demonstrated a role for p190 in regulating actin stress fiber and focal adhesion formation through a RhoA GTPase-mediated process [20,22,42,54, 55]. Indeed, Ridley and colleagues demonstrated a selective diminution of stress fibers and RhoA GTPase activity in fibroblasts upon microinjection of p190, relative to other RhoGAP proteins assayed [22]. Similarly, inhibition of p190 by overexpression of the amino terminus region of p120RasGAP resulted in attenuation of focal adhesion formation and reduced fibroblast cell adhesion [56]. Data from other studies suggest that upon focal adhesion formation transient inhibition of RhoA GTPase occurs via p190, thus permitting cell adhesion and migration [54]. Others have shown that keratinocytes devoid of FAK protein were adherent, however deficient in cell motility and polarization. This was due in part to attenuated p190 activity and resulting increased RhoA GTPase activity and formation of cortical stress fiber and focal adhesions within the cell periphery [57], suggestive of dysregulation of cell adhesion. In support of these previous findings, we also noted that upon overexpression of wild type p190, fewer actomycin stress fibers and focal adhesions were noted in the endothelial cells; effects which were shown to correlate with attenuation in the level of RhoA activity.

Interestingly, we showed that inhibition of p190 via siRNA-mediated protein suppression did not significantly affect endothelial cell stress fiber or focal adhesion formation, or barrier function in lung endothelial cells following exposure to rottlerin. Additionally, in cells with p190 protein suppressed or dominant negative p190 overexpressed we did not observe changes in stress fibers and focal adhesions or RhoA activity, respectively. These observations are similar to those noted by others using immortalized fibroblasts derived from p190 null mice [39] and siRNA suppression of p190 in human umbilical vein endothelial cell [58], NIH3T3 cells [30] and mouse lungs [25] where, in the absence of RhoA activation, attenuation of activity or protein expression of p190 had no significant effects on the cytoskeleton structures and

RhoA activity. However, in settings of RhoA activation, p190 attenuated the RhoA-mediated pathways, including LPS-induced lung edema [25] and fibroblast integrin engagement on fibronectin extracellular matrix protein [54]. Thus, it is likely in our study that p190 inhibition is unable to rescue rottlerin-induced stress fiber and focal adhesion disruption or endothelial barrier dysfunction because molecular and chemical inhibition of PKCδ independently causes RhoA inactivation. Furthermore, while we conclude that PKCδ most likely modulates RhoA-mediated signaling pathways through a mechanism independent of p190, it is possible that differential phosphorylation and/ or activation of p190 promotes its intrinsic GAP activity towards Rac GTPase [59]. Indeed, genetic ablation of Rac GTPase was shown to produce fewer and smaller focal adhesion contacts and promote actin stress fiber disruption in fibroblasts, independent of altered RhoA activity [60]. Alternatively, it is possible that p190 activation upon PKCδ inhibition is affecting other small GTPases similarly involved in regulating stress fiber and focal adhesion formation, such as Rap1 [61] and ARF6 [62].

In summary, we present new data demonstrating the ability of PKCδ to inversely affect the activity of p190 in lung microvascular endothelial cells. Our findings suggest that PKCδ may modulate p190 activity through direct interaction and/ or phosphorylation at distinct serine or threonine residues within the GTPase and FF domains. We further show that stress fiber and focal adhesion disruption and barrier dysfunction upon inhibition of PKCδ, is not mediated through p190-mediated inactivation of RhoA GTPase.

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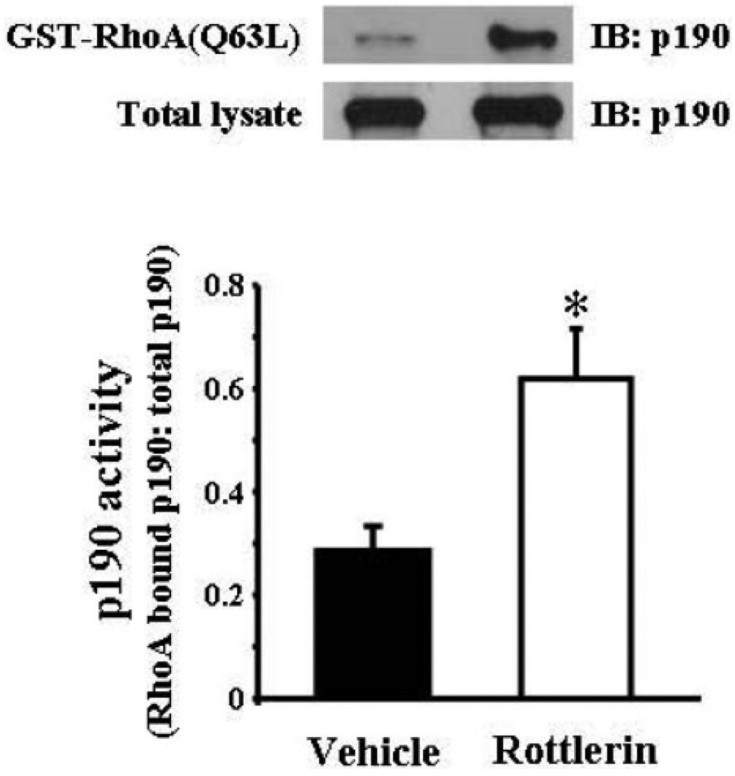

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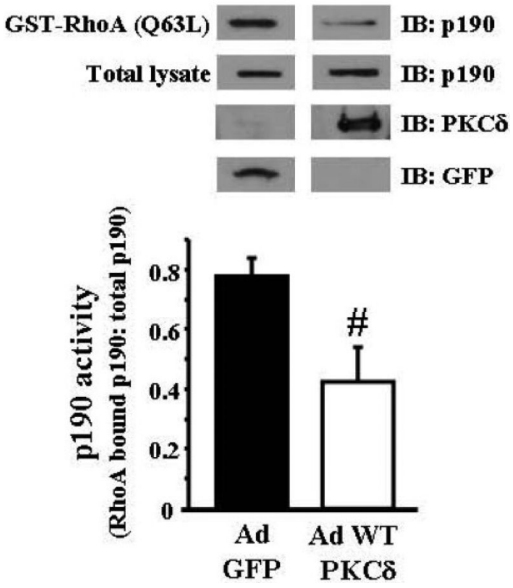
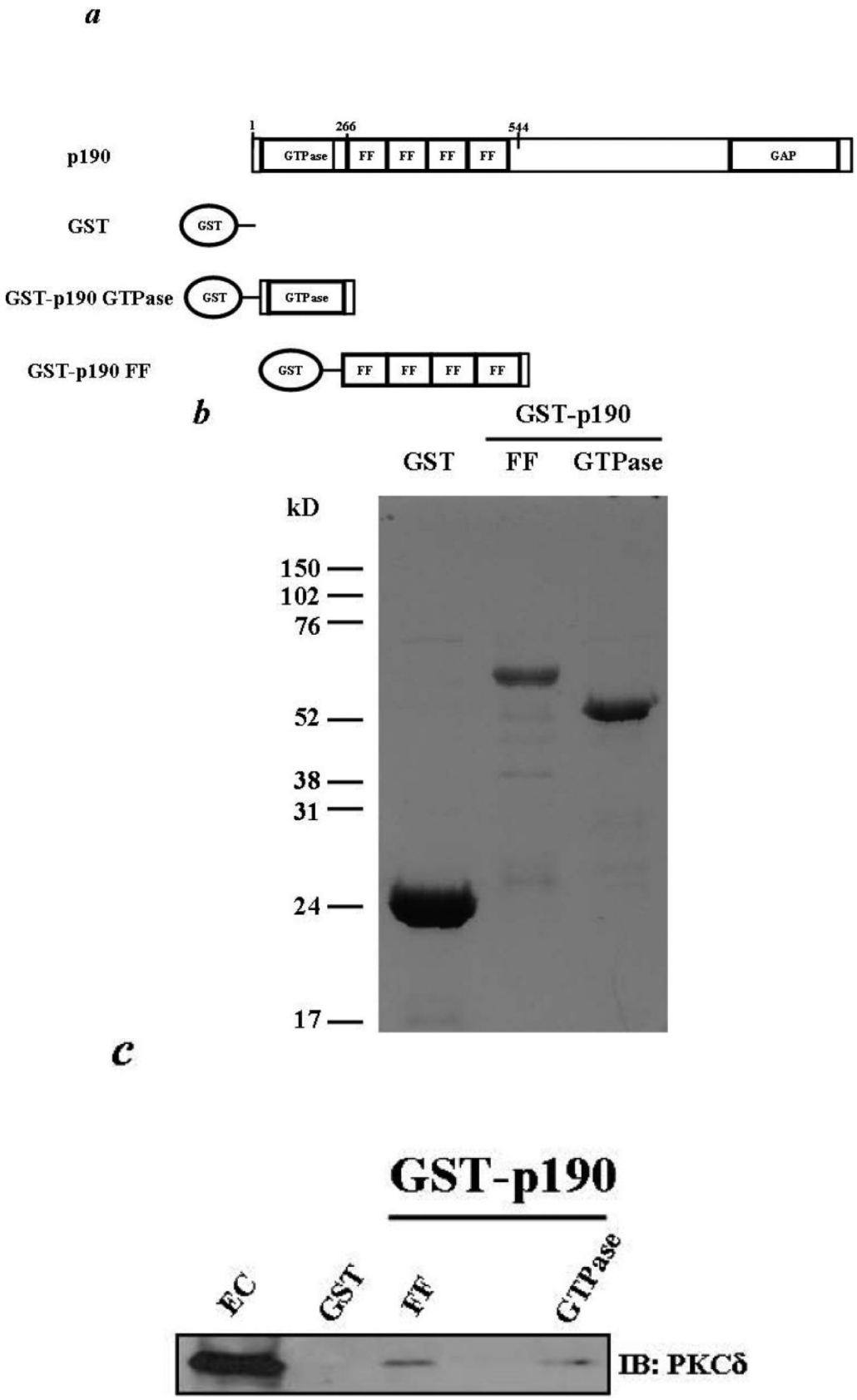


Figure 1. PKCδ activity inversely affects p190 activity

Confluent LMVEC were treated with vehicle (DMSO) or 10μM rottlerin for 30min (panel a) or infected with adenoviral vectors encoding GFP or wild-type PKCδ cDNA (panel b). Cells were harvested and p190 activity determined. The level of active p190 relative to total p190 was determined by densitometry. In panel b, GFP and PKCδ overexpression was confirmed in the transfected endothelial cells by immunoblot analysis. Data are presented as the mean ± SE. Panel a, n = 4; *$p<0.05$ vs. vehicle. Panel b, n = 7, $^{\#}p<0.05$ vs. GFP.



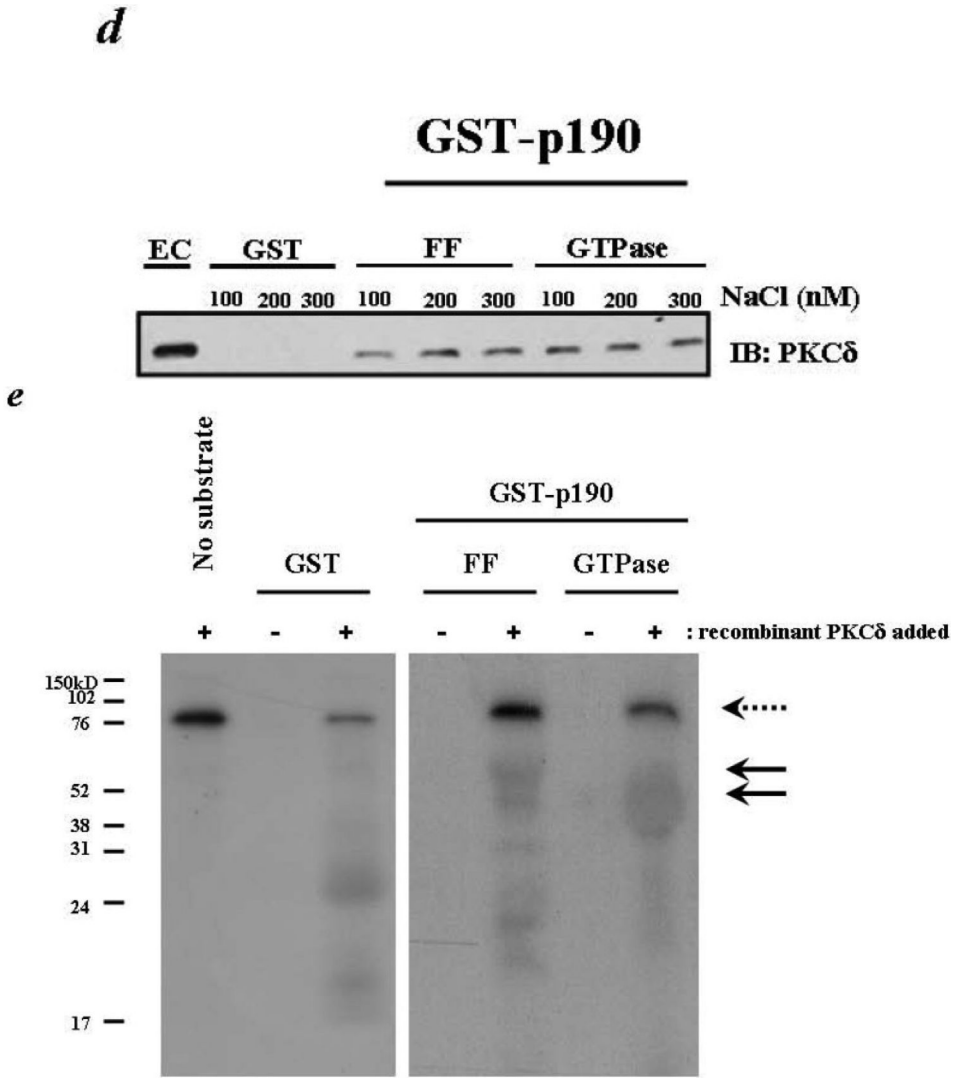
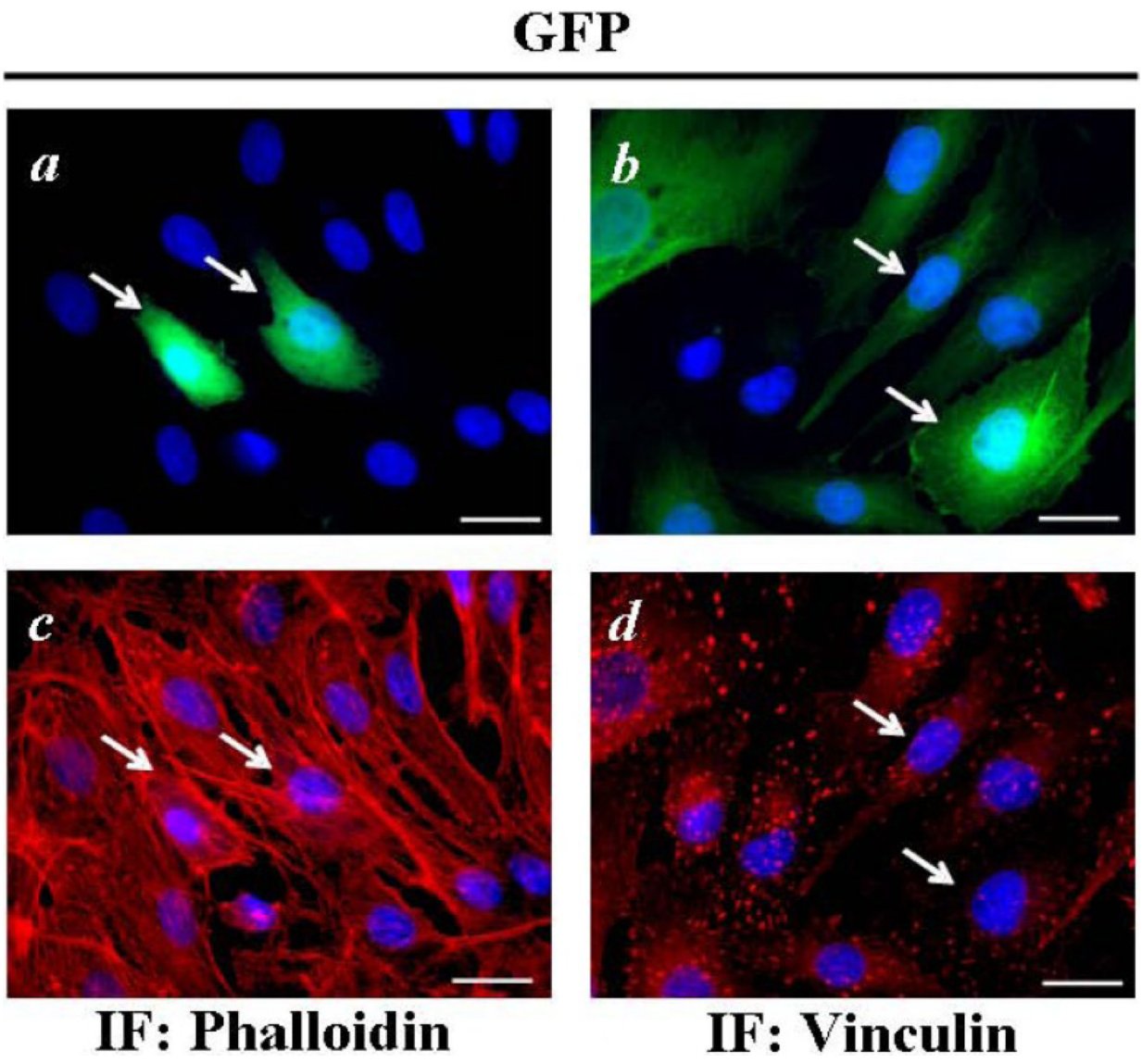
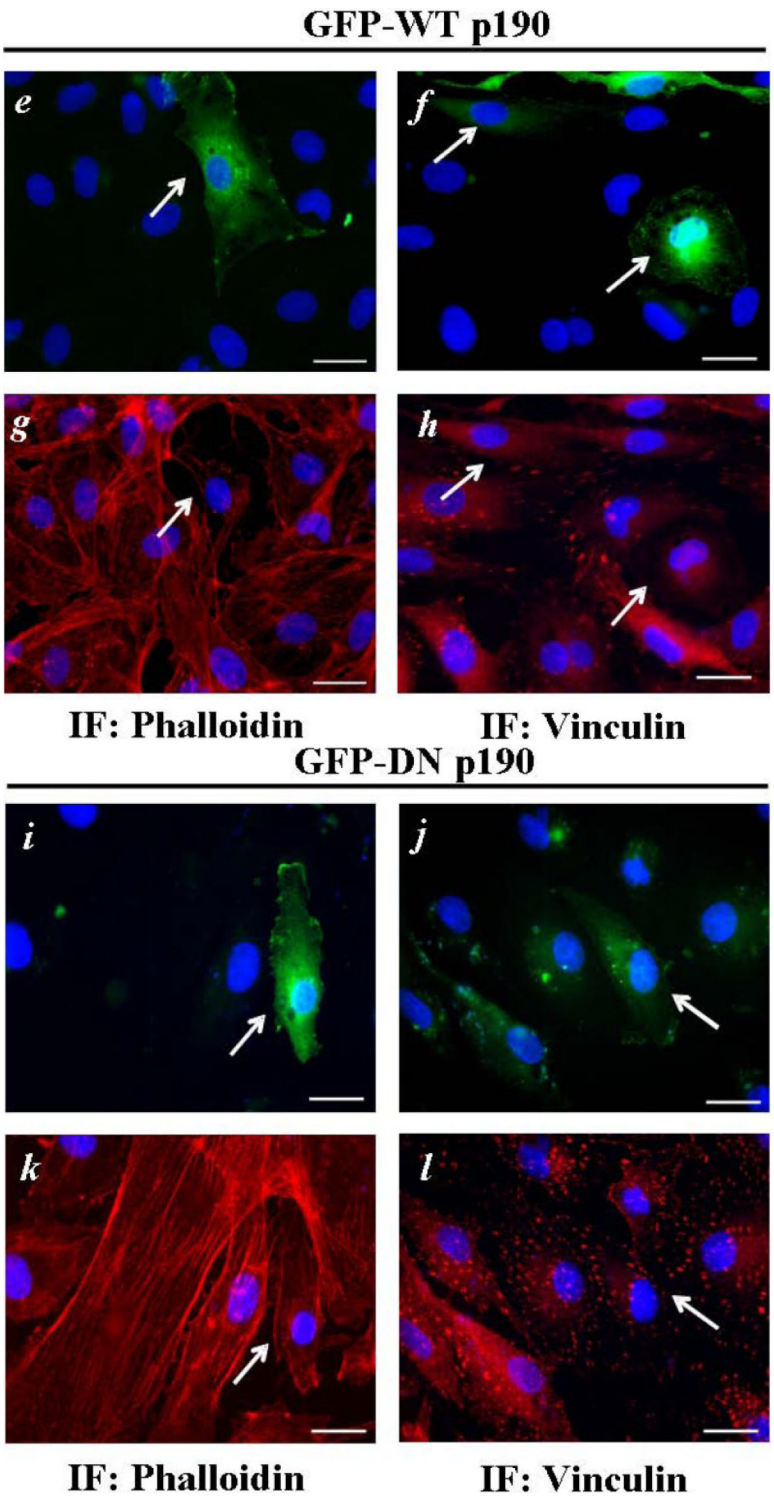


Figure 2. PKCδ interacts with and phosphorylates p190 FF and GTPase domains

Panel a, schematic representation of GST fused p190 FF and p190 GTPase domains. Panel b, bacterially expressed proteins encompassing the GST fused p190 FF (~60kDa) or p190 GTPase domains (~54kDa), or GST protein (~27kDa) were purified, resolved by SDS-PAGE and visualized via Coomassie blue staining. The purified GST-fused proteins were subsequently incubated with equivalent amounts LMVEC lysates (panel c) or equivalent amounts of LMVEC lysates containing increasing NaCl concentrations (panel d). Protein precipitates were resolved via SDS-PAGE and membranes were immunoblotted for PKCδ. In parallel, endothelial cell (EC) lysates were resolved as positive control for immunoblot analysis of PKCδ. Panel e, GST, GST p190 FF or GST p190 GTPase proteins were incubated with (+) or without (−) recombinant, enzymatically active PKCδ. In parallel, enzymatically active PKCδ was incubated in the absence of any exogenous substrate (No substrate). All reactions were incubated for 30min at 30°C. Proteins were resolved on SDS-PAGE and membranes autoradiographed. Solid arrows indicate phosphorylated p190 domains. Dashed arrow indicates PKCδ autophosphorylation. Representative gels or immunoblots are shown in panels b-d and representative autoradiograph is shown in panel e. n = 2−5.





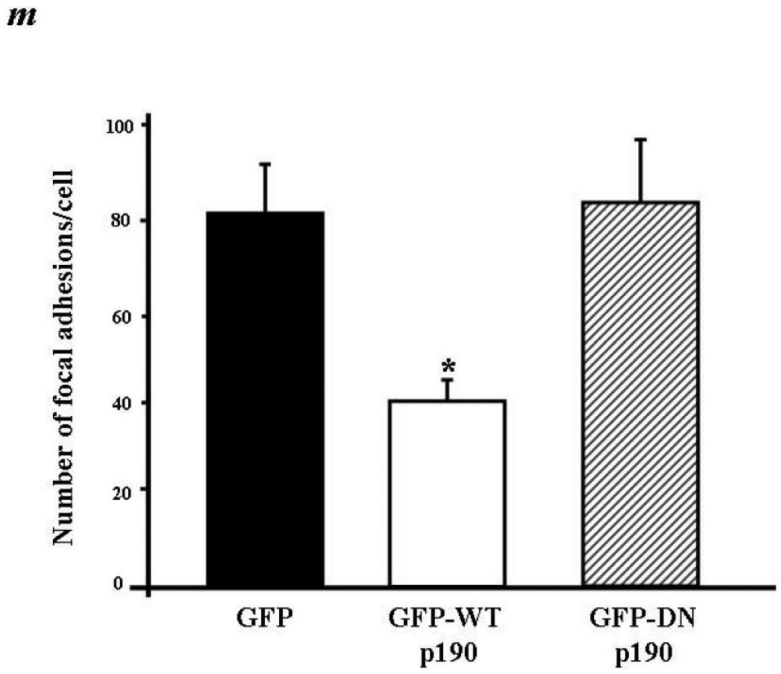


Figure 3. Overexpression of 190 results in diminished stress fiber and focal adhesion formation

PAEC were transfected cDNA encoding with GFP (panels a-d), GFP-conjugated wild type (wt) p190 (panels e-h), or GFP-conjugated dominant negative (dn) p190 (panels i-l). At 48h post-transfection, endothelial cells were fixed and immunofluorescently stained for vinculin using Texas Red-conjugated secondary antibodies directed against vinculin antibodies (panels d, h, l) or filamentous actin using Texas Red-conjugated phalloidin (panels c, g, k). Nuclei were counterstained using DAPI. Endothelial cells were visualized via fluorescence microscopy. Arrows indicate representative transfected endothelial cells. Images are representative of three independent experiments and 6−9 images were obtained for each experiment. Scale bars = 20μm. Panel m, number of focal adhesions per endothelial cell was quantitated as the number of immunofluorescently stained vinculin structures relative to total number of transfected endothelial cells for each construct. Data is presented as the mean ± SEM. n = 3; * $p<0.05$ vs. GFP or GFP-DN p190.

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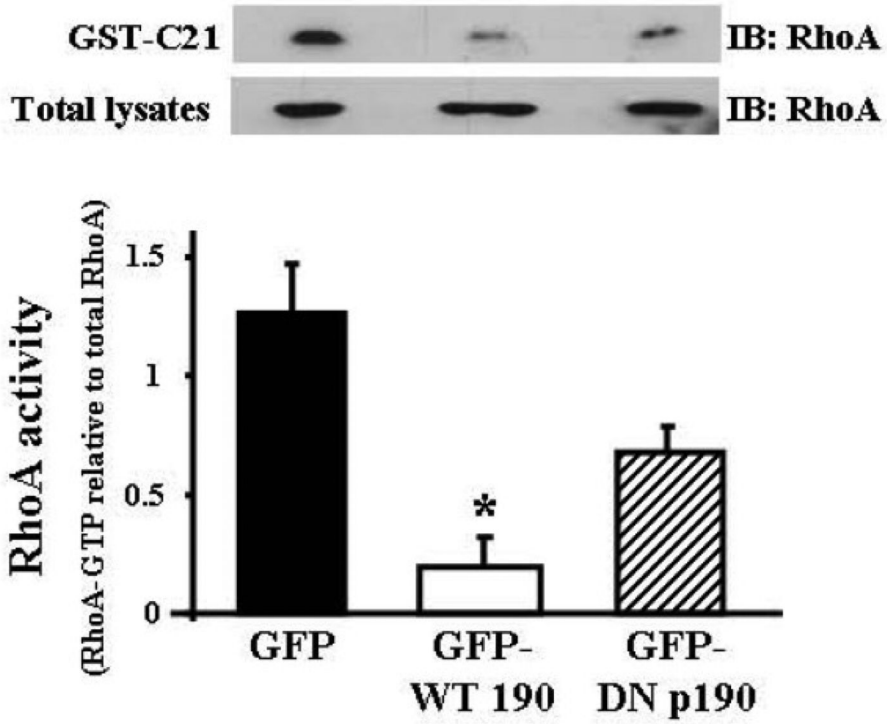

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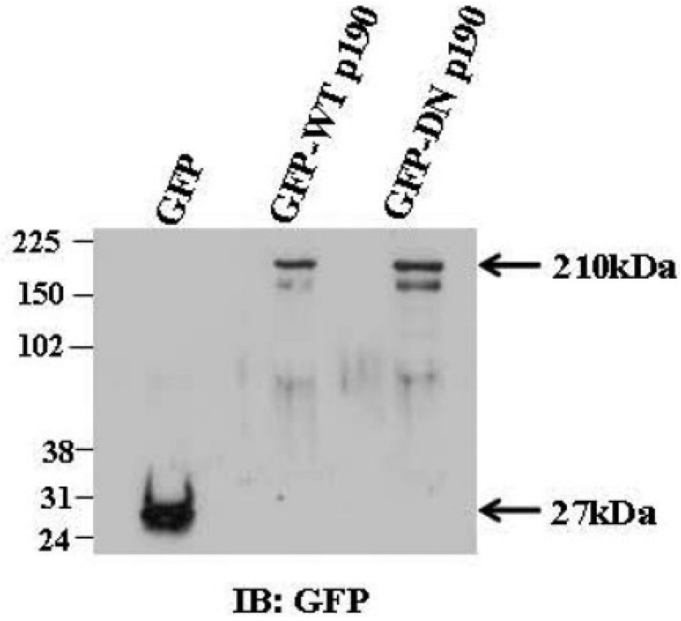

Figure 4. p190 overexpression inhibited RhoA activity

PAEC were transfected with cDNA encoding GFP, GFP-conjugated wild type (WT) p190, or GFP-conjugated dominant negative (DN) p190. At 48h posttransfection, GFP-positive cells were purified from untransfected endothelial cells using FACs analysis (panel a). Purified endothelial cells overexpressing the indicated construct were harvested and RhoA activity determined. Densitometric analysis was used to quantitate the level of active RhoA relative to total RhoA. Data is presented as mean ± SEM. n = 3; * $p < 0.05$ vs. GFP or GFP-DN p190. $p = 0.104$, GFP vs GFP-DN p190. Panel b, in parallel, lysates were resolved by SDS-PAGE, transferred to membrane, and immunoblotted for GFP to demonstrate protein overexpression.

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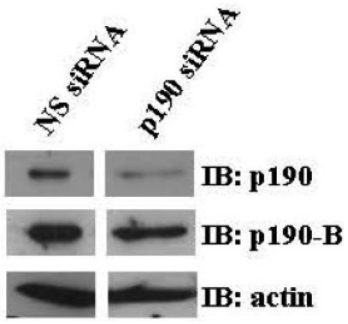

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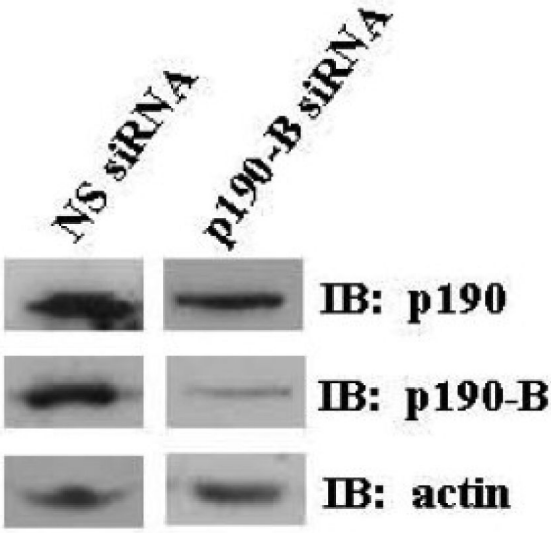

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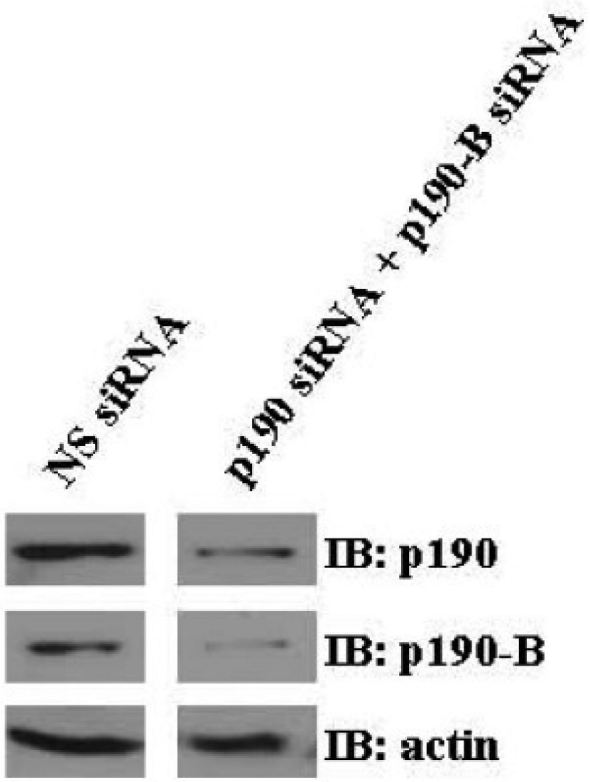
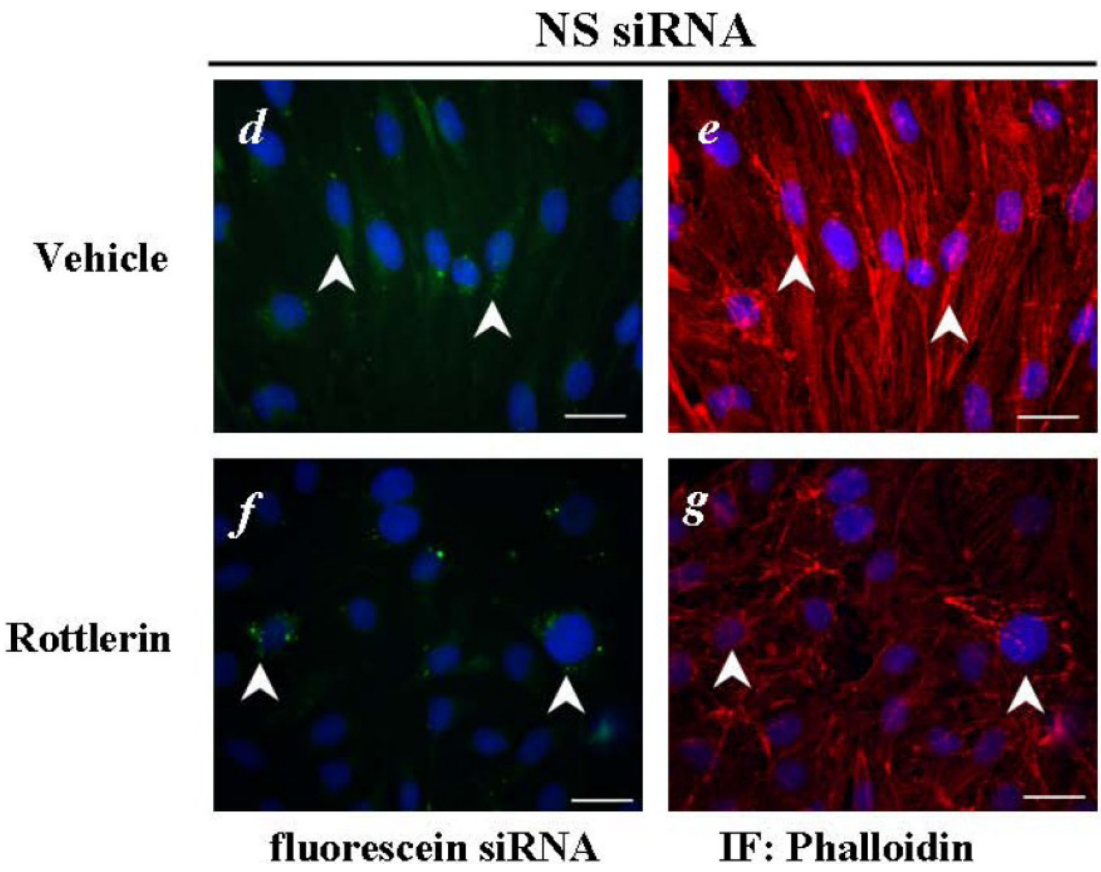
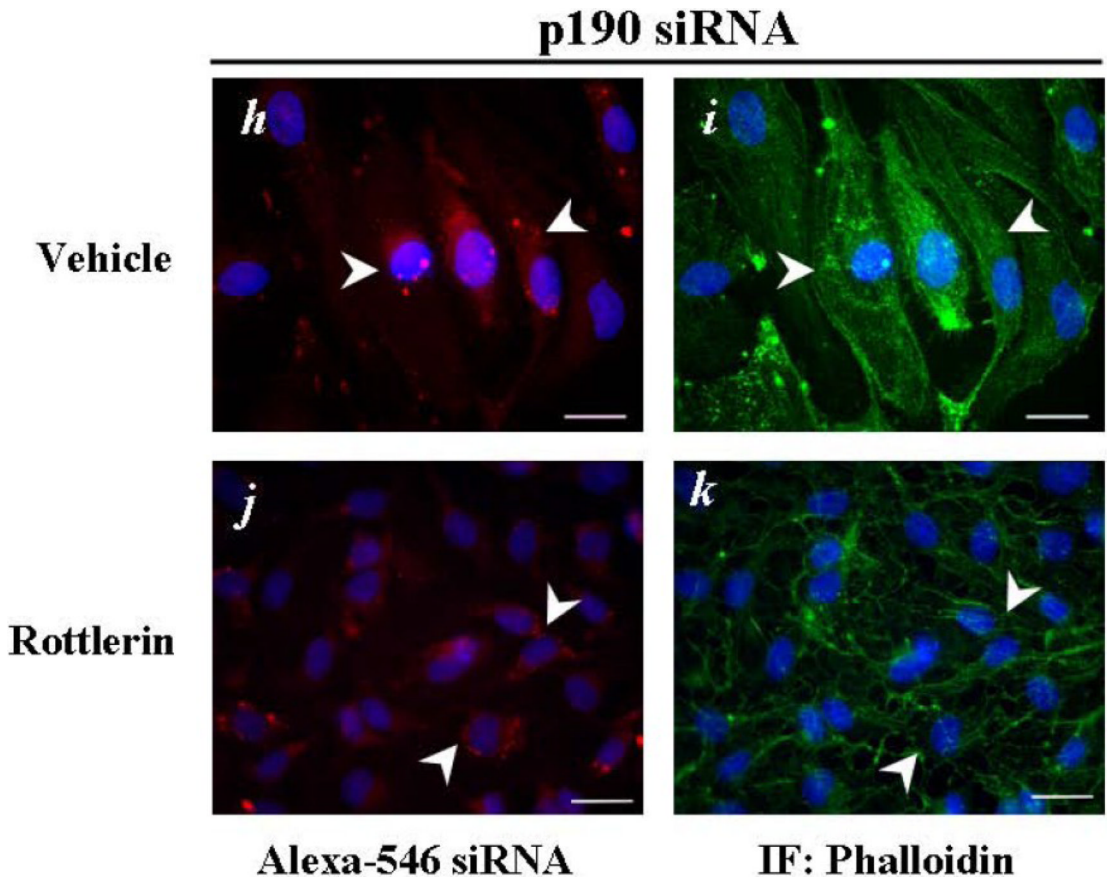






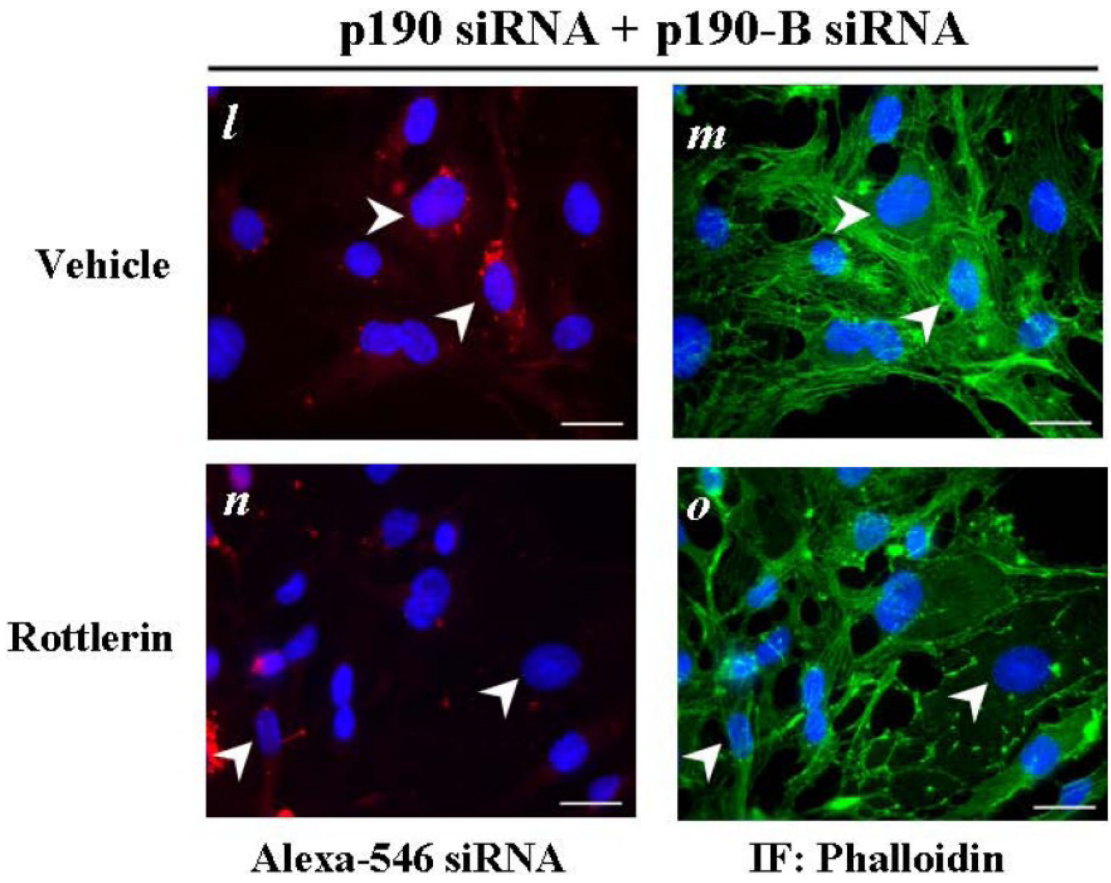
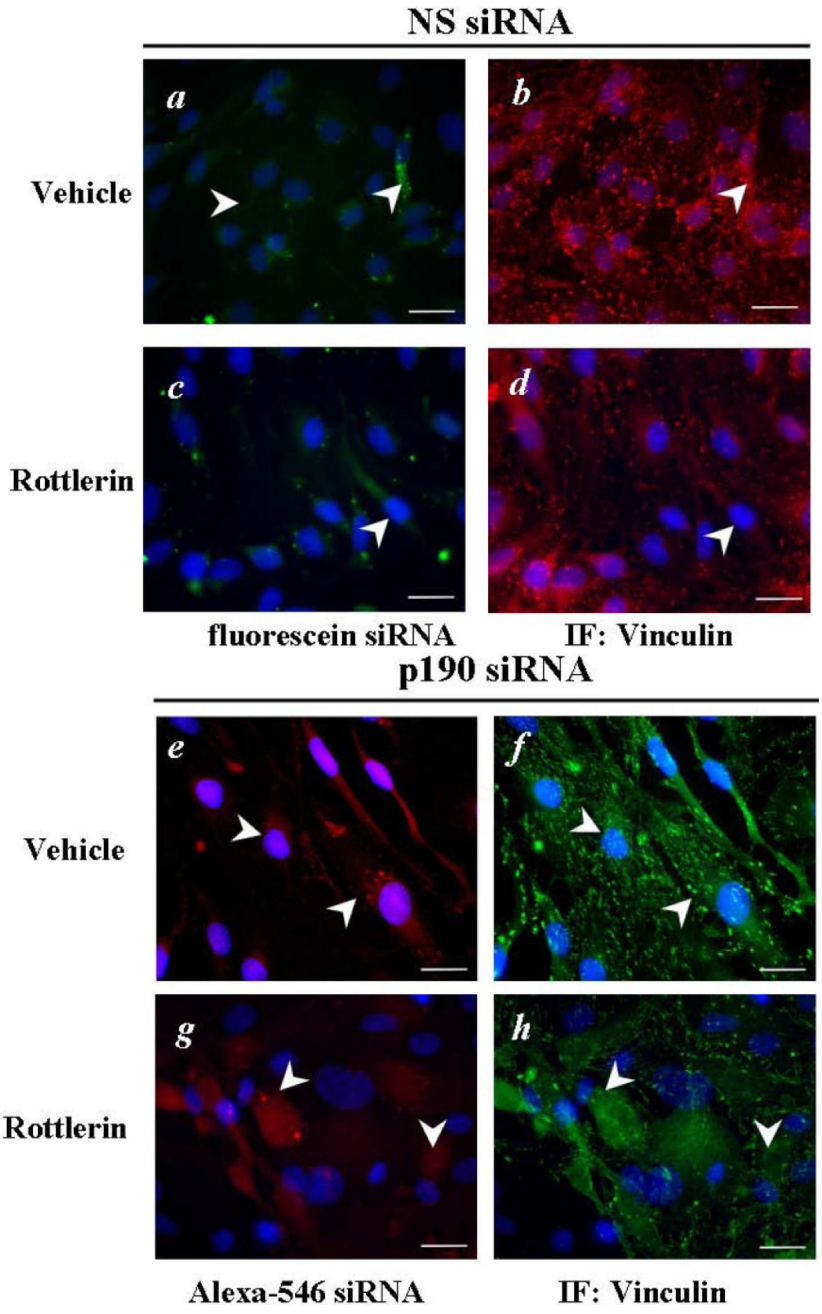


Figure 5. p190 protein suppression does not abrogate stress fiber disruption in PKCδ inhibited endothelial cells

Panels a-c, equivalent amounts (50μg) lysates harvested from LMVEC transfected with non-silencing siRNA or p190-directed siRNA were resolved by SDS-PAGE and immunoblotted for indicated protein. Membranes were stripped and reprobed for actin. Equivalent numbers of LMVEC were transfected with fluorescein conjugated non-silencing siRNA (panels d-g), Alexa-546 conjugated p190 siRNA (panels h-k), or Alexa-546 conjugated p190 siRNA plus p190-B siRNA (panels l-o) and plated onto collagen coated glass cover slips. At 96h posttransfection, the endothelial cells were exposed to vehicle or 5μM rottlerin for 30min. The endothelial cells were fixed and immunoflurorescently stained for filamentous actin using phalloidin (panels e, g, i, k, m, o). Nuclei were counter-stained with DAPI. Endothelial cells were visualized via fluorescence microscopy. Arrowheads indicate endothelial cells which took up fluorescently tagged siRNA. Experiments were performed in duplicate, and 4−5 images were obtained on each slide. Representative images are shown. Scale bars = 20μm. n = 3.



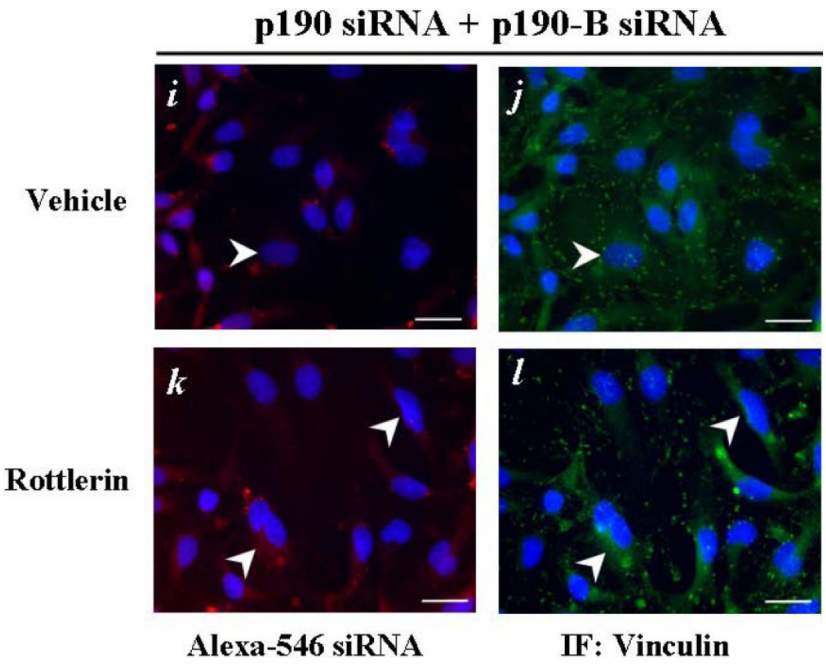
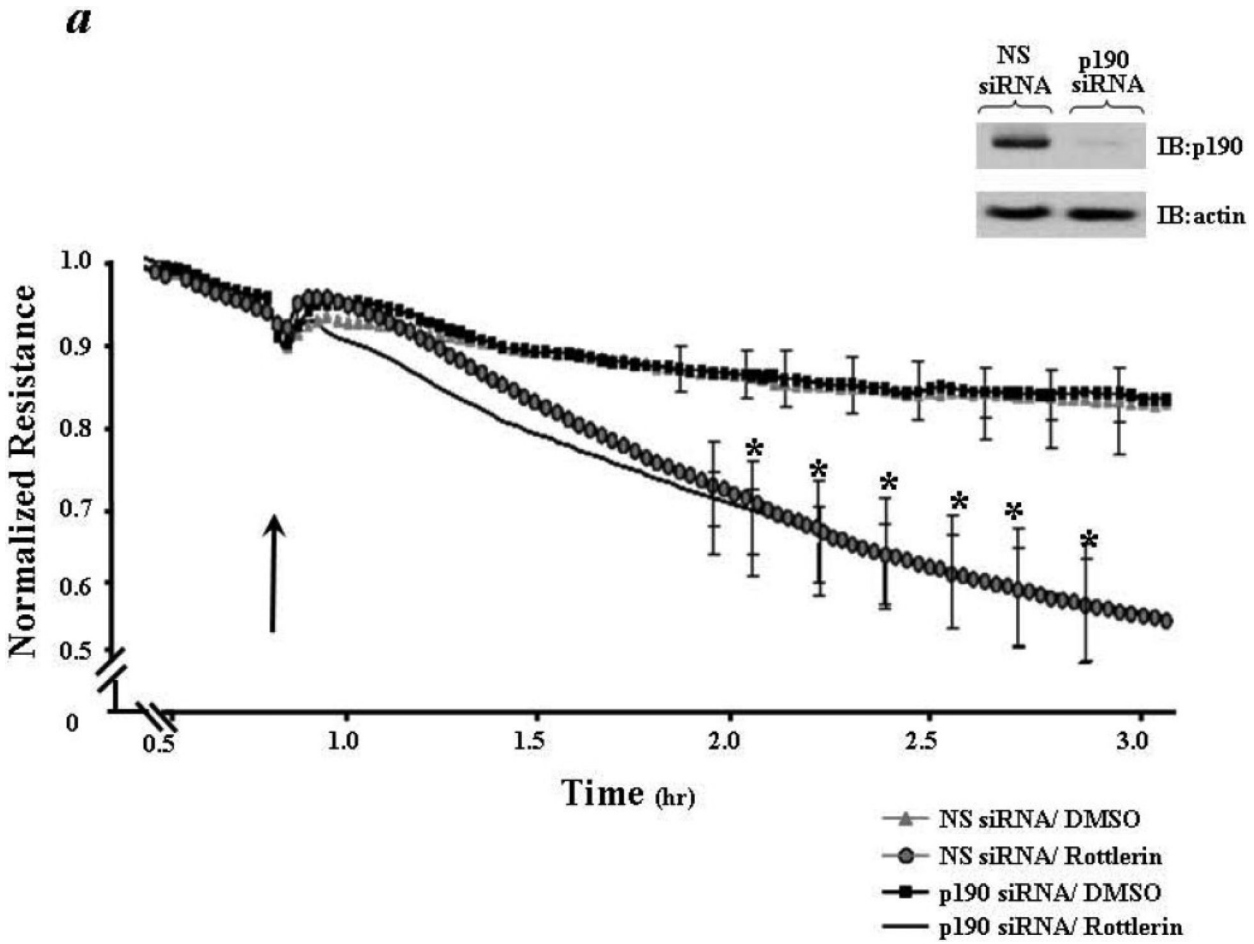
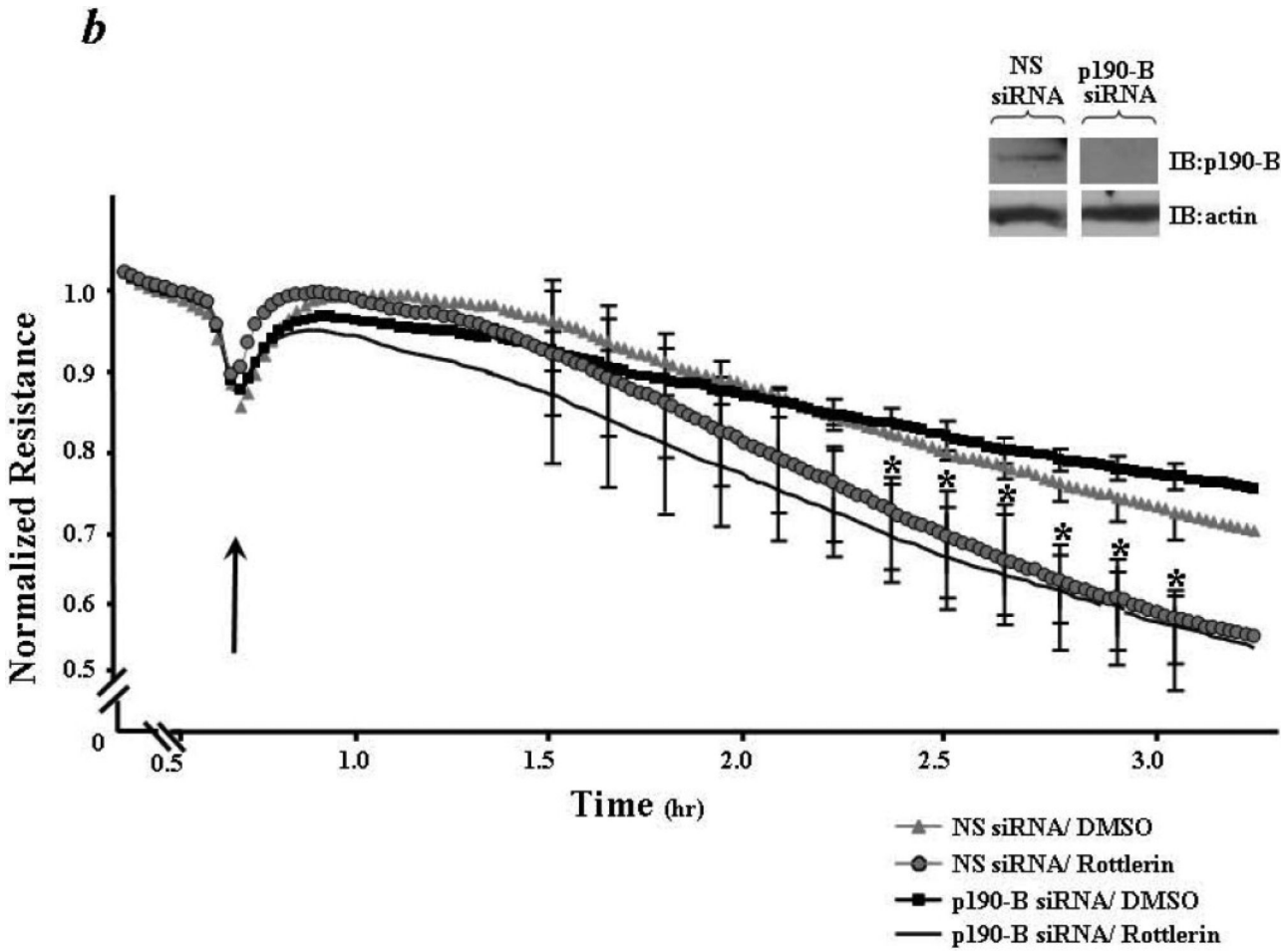


Figure 6. p190 suppression does not protect against focal adhesion disruption upon PKCδ inhibition

Equivalent numbers of LMVEC were transfected with fluorescein non-silencing siRNA (panels a-d), Alexa-546 conjugated p190 siRNA (panels e-h) or Alexa-546 conjugated p190 siRNA plus p190-B siRNA (panels i-l) and plated onto collagen coated glass cover slips. At 96h, endothelial cells were exposed to vehicle or 5μM rottlerin for 30min. Cells were fixed and immunofluorescently stained for vinculin (panels b, d, f, h, j, l). Nuclei were counter-stained with DAPI. Endothelial cells were visualized via fluorescence microscopy. Arrowheads indicate endothelial cells which took up fluorescently tagged siRNA. Experiments were performed in duplicate, and 4−5 images were obtained on each slide. Representative images are shown. Scale bars = 20 μm. n = 3.





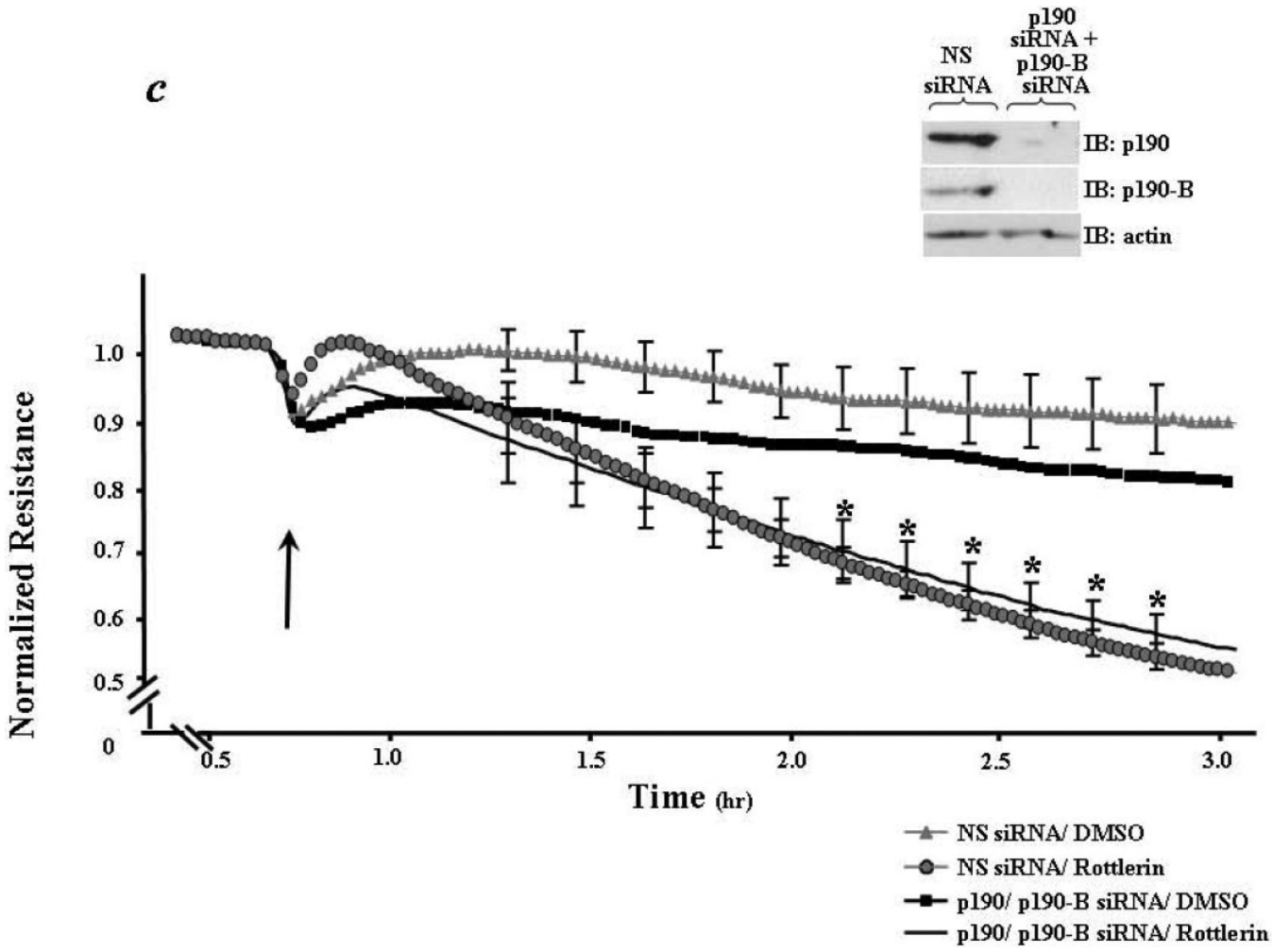


Figure 7. Suppression of p190 and p190-B individually or concurrently does not attenuate increased permeability upon PKCδ inhibition

Equivalent numbers of LMVEC were transfected with p190 siRNA (panel a), p190-B siRNA (panel b), both p190 and p190-B siRNA (panel c). In parallel, in each panel equivalent numbers of LMVEC were transfected with non-silencing (NΣ) σιPNA. Αλλ τρανσφεχτεδ ενδοτηελιαλ χελλσ ωερε πλατεδ οντο χολλαγεν χοατεδ ΕΧΙΣ αρραψσ. Ατ 96η ποσττρανσφεχτιον, ενδοτηελιαλ χελλσ ωερε τρεατεδ ωιτη ωεηιχλε (ΔΜΣΟ) ορ 5μM rottlerin and changes in resistance across endothelial monolayer were measured. Values are means ± SE of the normalized resistance. Arrow indicates time of addition of vehicle or rottlerin. n = 3−7; *p<0.05 vs. vehicle. Insert: endothelial cells were collected following each experiment and equivalent amounts of lysates were resolved via SDS-PAGE and immunoblotted for p190 to confirm protein suppression. Membranes were stripped and reprobed for actin to confirmed equal protein loading.

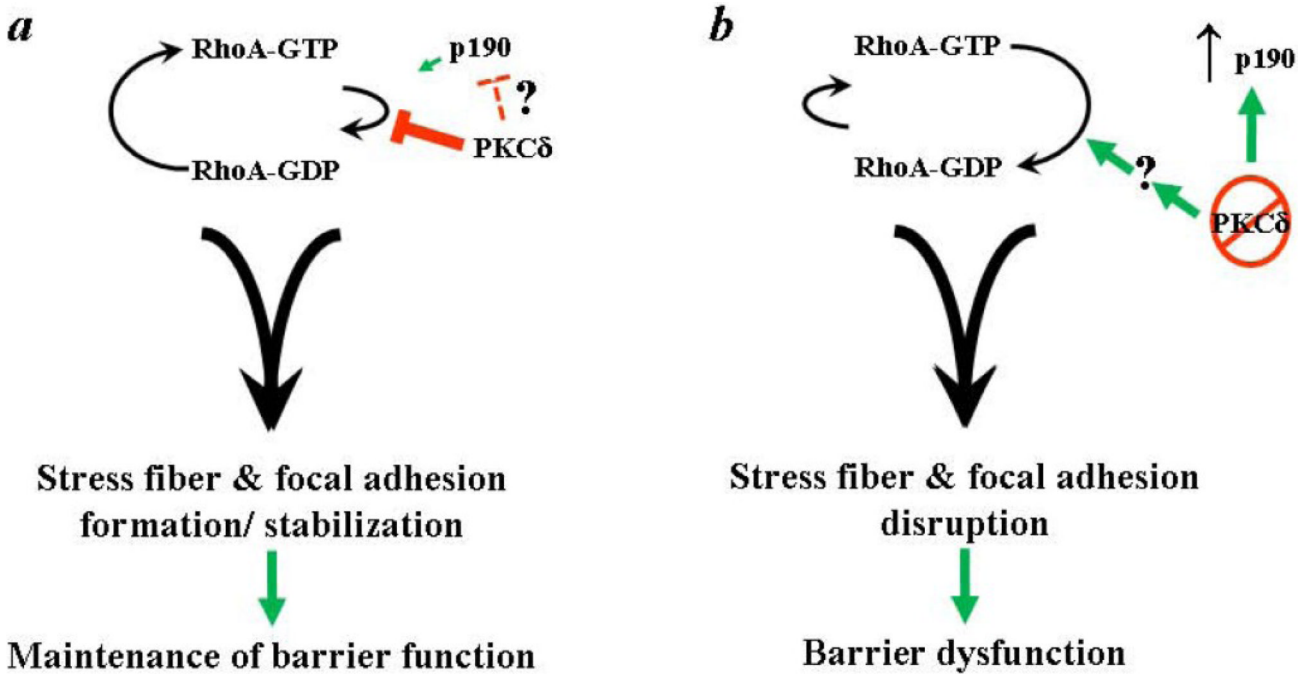


Figure 8. Schematic showing PKCδ- and p190-mediated regulation of endothelial stress fibers, focal adhesions, and RhoA activity

Panel a, under basal settings, PKCδ signals to maintain endothelial barrier function and stress fiber and focal adhesion formation by maintaining a level of active RhoA. Panel b, in settings of PKCδ inhibition, RhoA activity is diminished resulting in stress fiber and focal adhesion disruption and increase monolayer permeability, independent of the augmented p190 activity.