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RHOB LINKS PDGF SIGNALING TO CELL MIGRATION BY COORDINATING ACTIVATION AND LOCALIZATION OF CDC42 AND RAC

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Abstract

The small GTPase RhoB regulates endocytic trafficking of receptor tyrosine kinases (RTKs) and the non-receptor kinases Src and Akt. While receptor-mediated endocytosis is critical for signaling processes driving cell migration, mechanisms that coordinate endocytosis with the propagation of migratory signals remain relatively poorly understood. In this study, we show that RhoB is essential for activation and trafficking of the key migratory effectors Cdc42 and Rac in mediating the ability of platelet-derived growth factor (PDGF) to stimulate cell movement. Stimulation of the PDGF receptor- β on primary vascular smooth muscle cells (VSMCs) results in RhoB-dependent trafficking of endosome-bound Cdc42 from the perinuclear region to the cell periphery, where the RhoGEF Vav2 and Rac are also recruited to drive formation of circular dorsal and peripheral ruffles necessary for cell migration. Our findings identify a novel RhoB dependent endosomal trafficking pathway that integrates RTK endocytosis with Cdc42/Rac localization and cell movement.

Keywords

RhoB; Cdc42; Rac; Endosome; platelet-derived growth factor receptor; endocytic trafficking; vascular smooth muscle cells; cell migration

Cell migration is an important part of physiological and pathological processes that support embryonic morphogenesis, tissue repair/regeneration, immune surveillance, atherosclerosis, arthritis and cancer progression [Raftopoulou and Hall, 2004; Ridley et al., 2003]. Critical signaling events that promote cell migration are triggered by cell surface receptors resulting in fine alterations in the organization of the actin cytoskeleton. Among the many effector signaling molecules that alter actin organization, the Rho GTPases play pivotal roles in regulating cell migration. This class of molecules, which includes Cdc42, Rac and Rho, function as binary switches that trigger formation of different cytoskeletal actin structures required for migratory behaviors. Specifically, Cdc42, Rac and Rho promote the formation of filopodia, lamellipodia and stress fibers, respectively, different structures required to drive cell movement [Hall, 1998; Heasman and Ridley, 2008]. Cdc42 and Rac are particularly important at the leading edge of the cell to regulate localized actin

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polymerization and membrane protrusions. Actin remodeling at sites on the leading edge is thought to occur as a result of a redistribution of signaling molecules to spatially restricted areas in response to extracellular cues. How this redistribution occurs is incompletely understood, but receptor-mediated endosome formation and trafficking have been implicated in the process [Maxfield and McGraw, 2004; Polo and Di Fiore, 2006].

Endocytosis of receptor tyrosine kinases (RTKs) such as the PDGFR not only promotes cell proliferation but also actin remodeling and cell migration. PDGFR promotes formation of migratory cellular protrusions, such as peripheral ruffles and circular dorsal ruffles by stimulating rearrangement of actin filaments [Andrae et al., 2008; Buccione et al., 2004]. As the major driving force in migration, the extension of leading edge lamellipodia formed by Rac activation serve as pliable and dynamic structures. As another necessary part of the actin dynamic at the leading edge, dorsal circular ruffles function as important sites in directing spatially restricted actin remodeling adjacent to lamellipodia extension [Buccione et al., 2004]. How receptor-mediated endocytosis regulates these processes remains elusive. It has been shown that the regulators of endocytosis, such as dynamin and Rab5, are required for actin dynamics leading to the formation of circular ruffles in response to RTK stimulation [Lanzetti et al., 2004; Orth and McNiven, 2003]. One recent study has suggested that after stimulation, Rab5-mediated endocytic trafficking of Rac is important for the spatial restriction of signaling in cell migration [Palamidessi et al., 2008]. These studies support the concept that during receptor-mediated endocytosis the endosomal system serves as an important assembly site for the formation of signaling complexes that direct migration.

RhoB, a member of the Rho small GTPase family, has selective functions in endosomal trafficking. RhoB localizes to both the plasma membrane and the membrane of early and late endosomes [Adamson et al., 1992; Fernandez-Borja et al., 2005; Mellor et al., 1998; Rondanino et al., 2007]. Studies demonstrating the role of RhoB in the endocytic pathway show that, in different settings, RhoB facilitates the trafficking of signaling molecules including RTKs, Akt and Src to the cell surface, the nucleus, or the lysosome [Adini et al., 2003; Gampel et al., 1999; Neel et al., 2007; Sandilands et al., 2004; Wherlock et al., 2004]. In recent work, we reported that RhoB is required for PDGF-stimulated proliferation of primary vascular smooth muscle cells (VSMCs) by promoting the endosomal trafficking of active Akt, ERK and Src into the nucleus [Huang et al., 2007]. RhoB loss did not alter PDGFR protein levels, but did affect the phosphorylation and trafficking of this receptor. While the important role of PDGF signaling in migration of smooth muscle cells has been well documented, the mechanism has been obscure. Here, we demonstrate that RhoB exerts an essential function in PDGF-induced formation of the dorsal and peripheral ruffles and plays a role in the localization as well as activation of Cdc42/Rac.

MATERIALS AND METHODS

Animal and Culture of VSMCs and macrophages

The generation and characterization of RhoB heterozygous (+/-) and RhoB nullizygous (-/-) mice, which are fertile and lack apparent developmental defects, have been described previously [Liu et al., 2001]. Animal work was performed with Institutional Animal Use and Care Committee approval. The isolated aortas from RhoB +/- and -/- mice served as a source for preparing of VSMCs as previously described [Huang et al., 2007]. VSMCs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified 5% CO₂ atmosphere. For all experiments, cells of only 3-5 passages were used. Peritoneal macrophages were harvested from the peritoneal cavity of RhoB +/- and -/- mice and cultured in DMEM supplemented with 10% FBS by standard methods.

Cell migration assay

The migration assay was performed in transwell plates of 6.5-mm diameter with polycarbonate membrane filters containing 8 μm or 5 μm pores (Corning, Corning, NY). Cells were trypsinized and resuspended in serum-free medium (SFM). 600 μl SFM containing PDGF-BB (10 ng/ml) (Cell Signaling Technology, Beverly MA) was added to the lower wells of the chambers and aliquots of 3×10^4 VSMCs or 4×10^5 macrophages in 100 μl SFM were seeded into the upper wells of the cell inserts. After 16 to 20 hours of incubation at 37°C for VSMC, and 3 hours for macrophages, the inserts were washed with PBS, and the nonmigrating cells were removed from the upper surface of the membrane using a cotton swab. The cells on the lower surface of the membrane were fixed with ice-cold methanol for 10 minutes, rinsed with PBS, and then stained with crystal violet (0.5% crystal violet and 20% methanol). The number of migrated cells from four randomly chosen high-power fields (200 magnification) was counted in each well by microscopy. The migration assay was performed in at least three independent experiments.

Western blot analysis

Cells were harvested by scraping and washing twice in PBS, then lysed in RIPA buffer containing protease and phosphatase inhibitors. Equal protein for each sample (typically 30 $\mu\text{g}/\text{lane}$) was separated by SDS-PAGE and blotted to Immobilon-P transfer membrane (Millipore, Billerica MA). Blots were incubated with primary antibodies as recommended by the vendor and were detected with HRP conjugated secondary antibodies using the ECL reagents from PIERCE according to the manufacturer's instructions. Primary antibodies to the following antigens were used: RhoB (Bethyl Laboratories, Montgomery TX); PDGFR- β , actin, Cdc42, Vav2, phospho-y172-Vav2 and Tiam1 (Santa Cruz Biotechnology, Santa Cruz CA); phospho-y751-PDGFR β , Cdc42 and RhoA (Cell Signaling Technology, Beverly MA); β PIX (BD Biosciences, San Diego, CA); Cortactin and Rac (Upstate, Temecula, CA).

PAK1-PBD pull down assay

Cdc42/Rac activation assays were performed using a Rac /cdc42 assay kit (Millipore, Temecula, CA) according to the manufacture's protocol. Briefly, cell lysates were precipitated with a GST fusion-protein corresponding to the p21-binding domain (PBD, residues 67-150) of human PAK1 bound to glutathione-agarose, run on SDS-PAGE, and Western blotted using Cdc42 or Rac antibodies noted above.

Immunofluorescence studies

Cells plated onto coverslips were fixed in 4% paraformaldehyde, washed with PBS, and permeabilized with PBS/0.1% Triton X-100. After blocking with PBS plus 10% goat or donkey serum, cells were incubated with primary antibodies overnight. In addition to the antibodies noted above, anti-LAMP-1 (BD Transduction Laboratories, San Jose, CA) was used to detect late endosome/lysosomes and anti-EEA-1 (Santa Cruz Biotechnology, Santa Cruz CA; or Upstate Biotechnology, Lake Placid, NY) to detect early endosomes. Nonconjugated antibody was detected by 1 hr incubation with species-specific FITC, Cy3 or Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove PA). To visualize nuclei, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) in the mounting medium, before examination by fluorescence microscopy. Fluorescent signal was analyzed using a Zeiss Axiovert 220 microscope powered by Axiovision 4.0 software with multi-channel/Z-stack acquisition and 3D-deconvolution modules.

Transient transfection

The vascular smooth muscle cells isolated from RhoB null mice were seeded the day before transfection in 100-mm plates until reaching 90% confluence. Expression constructs for

wild-type RhoB (RhoB-WT), was generated as described previously [Lebowitz et al., 1995]. The RhoB containing vector or an empty vector control were introduced into the cells using lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. The cells were used in biological assays 48 h after transfection.

Statistical Analysis

Data were analyzed by unpaired Student's t-test and expressed as means $\pm$ standard deviation. The differences were considered to be statistically significant at p-value of < 0.05 .

RESULTS

RhoB is required for migration of VSMCs stimulated by PDGF

Using a RhoB knockout mouse model we previously showed that RhoB plays a critical role in PDGF induced VSMC proliferation [Huang et al., 2007]. It is well accepted that PDGF is an important regulator of both cell proliferation and migration in VSMCs. PDGF stimulation results in a robust motility response in culture cells [Heldin et al., 1998]. Therefore, we investigated whether RhoB was also necessary for PDGF-induced cell migration using a modified Boyden chamber assay. Cells seeded into this system were stimulated with PDGF-BB and cells migrating through the chamber were stained with crystal violet and visualized by microscopy. While quiescent (serum-deprived) RhoB $+/-$ and $-/-$ VSMCs did not migrate into the lower chamber, loss of RhoB dramatically reduced the migratory potential of VSMCs in response to PDGF-BB (10 ng/ml) stimulation (Fig. 1A, B). Similar results were obtained in cell migration assays using Boyden chambers that employed collagen-coated membranes (data not shown). Furthermore, transient expression of RhoB in RhoB null cells at 10% of endogenous wildtype levels, resulted in a significantly increased proportion of cells exhibiting migratory activity in response to PDGF-BB stimulation as compared to untransfected or RhoB null cells transfected with the control vector (Fig. 1C, D and E). To confirm the role of RhoB in migration of another cell type, we utilized primary cultured macrophages derived from RhoB $+/-$ and $-/-$ mice. Results from the Boyden chamber migration assay confirmed that the loss of RhoB also inhibited the migratory activity of macrophages in response to PDGF stimulation (Fig. S1A). These results suggest that RhoB is an important mediator for PDGF-stimulated cell migration.

Loss of RhoB decreases PDGF induced formation of membrane ruffles

Activation of RTKs leads to the formation of peripheral ruffles and circular dorsal ruffles, both of these actin-based structures at the cell surface are required for cell migration [Hall, 1998; Suetsugu et al., 2003]. To test whether RhoB is necessary for PDGF-induced formation of membrane protrusions we examined the plasma membrane ruffling in RhoB $+/-$ and $-/-$ VSMCs by immunofluorescent staining of cortactin and F-actin. Staining of the F-actin binding protein cortactin served as a marker to identify membrane ruffling that primarily localized to dynamic actin structures driven by Arp2/3 complex [Boyle et al., 2007; Kaksonen et al., 2000]. Western blotting showed that the cortactin protein expression levels were not significant different between RhoB $+/-$ and $-/-$ VSMCs (data not shown). Consistent with the role of PDGF in the formation of membrane protrusions, we observed the formation of cortactin containing peripheral ruffles and circular dorsal ruffles which translocated from a perinuclear region in RhoB $+/-$ cells after 5, 15 and 30 minutes of PDGF treatment (Fig. 2). In contrast, loss of RhoB led to a dramatic decrease in the formation of both peripheral and circular ruffles, and cortactin remained localized around the nuclear region after PDGF stimulation at each time point (Fig. 2 and Fig. S2).

The presence of Cdc42 and Rac at the leading edge of the migrating cell is important to regulate localized actin polymerization and membrane protrusions. Additional, Rac controls

the translocation of cortactin into the membrane ruffles [Weed et al., 1998]. We next investigated the localization of Cdc42 and Rac by immunofluorescent staining to evaluate their association with actin filaments in the membrane ruffles. In quiescent RhoB^{+/-} and ^{-/-} VSMCs, Cdc42 and Rac were detected diffusely within the cells (Fig. S3). Co-localization of Cdc42 or Rac with F-actin was readily observed in circular and peripheral ruffles in RhoB^{+/-} VSMCs but not in RhoB^{-/-} VSMCs treated with PDGF for various time points (Fig. 3). These results suggest that RhoB activity promotes Cdc42/Rac-mediated formation of circular and peripheral ruffles that are essential for PDGFR stimulated cell migration.

Loss of RhoB prevents PDGF-induced activation of Cdc42 and Rac

Because decreased protein levels would account for altered membrane ruffling in the RhoB null VSMC, we next examined the protein levels of Cdc42, Rac and RhoA, the principal effectors of actin remodeling. Immunoblotting assays revealed a markedly lower level of Cdc42 protein expression in unstimulated RhoB deficient cells, and the level of Rac was also significantly reduced (Fig. 4A, B). However PDGF stimulation did not change the Cdc42 or Rac protein levels in either RhoB^{+/-} and ^{-/-} VSMCs. Like all small GTPases, Cdc42 and Rac switch between an inactive GDP bound form and an active GTP-bound form allowing effector protein interactions and downstream signal transduction events to promote actin remodeling. Therefore, we also asked whether RhoB is required for PDGF-induced activation of Cdc42 and Rac. By using a PAK-1 PDB pulldown assay we observed that loss of RhoB in VSMCs greatly prevented the PDGF-induced increase of Cdc42-GTP and Rac-GTP levels observed in RhoB^{+/-} cells (Fig. 4C, D). In a similar experiment, we confirmed that RhoB was also needed for PDGF-stimulated activation of Cdc42 and Rac in macrophages (Fig. S1B). Furthermore, when RhoB expression was restored in RhoB^{-/-} VSMCs by retroviral infection with a RhoB-WT vector, the protein levels of Cdc42 and Rac were increased when compared with the empty vector control (Fig. S4A), and the activity status of Cdc42 and Rac in response to PDGF-BB stimulation was also restored (Fig. S4B). These results further suggest that RhoB is critically involved in the mechanism by which PDGF activated Cdc42 and Rac.

To gain insights into the mechanism by which RhoB regulates Cdc42 and Rac, we evaluated the RhoGEFs that may be involved in PDGF-stimulated migration of VSMCs. Immunoblot analyses showed that loss of RhoB attenuated the level of phosphorylated Vav2 in response to PDGF stimulation (Fig. 4E, F). Indirect immunofluorescence experiments also revealed that phosphorylated Vav2 co-localized with actin filaments in the membrane ruffles of RhoB^{+/-} cells, while it was difficult to discern Vav2 containing ruffles in RhoB deficient cells (Fig. S5A). The Vav2 co-localization appeared to be specific, as we did not observe any co-localization of β PIX, another important RhoGEF that can active Cdc42/Rac, with F-actin in the membrane ruffles after PDGF treatment (data not shown). In addition, we observed co-localization of phosphorylated Vav2 with Cdc42 or Rac at the PDGF-induced membrane ruffles in RhoB^{+/-} cells, but not RhoB null cells (Fig. S5B, C). Knock down of Vav2 by shRNA (Fig. S6A, B) inhibited the migration of VSMCs (Fig. S6C, D), and PDGF-induced Cdc42/Rac activation (Fig. S6E). Taken together, these results pointed to the possibility that Vav2 is a GEF for RhoB dependent PDGF-induced Cdc42/Rac activation.

RhoB is linked to the early endosomal trafficking of Cdc42

Endosomal trafficking has emerged as an important mechanism to control the spatial localization of small GTPase Rac [Sadowski et al., 2008]. It is worth noting that Cdc42 localizes to early endosome compartment [Balklava et al., 2007; Grovdal et al., 2008], and plays a role in regulating vesicular trafficking [Balklava et al., 2007; Harris and Tepass, 2008; Landry et al., 2009; Leibfried et al., 2008]. To determine whether RhoB-directed Cdc42/Rac signaling at peripheral membranes was associated with endosomes, we used

indirect immunofluorescence to evaluate associations of the observed protein complexes with different endosomal compartments. We first examined the possibility that Cdc42 trafficking occurred through late endosomes by co-staining with LAMP1, a specific marker for this endosomal compartment. However, there was no evidence of Cdc42 localizing to peripheral membranes by late endosome trafficking after PDGF stimulation for 5 minutes to 4 hours (data not shown). We also investigated whether the early endosomal compartment was relevant to RhoB-dependent trafficking of the Cdc42. In quiescent RhoB $+/+$ VSMCs, Cdc42 exhibited a perinuclear localization in EEA1- early endosomes. After 5 to 30 minutes of PDGF stimulation, we observed the Cdc42 bound EEA1-early endosomes redistribute from the perinuclear area to the peripheral region, and particularly localized to circular dorsal and peripheral ruffles (Fig. 5). In contrast, we did not observe dynamic and spatial trafficking of Cdc42 in early endosomes in RhoB $-/-$ VSMCs (Fig. 5). To confirm that the endosomal trafficking of Cdc42 was associated by PDGFR endocytosis, we examined PDGFR localization and found PDGFR localized to early endosomes with Cdc42 in RhoB $+/+$ cell membrane ruffles (Fig. S7). Western blotting analysis of EEA1 protein levels showed no significant difference in EEA1 expression between RhoB $+/+$ and $-/-$ VSMCs (data not shown), suggesting that the difference in cellular localization of GTPase-bound EEA1 positive endosomes is not due to EEA1 protein levels. Together, these observations support the concept that RhoB links PDGFR stimulation to early Cdc42-endosomal trafficking and membrane ruffle formation, both of which drive actin organization events crucial for migration.

Loss of RhoB impaired the localization of Rac and Vav2 in endosomes

The above results suggest that Cdc42 localizes to early endosomes which are transported to specific locations within PDGF-stimulated cells in a RhoB-dependent manner. In a set of similarly designed co-immunostaining experiments, we also investigated whether Rac was shuttled through the early endosomal system in a RhoB-dependent manner in response to PDGF stimulation. In quiescent RhoB $+/+$ and RhoB $-/-$ VSMCs, Rac displayed a diffuse localization pattern in the cytoplasm and was not co-localized with the early endosomal marker EEA1. After 5 minutes of PDGF stimulation, Rac was not observed at peripheral cell areas in early endosomes (data not shown). However, after 15 min of PDGF stimulation, we observed that Rac was detected in EEA1-positive endosomes in membrane ruffles of RhoB $+/+$ VSMCs (Fig. 6A). Similarly, phosphorylated Vav2 was found to be present in early endosomes localized at membrane ruffles of RhoB $+/+$ VSMCs (Fig. 6B). At the same time points, we observed co-localization of phosphorylated Vav2 with Cdc42 or Rac in membrane ruffles of RhoB $+/+$ VSMCs (Fig. S5B,C). However, loss of RhoB impaired the localization of both Rac and Vav2 in early endosomes (Fig 6). To rule out the possibility of competition between RhoGEF proteins in VSMC, we determined the localization of Tiam, a RhoGEF that can activate Rac in early endosomes [Palamidessi et al., 2008]. We did not observe Tiam localization to EEA1 stained endosomes in PDGF stimulated VSMCs (data not shown), suggesting that this RhoGEF is not involved in migration stimulated by PDGF. Taken together, our results suggest that RhoB coordinates cellular migration by 1) regulating the Vav2-mediated activation of Cdc42/Rac, 2) endosomal delivery of these key signaling molecules to the plasma membrane, and 3) directing actin remodeling and ruffle formation. Figure 7 presents a model illustrating the role of RhoB during PDGF-stimulated endosomal signaling that mediates smooth muscle cell migration.

DISCUSSION

Rho family small GTPases Cdc42 and Rac have been implicated as critical proteins governing directional cell movement. However, the mechanisms underlying coordinated regulation of their activities at specific intracellular sites, in response to extracellular cues,

are not well defined. One appealing hypothesis is that endosomes, formed in response to receptor stimulation, may act as organizers within the cell to carry signaling molecules to specific sites, ultimately directing distinct cellular behaviors, such as cell migration [Maxfield and McGraw, 2004; Polo and Di Fiore, 2006]. Indeed, a recent study has demonstrated the importance of Rab5-regulated endocytic trafficking in the localization and activation of Rac in cell migration [Palamidessi et al., 2008]. However, there is no report linking the regulation of Cdc42-associated cell migration and endosomal trafficking. RhoB has been implicated in endosome trafficking associated with cell polarity and directional cell movement controlled by Wnt signaling downstream of adhesion receptors [Witze et al., 2008]. Accumulating evidence suggests that RhoB is a regulator of endocytic pathways. The work of several laboratories has identified important roles for RhoB in the endocytic trafficking of RTKs, such as EGFR and PDGFR, along with key downstream signaling molecules Src, Akt and ERK [Adini et al., 2003; Huang et al., 2007; Sandilands et al., 2004; Wherlock et al., 2004]. A study on macrophages has reported that RhoB affects cell adhesion and migration on different substrates in response to colony stimulating factor-1 (CSF-1) stimulation by regulating cell surface expression of integrins [Wheeler and Ridley, 2007]. However, the role of RhoB in growth factor induced cell migration has not been studied in detail. Interestingly, we demonstrate here that RhoB is required for the activation of Cdc42 and Rac, and RhoB-dependent endosomal trafficking is linked to the localization of Cdc42 and Rac, central regulators of actin cytoskeleton reorganization events vital for cell migration. Our findings extend the role of RhoB to coordinating responses induced by RTK activation, and reveal the mechanism by which RhoB synchronizes cell migration. Our data show that loss of RhoB results in the decrease of Cdc42 and Rac protein levels, and their active form after PDGF stimulation, however, the mechanism causing this change needs to be defined. Recent studies reported that depletion of RhoGDI1 promotes the degradation of RhoGTPases [Boulter et al., 2010], and loss of caveolin-1 modulates active and non-active Rac protein levels by regulating Rac degradation [Nethe et al., 2010]. Future studies will explore the RhoB-mediated mechanisms that regulate Cdc42/Rac protein synthesis and degradation.

Although the Rab and Arf families of the small GTPase superfamily are better known for their role in vesicle trafficking within the endocytic and secretory pathways [Donaldson and Honda, 2005; Zerial and McBride, 2001], important roles for Rho small GTPases in endocytic pathways are also emerging [Qualmann and Mellor, 2003]. RhoB localizes to both early and late endosomes [Adamson et al., 1992; Fernandez-Borja et al., 2005; Mellor et al., 1998; Rondanino et al., 2007] and regulates RTK trafficking in the late endosomal compartment [Gampel et al., 1999; Huang et al., 2007; Wherlock et al., 2004]. Here our findings suggest that RhoB-dependent activation and trafficking of Cdc42 and Rac most likely occur within the early endosomal system. Based on our observations, we suggest that upon PDGF stimulation, Cdc42 associates with the early endosomal compartment that travels from the perinuclear region to the peripheral cell area. The peripheral endosomal compartment then localizes to the sites where dorsal circular ruffles and edge membrane ruffles are initiated. Subsequent to Cdc42 trafficking, Rac recruitment to early endosomes was observed in the ruffles. Cdc42 is known to function in chemotaxis and directed migration in a variety of cell types. Studies have shown that inhibition of Cdc42 decreases Rac localization and activation at the leading edge, thereby reducing the stability of lamellipodia [Heasman and Ridley, 2008]. Also, Cdc42-deficient cells have reduced Rac activity [Czuchra et al., 2005; Wu et al., 2007], and increased Rac activation occurs downstream of Cdc42 in response to RTK stimulation [Bosse et al., 2007]. Taken together, these studies suggest that Cdc42 is necessary for Rac activation at the leading edge of membrane ruffle formation. However, the precise mechanism of how Cdc42 regulates Rac localization and activation was not yet well defined. Cdc42 has been reported to localize to early endosomes [Balklava et al., 2007; Grovdal et al., 2008], and as a regulator of endocytic

trafficking [Balklava et al., 2007; Harris and Tepass, 2008; Landry et al., 2009; Leibfried et al., 2008]. Our data support the concept that RhoB can act directly or with Cdc42 to recruit Rac to the cell periphery through the endosomal recycling pathway. On the basis of our observations in VSMCs and macrophages, we propose that Rac localization and activation occurs downstream of Cdc42 but in a manner that relies on RhoB-dependent endosomal trafficking triggered by RTK stimulation.

RhoGEFs activate Rho proteins by facilitating the exchange of GDP for GTP. Among the RhoGEFs, Vav2 is known to associate with RhoB in endosomal pools from EGF-stimulated cells [Gampel and Mellor, 2002], and it is known to play an important role in several cell types to regulate actin remodeling in cell spreading and migration [Hornstein et al., 2004; Marignani and Carpenter, 2001]. The tyrosine phosphorylation of Vav proteins is necessary for their GEF activity [Schuebel et al., 1998]. It has been shown that Vav2 is phosphorylated in response to growth factors EGF and PDGF, and activates Cdc42 and Rac downstream from growth factor receptor stimulation [Garrett et al., 2007; Pandey et al., 2000]. Following EGF or PDGF stimulation, Vav2 binds to the tyrosine-phosphorylated growth factor receptor via its SH2 domain [Moore et al., 2000; Pandey et al., 2000], and this interaction results in tyrosine phosphorylation of Vav2 [Tamas et al., 2001]. Also, products of PI3K may participate in the mechanism of activation of Vav2 because PI3K inhibitors significantly inhibit Vav2 exchange activity [Tamas et al., 2003]. We have previously shown that RhoB is required for regulation of PDGFR trafficking and signaling to PI3K pathway. The level of active PI3K induced by PDGF is significantly reduced in VSMCs isolated from RhoB null animals [Huang et al., 2007]. The dysregulation of PDGFR trafficking and interruption of downstream PI3K signal pathway by RhoB deletion potentially results in the decrease and mislocalization of phosphorylated Vav2. Indeed, our results here demonstrated that loss of RhoB attenuated the level of phosphorylated Vav2 in response to PDGF stimulation, and impaired the localization of phosphorylated Vav2 in early endosomes, as well as the co-localization of phosphorylated Vav2 with Cdc42 and Rac in membrane ruffles. Our findings suggest that RhoB regulates an endosomal trafficking pathway that permits a spatially restricted assembly of Vav2 and Cdc42/Rac, activation of Cdc42/Rac, and formation of circular and peripheral ruffles that mediate directed cell migration. This characterization of endosomal trafficking events further defines the role of RhoB in cellular responses orchestrated by tyrosine-phosphorylated growth factor receptor stimulation.

Recent studies on the mechanism of RhoB regulated endocytic trafficking of the EGFR have shown that mDia proteins [Fernandez-Borja et al., 2005; Wallar et al., 2007] and MAP1A/LC2 [Lajoie-Mazenc et al., 2008] are the important RhoB effector molecules that coordinate with RhoB to facilitate trafficking of endosomes and signaling molecules. mDia1 and mDia2 can regulate actin dynamics to form the actin coat around endosomes that is essential for RhoB directed endosome trafficking [Fernandez-Borja et al., 2005; Wallar et al., 2007]. It has been suggested that interaction of RhoB and MAP1A/LC2 stabilizes microtubules, thus resulting in the control of endosomal trafficking [Lajoie-Mazenc et al., 2008]. It is therefore possible and yet to be determined that the RhoB-mediated recruitment of mDia to endosomes and interaction of RhoB with MAP1A/LC2 on microtubules, is relevant to the observed RhoB-dependent endosomal trafficking of PDGFR and downstream signaling molecules.

In the context of the existing literature, our studies highlighting RhoB effector molecules in cell migration shed additional light on the importance of the endosomal trafficking pathway identified here to couple RTK activation and the endosomal/actin reorganization events, thus driving cell migration. Specifically, our findings reveal a RhoB-dependent endosomal trafficking pathway controlling Cdc42/Rac redistribution and actin remodeling, adding insight into the understanding of cell migration stimulated by growth factors. Together with

RhoB's function to promote VSMCs proliferation, this study suggests that RhoB plays an important role in governing stress responses in vascular cells. Because VSMC migration and proliferation are necessary for tumor-associated angiogenesis and the development of cardiovascular disease, this study supports the concept of therapeutically targeting RhoB to treat vascular disorders associated with aberrant cellular responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. RhoB is required for PDGF-stimulated migration of VSMCs. (A) Cell migration of quiescent mouse RhoB ^{+/+} and ^{-/-} VSMCs in response to serum-free medium (SFM) with or without recombinant PDGF-BB (10 ng/ml) was measured by modified Boyden chamber assay. After 16-20 hours, cells that migrated onto the lower surface of the membrane were fixed, stained and examined (magnification x100). Three independent experiments were performed with different batches of VSMCs and a representative analysis is shown. (B) Quantitative analysis of VSMC migration. Cells present in four different areas of the filter were counted for each data point. The number of migrated cells was graphed relatively to RhoB ^{+/+} cells. Values are mean $\pm$ SD (n=3). ***P* < 0.01, vs RhoB ^{+/+}. (C) Restoring expression of RhoB in null VSMCs increases the number of cell migration in response to PDGF stimulation. The results represent one of three independent experiments. (D) Quantitative analysis of migrated cell number from RhoB ^{-/-} control and empty vector or RhoB-WT transfected VSMCs. Values are mean $\pm$ SD (n=3). **P* < 0.05; ***P* < 0.01, vs RhoB-WT. (E) Immunoblot analysis of RhoB. The cell extracts were prepared from the RhoB ^{+/+} and RhoB ^{-/-} VSMCs, as well as RhoB null cells transfected with either empty vector or RhoB-WT.

Fig. 2.

Loss of RhoB decreases PDGF-induced membrane ruffle formation. RhoB $+/+$ or $-/-$ vascular SMCs stimulated with recombinant PDGF-BB (10 ng/ml) for indicated times, and double immunofluorescence staining was performed with fixed and permeabilized cells using cortactin antibody and AlexaFluor-Phalloidin. Examples of peripheral (arrows) and dorsal circular (arrowheads) ruffles are shown. Scale bars, 100 μ m.

Fig. 3.

Loss of RhoB impairs PDGF-induced Cdc42/Rac localization in membrane ruffles. Cells were incubated with fresh SFM containing PDGF-BB (10 ng/ml) for 15 minutes. Immunofluorescence was performed on fixed and permeabilized cells using anti-Cdc42 (A) or Rac (B) antibodies detected with goat anti-mouse CY3 and co-stained with AlexaFluor-Phalloidin. Scale bars, 50 μm. White boxed areas are magnified.

Fig. 4.

Loss of RhoB attenuates the protein level of Cdc42/Rac and PDGF-stimulated activation of Cdc42/Rac. (A) Quiescent RhoB ^{+/+} or ^{-/-} VSMCs were stimulated with recombinant PDGF-BB (10 ng/ml) for indicated times, and protein levels of Rho small GTPases (Cdc42, Rac and RhoA) were measured by Western blotting. The results represent one of three independent experiments. (B) Quantitative analysis of Cdc42 and Rac in RhoB ^{+/+} or ^{-/-} VSMCs. The levels of proteins were quantified by densitometric analysis of western blots and the amount of Cdc42 or Rac was normalized to the amount of actin. The amount of proteins were graphed relatively to RhoB ^{+/+} cells at 0 time point. Values are mean $\pm$ SD (n=3). **P*<0.05; ***P*<0.01 compared with RhoB ^{+/+}. (C) Immunoblot analysis of active Cdc42 and Rac in RhoB ^{+/+} or ^{-/-} VSMCs. The cells were incubated with serum-free medium and PDGF-BB (10 ng/ml) for 5 minutes before cell lysates were prepared for the pull down assay with PAK-1 PDB agarose, and analyzed by Western analysis using the antibodies indicated to detect the activation status. (D) Quantitative analysis of active Cdc42 and Rac. Active Cdc42 or Rac was normalized to the levels of total Cdc42 or Rac. The active GTPases were graphed relatively to control cells. Values are mean $\pm$ SD (n=5). **P*<0.05, vs control, ***P*<0.01, vs control. (E) Immunoblot analysis of protein levels of phospho-Vav2, Vav2 and β -PIX in RhoB ^{+/+} and ^{-/-} VSMCs. The results represent one of three independent experiments. (F) Quantitative analysis of phospho-Vav2 in RhoB ^{+/+} and ^{-/-} VSMCs. The amount of protein was normalized by amount of actin. The amount of phospho-Vav2 were graphed relatively to RhoB ^{+/+} VSMCs at 0 time point. Values are mean $\pm$ SD (n=3). **P*<0.05; ***P*<0.01 compared with RhoB ^{+/+}.

Fig. 5.

RhoB controls PDGF induced endosomal trafficking of Cdc42 to membrane ruffles. Quiescent VSMCs were incubated in SFM or SFM containing PDGF-BB (10 ng/ml) for 15 and 30 minutes. Cells were double stained with anti-Cdc42 (red) and anti-EEA1 (green) antibodies. The two images were merged to show the co-localization. Scale bars, 50 μ m. White boxed areas are magnified.

Fig. 6.

RhoB is required for endosomal recruitment of Rac and phospho-Vav2 to membrane ruffles stimulated by PDGF. Cells were incubated in SFM containing PDGF-BB (10 ng/ml) for 15 minutes. Immunofluorescence was performed on fixed and permeabilized cells using anti-Rac (A) or phospho-Vav2 (B) antibodies, and anti-EEA1 antibody for double staining. Scale bars, 50 μ m. White boxed areas are magnified.

Fig. 7.

Model of a RhoB-dependent endosomal trafficking pathway that couples RTK stimulation to Cdc42/Rac activation, localized actin dynamics, and cell migration. RhoB localizes to endosomal compartments and mediates endosomal trafficking of Cdc42 and Rac in response to PDGFR stimulation. Through the recruitment of Vav2, RhoB controls activation of Cdc42 and Rac in early endosomes and delivers active-Cdc42/Rac to the plasma membrane resulting in actin remodeling events that are essential for cell migration.