

p68RacGAP Is a Novel GTPase-activating Protein That Interacts with Vascular Endothelial Zinc Finger-1 and Modulates Endothelial Cell Capillary Formation*

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The endothelium is required for maintenance of vascular integrity and homeostasis during vascular development and in adulthood. However, little is known about the coordinated interplay between transcription factors and signaling molecules that regulate endothelial cell-dependent transcriptional events. Vascular endothelial zinc finger-1 (Vezf1) is a zinc finger-containing transcription factor that is specifically expressed within the endothelium during vascular development. We have previously shown that Vezf1 potently activates transcription of the endothelin-1 promoter. We now report the identification of p68RacGAP, a novel Vezf1-interacting 68-kDa RhoGAP domain-containing protein. p68RacGAP mRNA is highly expressed in vascular endothelial cells by Northern blot analysis, and immunohistochemical staining of adult mouse tissues identified p68RacGAP in endothelial cells, vascular smooth muscle cells, and epithelial cells *in vivo*. Rac1 and Vezf1 both bind avidly to p68RacGAP, suggesting that p68RacGAP is not only a GTPase-activating protein for Rac1 but that p68RacGAP may also be part of the protein complex that binds to and modulates Vezf1 transcriptional activity. Functionally p68RacGAP specifically activates the GTPase activity of Rac1 *in vivo* but not Cdc42 or RhoA. In addition, p68RacGAP potently inhibits Vezf1/DB1-mediated transcriptional activation of the human endothelin-1 promoter and modulates endothelial cell capillary tube formation. Taken together, these data suggest that p68RacGAP is a multifunctional regulatory protein that has a Rac1-specific GTPase-activating activity, regulates transcriptional activity of the endothelin-1 promoter, and is involved in the signal transduction pathway that regulates endothelial cell capillary tube formation during angiogenesis.

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Angiogenesis, the sprouting of new blood vessels from an existing primary vascular complex, is a highly regulated process under normal conditions; it accounts for neovascularization that occurs during wound healing and during the female reproductive cycle. However, many disease processes are driven by persistent unregulated angiogenesis. These include diabetic retinopathies, inflammatory states such as rheumatoid arthritis, and tumor growth and metastasis (1). The sprouting of new blood vessels during angiogenesis requires cell migration, a principal feature not only in embryonic development, where cell migration is required for movement of differentiating stem cells and for the coordinated movement required for proper vascular and organ formation, but also in maintenance of tissue integrity after an insult and in immune surveillance.

Cell migration is usually initiated in response to extracellular cues that stimulate transmembrane receptors to initiate intracellular signaling. Cell movement requires the dynamic reorganization of the actin cytoskeleton, a process that is regulated by Ras homology (Rho)¹ proteins. Rho proteins form a subgroup of the Ras superfamily of 20–30-kDa GTP-binding proteins that regulate a wide spectrum of cellular activities (2). To date, 25 mammalian Rho GTPases have been identified with RhoA, Rac1, and Cdc42 being the most characterized (3). These proteins act as molecular switches that cycle between an inactive, GDP-bound and an active, GTP-bound form. Among the regulatory proteins crucial for Rho GTPase function are guanine nucleotide exchange factors, which promote activation of Rho GTPase through GDP-GTP exchange, and GTPase-activating proteins (GAPs), which stimulate intrinsic GTPase activity and inactivate Rho GTPases (4, 5). The net effect of the activation and deactivation signal is a tightly regulated transduction mechanism. The RhoGAP family is defined by the presence of a 150-amino acid homology region, designated the RhoGAP domain, which is necessary and sufficient for GAP activity and shares at least 20% sequence identity among family members (6). Specific roles for RhoGAP family members in endothelial cell events have not been clearly defined, although members of this family are likely to be crucial regulators of

¹ The abbreviations used are: Rho, Ras homology; Vezf1, vascular endothelial zinc finger-1; GAP, GTPase-activating protein; PBD, GTP-dependent binding domain for PAK1; RBD, Rhotekin binding domain; GST, glutathione S-transferase; GFP, green fluorescent protein; CMV, cytomegalovirus; MEC, myocardial endothelial cell; HUVEC, human umbilical vein endothelial cell; Ura, uracil; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate; ERM, ezrin-radixin-moesin; PDGF, platelet-derived growth factor; LPA, lysophosphatidic acid; BCR, Breakpoint cluster region.

endothelial migratory events that are required during angiogenesis.

The GTPase-induced GTP hydrolysis is a key event in intracellular signal transduction that controls numerous vital processes, including actin reorganization; gene transcription; vessel trafficking; cell proliferation, survival, and differentiation; and motility. The coordinated movement of cells depends on promotion of protrusions (lamellipodia) by Rac1 and formation of new cell adhesions to adjacent cell matrix at the leading edge. In addition, Rho-dependent actin-myosin contraction in the cell body and tail retraction are required to allow the body and rear of the cell to follow the extending front (7). Thus, the actin cytoskeleton provides the driving force for cell migration through its role in promoting both protrusive force toward and contraction and retraction away from a stimulus in response to extracellular cues.

Connolly *et al.* (8) demonstrated that Rac is required for endothelial cell capillary assembly *in vitro*. In addition, WAVE2, a Rac effector protein crucial for Rac-induced membrane ruffling, assists in the formation of lamellipodia at the leading edge, and WAVE2 knock-out mice die at embryonic day 10 due to impaired angiogenesis that is presumably secondary to impaired migration of endothelial cells (9). These observations are also consistent with studies demonstrating that Rac1-deficient embryos die before embryonic day 9.5 primarily due to deficient cell migration during and after gastrulation (10). Taken together, these observations indicate that tight coupling exists between signaling events and endothelial cell migration for proper blood vessel growth, creating a need for careful regulation of Rho and related proteins.

Vascular endothelial zinc finger-1 (Vezf1) was discovered by Stuhlman and colleagues (11) using a retroviral screen for developmentally regulated genes. Structurally Vezf1 and its human homologue, DB1 (12), have six N-terminal Cys₂/His₂-type zinc finger motifs as well as a C-terminal proline-rich region characteristic of a transcriptional activation domain. Vezf1 mRNA expression is detected from embryonic day 7.25 to 11.5 in mouse embryos, and its expression overlaps with other endothelial cell-specific markers (11). We have previously shown that endothelin-1 (a vasoactive peptide that is predominantly expressed in endothelial cells) is one of the downstream genes that are potently and specifically transactivated by Vezf1 (13). We have also shown that Vezf1 binds to the response element ACCCCC within the endothelin-1 promoter. Given the endothelial cell-specific expression of Vezf1, its overlapping expression with other endothelial cell-specific markers, and our previous characterization of Vezf1 transcriptional regulation of endothelin-1, we chose to use Vezf1 as a bait to screen for other proteins that associate with Vezf1 and regulate blood vessel formation.

In the process of searching for Vezf1-interacting partners, we discovered a novel RhoGAP protein, p68RacGAP. p68RacGAP is expressed predominantly in endothelial cells. It has a Rac1-specific GTPase activity *in vitro* and *in vivo*, and it dose-dependently inhibits Vezf1-dependent transcriptional activation of endothelin-1 promoter activity. p68RacGAP modulates endothelial cell shape changes and angiogenesis *in vivo* indicating that p68RacGAP is a potential link between transcriptional endothelial cell differentiation programs and cell signaling pathways required in adult angiogenesis.

MATERIALS AND METHODS

Plasmids—Plasmids pGL2-Basic, pGL2-Promoter, pCMV-βGal, pGL2-204/170, pME18S-DB1, GST-vector, GST-Vezf1, and GFP-Vezf1 have already been described (11, 13). Bacterial expression vectors encoding glutathione S-transferase (GST) fusion proteins of wild type (GST-RhoA, GST-RhoB, GST-Rac1, and GST-Cdc42) or GTPase-deficient, GAP-insensitive (GST-RhoAQ63L, GST-Rac1Q61L, and

GST-Cdc42Q61L) human Rho GTPases or the isolated GTP-dependent binding domain for PAK1 (GST-PBD) or Rhotekin (GST-RBD) or mammalian expression vectors encoding RhoGAPs (pCMV-p50RhoGAP and pCMV-p190RhoGAP) have been described elsewhere (14).

Full-length p68RacGAP was subcloned into the EcoRI/SalI site of pCMV-Tag3B (Stratagene) to generate Myc epitope-tagged Myc-p68RacGAP; Myc-p68RacGAP-R70A was subsequently generated by site-directed mutagenesis (see below). Full-length p68RacGAP was subcloned into the NotI/HindIII site of pAdTrack-CMV to generate an adenovirus that bicistronically expresses p68RacGAP and green fluorescent protein (GFP). pPC86 and pDBLeu were obtained from Invitrogen. pGADT7 and pGBKT7 were obtained from BD Biosciences.

Cells and Reagents—The culture of myocardial endothelial cells (MECs), NIH/3T3, COS-7, and C2C12 cells has been described previously (13). 293 and rabbit endothelial vascular cells were obtained from the Tissue Culture Facility at Lineberger Comprehensive Cancer at the University of North Carolina at Chapel Hill. HUVECs were obtained from Clonetics. Anti-Myc antibody 9E10 and Texas Red-conjugated phalloidin were obtained from Santa Cruz Biotechnology and Molecular Probes, respectively. Matrigel was obtained from BD Biosciences.

Yeast Two-hybrid Screening—A yeast two-hybrid screen was performed with the PROQUEST two-hybrid system (Invitrogen). The six zinc finger domain of Vezf1 was fused with the GAL4 DNA binding domain in pDBLeu and used as a bait. A mouse day 10.5 cDNA embryo library in pPC86, which expresses fusions with the GAL4 activation domain, was screened for potential prey in the yeast strain MaV203. A total of 1×10^7 independent clones was screened. The screen is dependent on the interaction between the bait and prey to complement His auxotrophy. Of 25 His⁺/Ura⁺ colonies, 11 were also LacZ⁺. pPC86-based plasmids from these colonies were isolated from yeast and transformed into bacteria. To eliminate false positives, these plasmids were separately transformed into yeast (MaV203) containing either bait, several negative control plasmids, or the p53 binding site, and transformants were tested for β-galactosidase activity using the colony lift assay; candidate cDNA inserts were sequenced and used to query the GenBank™.

RNA Preparation and Northern Blot Analysis—Total RNA from cells in culture was prepared by guanidinium isothiocyanate extraction and centrifugation by calcium chloride methods (15). The quality of the RNA was analyzed by agarose-formaldehyde gel electrophoresis, and quantification was performed spectrophotometrically. Purified RNA was separated by agarose-formaldehyde gel electrophoresis and transferred to a nitrocellulose membrane by capillary action. The membrane was subsequently subjected to hybridization in the Quickhyb hybridization buffer (Stratagene) with a [³²P]dCTP randomly labeled 1.8-kb cDNA fragment of p68RacGAP originally isolated from the yeast two-hybrid screen. An adult multiple organ polyadenylated RNA blot used to analyze tissue distribution was obtained from OriGene Technologies.

Binding Assays—To test the interactions between p68RacGAP and Vezf1 or GTPase proteins, binding assays were performed with 30 μg of Vezf1, the indicated GTPase proteins or control proteins as GST fusions bound to glutathione-Sepharose 4B beads, and 500 μg of lysates from COS-7 cells transfected with vector or Myc-p68RacGAP, Myc-p50RhoGAP, or Myc-p190RhoGAP. A GST pull-down and anti-Myc blot were performed as described previously (16).

To determine the GAP activity of p68RacGAP against putative small GTPase, 30 μg of freshly prepared GST-PBD or GST-RBD were incubated with 500 μg of COS-7 lysates transiently transfected with plasmids expressing Myc-Rac1-WT, Myc-Cdc42-WT, Myc-RhoA-WT, Myc-p68RacGAP, Myc-p68RacGAP-R70A, Myc-p50RhoGAP, Myc-p190RhoGAP, Myc-Rac1Q61L, Myc-RhoAQ63L, or Myc-Cdc42Q61L. Binding assays were performed by lysing the cells containing Rac1 or Cdc42 in Buffer B (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin) or cells containing RhoA in Buffer A (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin) with rocking for 30 min at 4 °C. Beads were pelleted at 14,000 rpm for 30 s and washed three times with Buffer B. The final pellet was resuspended in SDS sample buffer and separated by 15% SDS-PAGE. Gels were transferred to polyvinylidene difluoride membrane, and bound proteins were immunoblotted with monoclonal anti-Rac (1:1000), anti-Cdc42 (1:250), or anti-RhoA (1:250) from Transduction Laboratories.

Immunoprecipitation—To test the interaction between p68RacGAP and Vezf1 in endothelial cells, cell lysates from non-transfected MECs were incubated with polyclonal antibody to p68RacGAP as described previously (16) and blotted with anti-Vezf1 antibody (13).

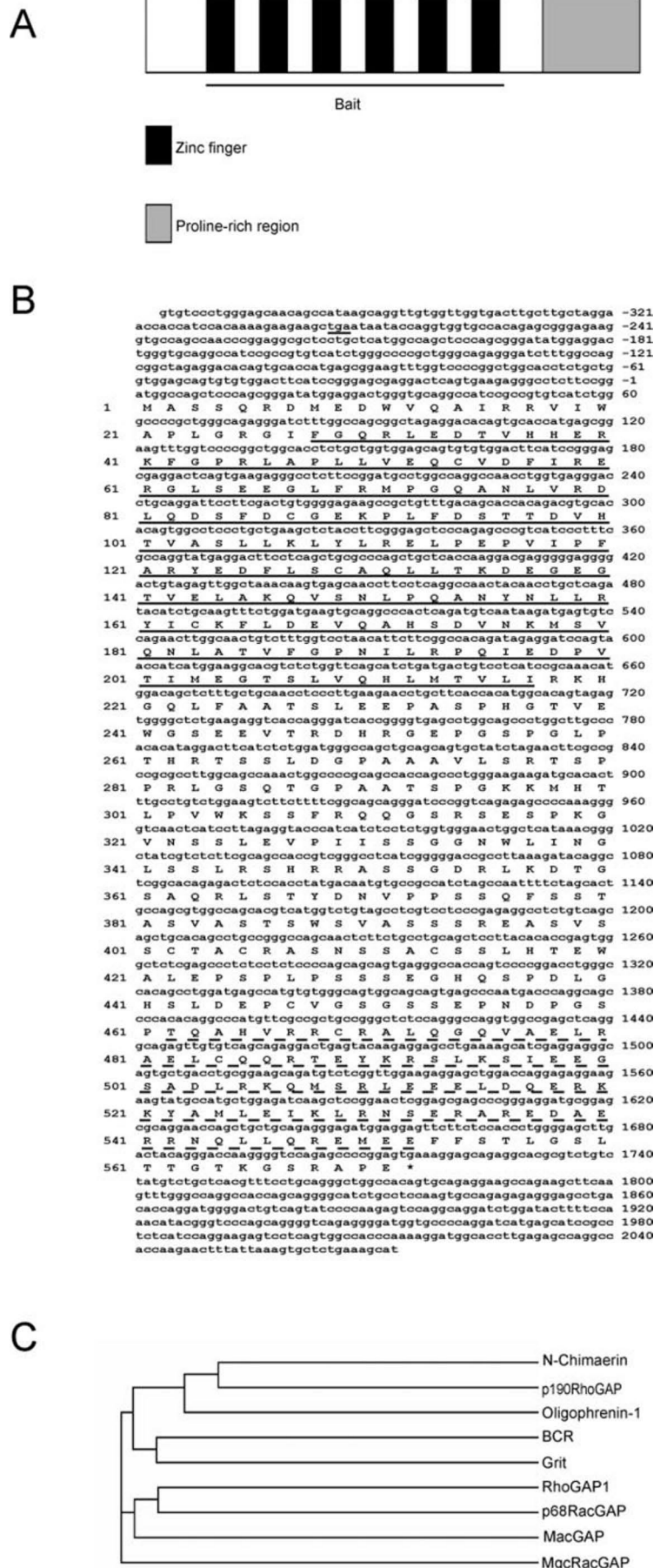


FIG. 1. Cloning and analysis of the p68RacGAP cDNA. *A*, schematic of *VeZF1* protein showing the zinc finger domains and the proline-rich region. A portion of the protein used for yeast two-hybrid screen is *underlined*. *B*, nucleotide (*upper row*) and deduced amino acid (*lower row*) sequences. The open reading frame contains 2,073 bases coding for 571 amino acids with a predicted molecular weight of 68 kDa. The upstream stop codon and the RhoGAP domain (*single line*) and the partial ERM domain (*dotted lines*) are *underlined*. *C*, phylogenetic tree produced by amino acid sequence alignment of the RhoGAP homology domain of p68RacGAP with the catalytic domain of eight other RhoGAP-containing proteins.

Mutagenesis—Site-directed mutagenesis of Myc-p68RacGAP was performed by PCR as described previously (13) to generate the plasmid Myc-p68RacGAP-R70A that lacks the critical arginine finger. The sequence CCTCTTCGGATGCCT was mutated to CCTCTTCGGATGCCT with the mismatched primers 5'-CAGTGAAGAGGGCCTTTC-CGGATGCCTGGCCAGG-3' and 5'-CCTGGCCAGGCATCCGGAAGAGGCCCTTTCACACTG-3'. The sequence of the mutated PCR fragment was confirmed by the dideoxy chain termination method.

Intracellular Localization—To test for co-localization of p68RacGAP and Vezf1, NIH/3T3 cells (grown in 6-well plates on coverslips) were transiently transfected with GFP-Vezf1 and pCMV-p68RacGAP using the LipofectAMINE method (Invitrogen). Twenty-four hours after transfection, cells were washed three times with 2 ml of phosphate-buffered saline and permeabilized with blocking buffer (1% bovine serum albumin, 0.5% Triton X-100 in phosphate-buffered saline) for 10 min. Blocking buffer was washed two times with phosphate-buffered saline, and cells were incubated in primary antibody (1:1000 anti-Myc 9E10 in blocking buffer) for 2 h. Cells were washed again and subsequently incubated for 1 h with 1:400 TRITC-conjugated goat anti-mouse antibody. Cells were visualized by confocal microscopy.

Antisera—Polyclonal antisera were generated in rabbits against a conserved peptide (RSHRRASSGDRLKD) conjugated via an added cysteine residue at the N-terminal region (Sigma Genosys). The third bleed was affinity-purified.

Endothelial Cell Capillary Tube Formation Assays—HUVECs (8×10^5 in 100-mm dishes) were infected with either pAdTrack-CMV-p68RacGAP or pAdTrack-CMV (at a multiplicity of infection of 30) in serum-free medium. Three hours after infection, the medium was substituted with standard endothelial cell growth medium (Clonetics) and incubated for 48 h. HUVECs were then trypsinized, and 2.5×10^5 cells were plated in 6-well plates precoated with Matrigel and incubated. Twelve hours later, cells were fixed with blocking buffer as described above and visualized by fluorescence microscopy with or without a GFP filter.

Transfection, Luciferase Assays, and Immunohistochemistry—Vezf1 was co-transfected with human endothelin-1 promoter fragment (pGL2-204/+170) and increasing concentrations of p68RacGAP into MECs using luciferase as our reporter gene as described previously (13). Adult mouse tissues were prepared as described previously (17) and incubated with purified polyclonal antibody generated against a conserved peptide of p68RacGAP.

RESULTS

Cloning of a Novel RhoGAP-containing Protein by Interaction with Vezf1 in a Yeast Two-hybrid Screen—The central paradigm of transcriptional regulation relies on the molecular interaction between a transcription factor and multiple regulatory proteins that unite to reconstitute an active transcription factor unit that binds to a downstream promoter or enhancer for transcriptional activation or repression. Little is known about regulatory proteins that interact with Vezf1 to mediate its transcriptional or signaling mechanisms. We hypothesized that such regulatory proteins will likely be expressed in a spatial and temporal relationship with Vezf1. Therefore, we utilized the zinc finger domains of Vezf1 (Fig. 1A) in a yeast two-hybrid system to screen 1×10^7 independent clones of an embryonic day 10.5 mouse embryo cDNA library. Of these, 11 potential positive clones survived selection. After plasmid rescue and sequencing, one of the 11 was found to contain a 1.8-kb fragment with a poly(A) tail that represents the 3' cDNA sequence of a novel gene in-frame with the GAL4 activation domain. This 1.8-kb SalI-NotI fragment was extended by 5'-rapid amplification of cDNA ends to obtain the full-length cDNA sequence of 2,073 bp (Fig. 1B). The cDNA contains a single open reading frame that initiates 276 base pairs downstream of an in-frame stop codon and encodes a protein of 571 amino acids with a predicted molecular mass of 68 kDa (GenBank™ accession number AY541447). *In vitro* transcription and translation using this cDNA as a template produced a specific protein product of the expected size, validating the predicted open reading frame. The primary amino acid sequence contains a predicted RhoGAP domain at its N terminus and a partial ezrin-radixin-moesin (ERM) domain

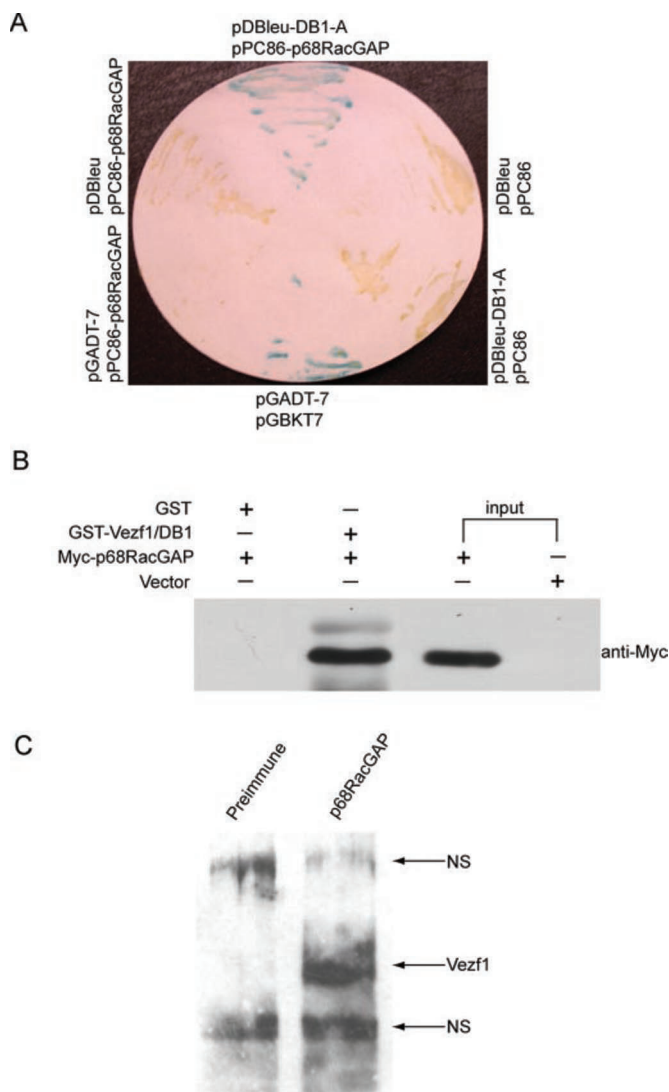


FIG. 2. Verification of *in vivo* interaction of p68RacGAP with Vezf1. A, yeast transfectants containing the indicated plasmids were grown under growth-resistant conditions (His^- , Ura^-). Growth and β -galactosidase activity were only detected in yeast transfected with p68RacGAP and Vezf1. B, binding assay of recombinant Vezf1 and p68RacGAP. Cell lysates from COS-7 cells transfected with vector or Myc-p68RacGAP were incubated with recombinant GST or GST-p68RacGAP. GST-Vezf1 binds avidly to Myc-p68RacGAP, but GST itself does not. C, interaction of p68RacGAP and Vezf1 in endothelial cells. Cell lysates from non-transfected MECs were incubated with a polyclonal antibody to p68RacGAP. Vezf1 specifically co-immunoprecipitated with p68RacGAP but was not pulled down with preimmune serum. NS, nonspecific.

typical of proteins involved in membrane recruitment at its C terminus. The RhoGAP domain, which is phylogenetically similar to other RhoGAP-containing proteins (Fig. 1C), contains the invariant arginine finger at position 70 that is characteristic of RhoGAP proteins and that is functionally critical for GTPase rate enhancement. For reasons explained subsequently, we have called this protein p68RacGAP.

We confirmed the specificity of our two-hybrid interaction with two stringent assays. First, we measured activation of the *lacZ* gene by activation domain fusion in yeast (Fig. 2A). β -Galactosidase activity was only detected when the p68RacGAP activation domain fusion was expressed in yeast containing the zinc finger domain of Vezf1 fused with the GAL4 DNA binding domain. It is notable that the strength of the p68RacGAP interaction with its binding partner is greater than that of T

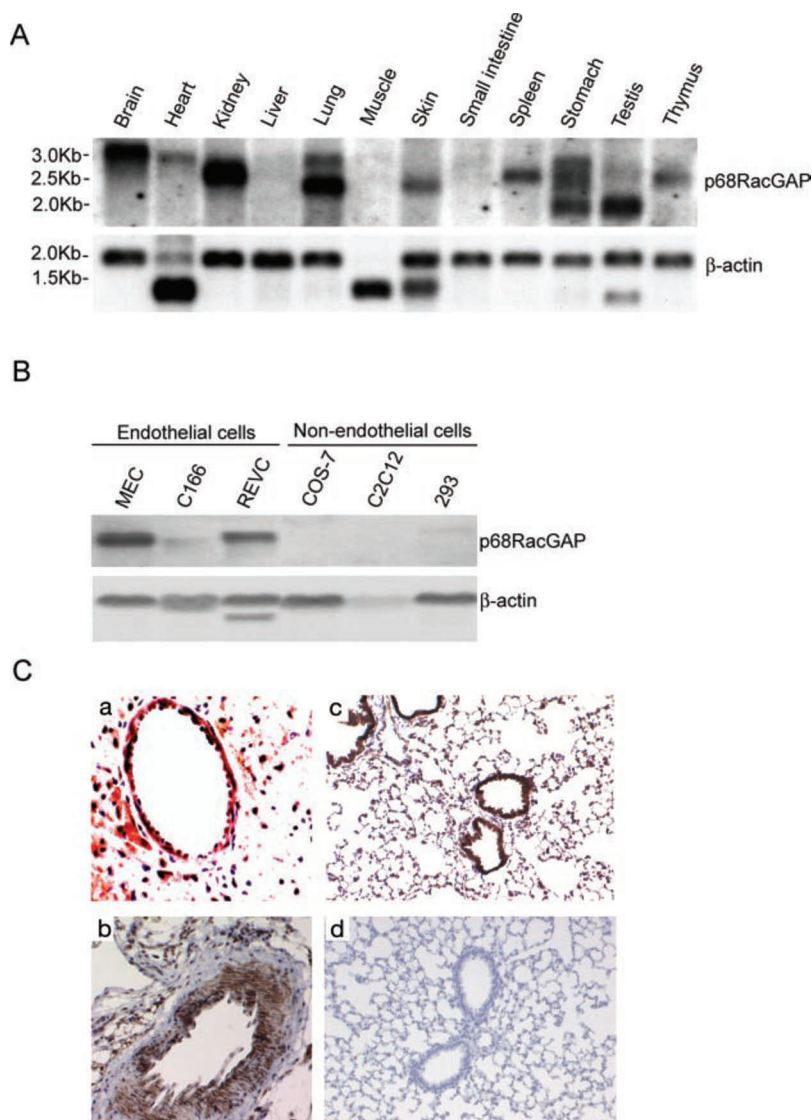


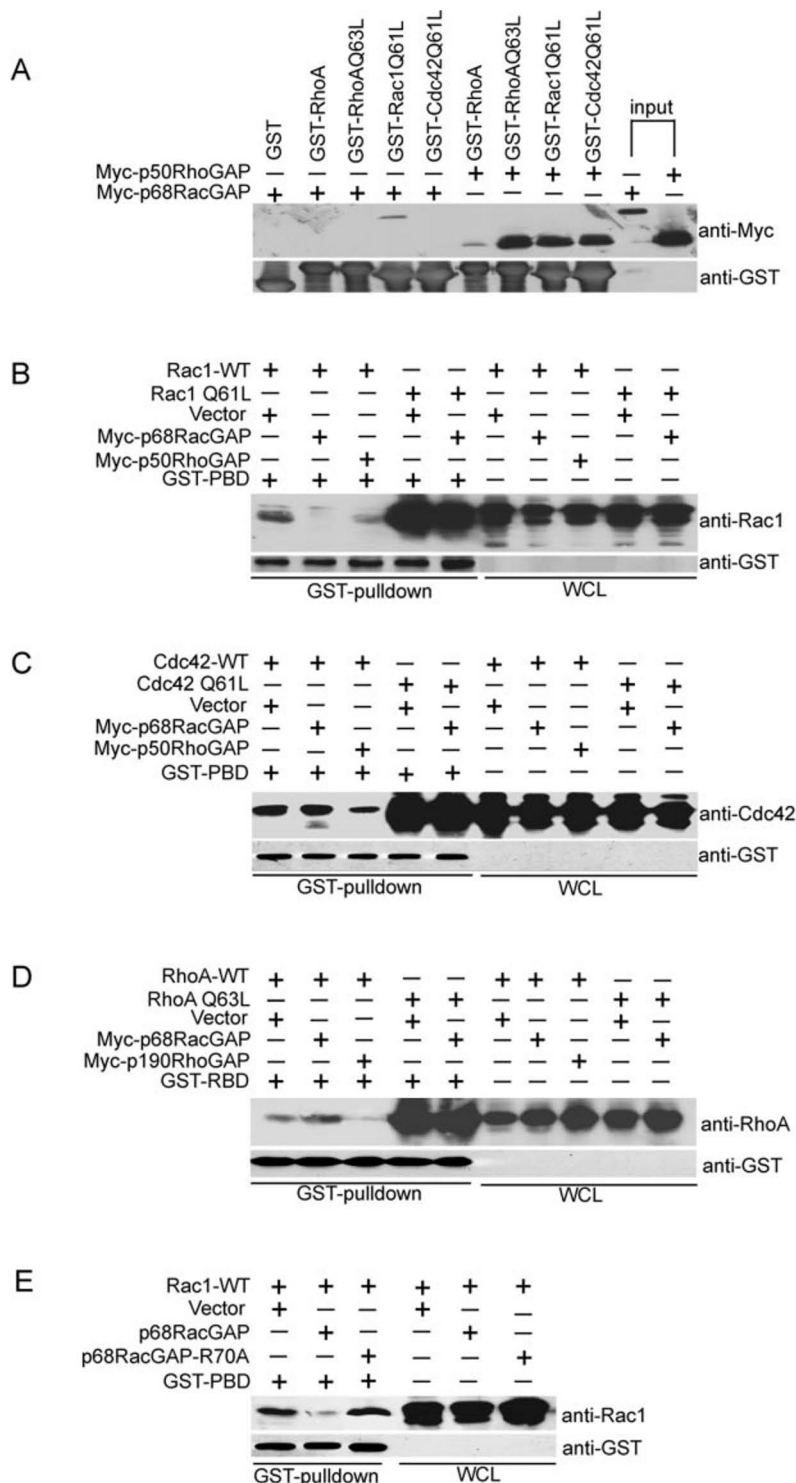
FIG. 3. Tissue and cellular distribution of p68RacGAP. *A*, Northern analysis with a p68RacGAP cDNA probe of polyadenylated RNA from adult mouse tissues. Filters were probed with β -actin to visualize RNA loading. p68RacGAP RNA was highest in the lung, kidney, and brain. *B*, Northern analysis with a p68RacGAP cDNA probe of total RNA from the indicated cell lines. p68RacGAP RNA was only detected in the endothelial cells tested but not in other cells. *C*, immunohistochemical analysis of adult mouse tissues with a polyclonal antibody (2.5 μ g/ml) to a conserved p68RacGAP peptide alone or antibody plus peptide. p68RacGAP is detected in endothelial cells of intramyocardial vessels (*a*) and bronchial epithelial cells (*b*). However, the p68RacGAP peptide (25 μ g/ml) efficiently competed and blocked binding of the antibody to the tissues (*c* and *d*). *REVC*, rabbit endothelial vascular cells.

antigen with p53, its well characterized interacting protein. Second, we tested the binding efficiency of p68RacGAP, expressed in mammalian NIH/3T3 cells, with recombinant Vezf1, expressed as a GST fusion protein in a GST binding assay. As shown in Fig. 2*B*, p68RacGAP avidly binds GST-Vezf1 but not GST alone. The result of this experiment confirms that p68RacGAP is a binding partner for Vezf1 and strengthens the validity of our two-hybrid screen. We tested the hypothesis that the interaction between endogenous Vezf1 and p68RacGAP occurs *in vivo* in endothelial cells by immunoprecipitating cell lysates from MECs with polyclonal antibody to a conserved peptide of p68RacGAP. As shown in Fig. 2*C*, Vezf1 is efficiently co-immunoprecipitated with p68RacGAP in endothelial cells. This experiment indicates that p68RacGAP is expressed in endothelial cells and that it interacts with Vezf1, suggesting that p68RacGAP might modify the transcriptional activity of this nuclear protein.

Tissue Distribution of p68RacGAP—As a first step in the characterization of p68RacGAP, we examined the expression of p68RacGAP mRNA in adult mouse tissues and cell lines by Northern blot analysis. Hybridization with the 1.8-kb C-terminal fragment originally obtained from our yeast two-hybrid screen (which lacks the RhoGAP domain) with polyadenylated mouse RNA resulted in bands of different sizes between 2.0 and 3.0 kb, indicating the occurrence of tissue-specific mRNA pro-

cessing (Fig. 3*A*). p68RacGAP mRNA was most abundant in two highly vascular organs (kidney and lung) as well as in brain; furthermore there was moderate expression of p68RacGAP in the heart and relatively little or no expression in skeletal muscle and liver (Fig. 3*A*). The robust expression of p68RacGAP in highly vascular tissues suggested that p68RacGAP might be differentially expressed in endothelial cells and possibly endothelial cell-enriched tissues. Hybridization to RNA from multiple cell lines showed that p68RacGAP was highly expressed in endothelial cell lines: MECs, C166, and rabbit endothelial vascular cells (Fig. 3*B*); however, we did not detect similar expression in the non-endothelial cell lines we examined, suggesting that p68RacGAP is predominately expressed in endothelial cells and/or their precursors. Immunohistochemical staining of large and medium sized arteries of adult mice with purified polyclonal antibody raised against a conserved peptide of p68RacGAP indicated that p68RacGAP is predominantly expressed in endothelial cells of myocardial tissues and epithelial cells of pulmonary tissues (Fig. 3*C*, *a* and *b*). We also noted expression of p68RacGAP in choroid plexus, Purkinje cells of the cerebellum, and in some myocardial cells (data not shown). P68RacGAP peptide efficiently competed with p68RacGAP antibody and prevented its binding to mouse tissues (Fig. 3*C*, *c* and *d*) indicating that p68RacGAP efficiently identifies tissues that express p68RacGAP.

FIG. 4. GTPase binding and *in vivo* RacGAP activity of p68RacGAP. *A*, lysates from COS-7 cells transfected with Myc-p68RacGAP or Myc-p50RhoGAP were incubated with the indicated GST fusion proteins. After precipitation with glutathione-Sepharose conjugates, immunoblotting was performed with anti-Myc antibodies to detect protein-protein interactions. p68RacGAP bound GTP-loaded Rac1 (GST-Rac1Q61L) in this assay but not Cdc42 or RhoA. *B–E*, COS-7 cells transfected with the indicated plasmids were incubated with GST-PBD, which binds GTP-loaded Rac1 or Cdc42 (*B*, *C*, and *E*), or GST-RBD, which binds GTP-loaded Rho (*D*). p68RacGAP could hydrolyze Rac1 efficiently (*B*) but not Cdc42 (*C*), RhoA (*D*), or the non-hydrolyzable Rac1, Cdc42, or RhoA (*B–D*). p68RacGAP, lacking an arginine finger (p68RacGAP-R70A), lacks the ability to hydrolyze Rac1 when compared with wild type protein (*E*). WCL, whole cell lysate.



p68RacGAP Preferentially Binds and Hydrolyzes Rac1—Since p68RacGAP has a putative RhoGAP domain, we chose to characterize its RhoGAP activity. As a first step, we investigated the effect of p68RhoGAP on binding to RhoA, Rac1, and Cdc42, the three best characterized members of the family. These binding assays are predicated on the observation that RhoGAPs binds preferentially to the GTP-bound GTPase. We incubated recombinant non-hydrolyzable (constitutively GTP-bound) GST-RhoA, -Rac1, or -Cdc42 with p68RacGAP- or p50RhoGAP-expressing cell lysates. p68RacGAP preferentially bound Rac1 but not RhoA or Cdc42 (Fig. 4A). As a positive control in these studies, p50RhoGAP bound RhoA, Rac1, and

Cdc42 indiscriminately as expected. These results clearly demonstrate that p68RacGAP specifically binds Rac1, suggesting that p68RacGAP may modulate Rac-specific cellular activity. To test the GAP activity of p68RacGAP and its possible specificity for Rac1 *in vivo*, we incubated cell lysates from COS-7 cells transiently transfected with wild type Rho proteins (RhoA-WT, Rac1-WT, or Cdc42-WT) or their genetically engineered GTPase-deficient and GAP-insensitive derivatives (RhoAQ63L, Rac1Q61L or Cdc42Q61L respectively) with p68RacGAP, p50RhoGAP, or vector. As shown in Fig. 4B, in the presence of p68RacGAP, a significantly smaller amount of GTP-Rac1-WT could be pulled down with GST-PBD (a target

binding protein that can only bind to the GTP-bound forms of Rac and Cdc42 but not RhoA) suggesting that p68RacGAP hydrolyzed GTP-Rac1 to GDP-Rac1 so that it cannot bind to GST-PBD. As expected, p68RacGAP did not have any effect on the GAP-insensitive form of Rac1. Interestingly p68RacGAP hydrolyzed GTP-bound Rac1 more efficiently than the well characterized p50RhoGAP that was used as a positive control. Furthermore p68RacGAP did not hydrolyze either wild type GTP-bound Cdc42 or RhoA, although these proteins were respectively hydrolyzed by their cognate GAPs p50RhoGAP and p190RhoGAP (Fig. 4, C and D). These results indicate that p68RacGAP has a functional RhoGAP domain with a potent *in vivo* RhoGAP activity that specifically targets Rac1 but not Cdc42 or RhoA.

The Arginine Finger Is Required for p68RacGAP in Vivo RacGAP Activity—It has been reported that an arginine finger is a critical residue that is required for the GTPase rate enhancement of RhoGAP-containing proteins. The arginine finger is an amino acid residue that points to the active site of its corresponding small GTP-binding protein and, through its juxtaposition to the active site, neutralizes the developing charges in the transition state of the reaction and stabilizes the critical glutamine residue, thus enhancing the intrinsic GTPase activity. The arginine finger is invariant within a subfamily of GAPs and is conserved in p68RacGAP, and when studied in other GAPs, mutations in this evolutionarily conserved residue drastically impair GAP activity without changing binding affinity (18). We tested the significance of the arginine finger of p68RacGAP by replacing arginine 70 with alanine (p68RacGAP-R70A). Consistent with the activity of other known GAPs, this single amino acid substitution abolished the *in vivo* GAP activity of p68RacGAP (Fig. 4E). Mutagenesis of the p68RacGAP arginine finger and the subsequent loss of Rac1 GAP activity provides further evidence that p68RacGAP is a *bona fide* GTPase-activating protein for Rac1 and that its GAP function is similar or identical to that of other RhoGAPs.

p68RacGAP Inhibits Rac1-dependent Lamellipodia Formation—One of the cardinal characteristics of Rho GTPases is their role in actin cytoskeletal reorganization. The activation of Rho, Rac, and Cdc42 by extracellular signals has been well characterized in a wide variety of cell types including 3T3 fibroblasts (2). In these cells, bradykinin activates Cdc42 to produce filopodia, platelet-derived growth factor (PDGF) activates Rac to elicit lamellipodia extensions and membrane ruffling, and lysophosphatidic acid (LPA) activates Rho leading to formation of actin stress fibers and formation of associated focal adhesion (19). Thus, Rho, Rac, and Cdc42 regulate three distinct signal transduction pathways linking plasma membrane receptors to the assembly of distinct filamentous actin structures. To test the effect of p68RacGAP on actin cytoskeletal reorganization, we infected NIH/3T3 cells with an adenovirus that bicistronically expresses p68RacGAP and GFP that was then compared with an adenovirus expressing GFP alone. After 16 h of serum withdrawal, cells were challenged for 10 min with 100 ng/ml bradykinin, 10 ng/ml PDGF, or 20 ng/ml LPA. Cells expressing p68RacGAP became insensitive to PDGF under conditions that readily enhance Rac1-induced lamellipodia formation (*arrows*) in the GFP expressing cells (Fig. 5); however, p68RacGAP did not have any effect on bradykinin-induced filopodia extension or LPA-induced stress fiber formation. This result indicates that p68RacGAP specifically inhibits Rac1-mediated cytoskeletal reorganization, further buttressing our observation that p68RacGAP has a Rac1-specific binding and GTPase-activating function.

p68RacGAP Co-localizes with Vezf1 and Inhibits Vezf1-dependent Transcriptional Activation of the Endothelin-1 Promoter—As stated above, p68RacGAP was cloned based on its

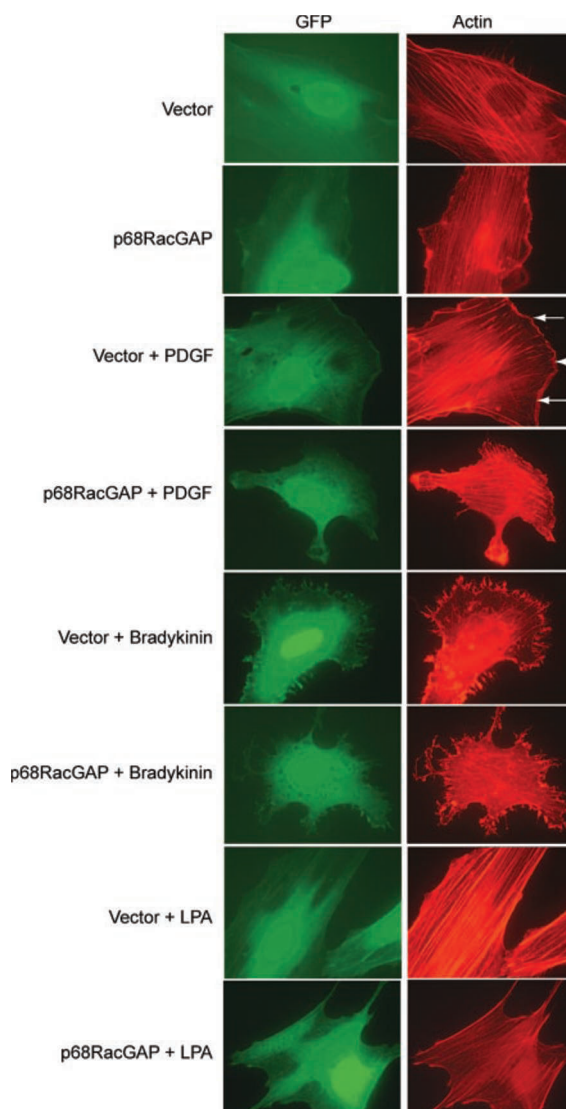


FIG. 5. Effect of p68RacGAP on actin cytoskeletal changes induced by PDGF, LPA, and bradykinin in NIH/3T3 cells. NIH/3T3 cells were infected with adenovirus that bicistronically expresses GFP and p68RacGAP or GFP alone. After 12 h of serum withdrawal, cells were incubated with 10 ng/ml PDGF, 20 ng/ml LPA, or 100 ng/ml bradykinin as indicated for 10 min prior to fixation. Filamentous actin was visualized with Texas Red-conjugated phalloidin staining (*left panels*). GFP was visualized by fluorescence microscopy (*right panels*). *Arrows* indicate lamellipodia.

interaction with the endothelial cell-specific transcription factor Vezf1. We have also shown that p68RacGAP avidly binds and interacts with Vezf1 in non-endothelial and endothelial cells (Fig. 2, B and C). Therefore, we hypothesized that a juxtaposition of both regulatory proteins, possibly by co-localization, might create an avenue for this *in vivo* interaction. This would provide a potential point of interaction between Rac signaling and transcriptional events within the endothelium. We tested this hypothesis by co-transfecting NIH/3T3 cells with GFP-Vezf1 and p68RacGAP or a closely related protein, p50RhoGAP (also known as RhoGAP1, see Fig. 1C) and observing their localization by confocal microscopy. Consistent with our previous observations (13), Vezf1 is localized to the nucleus (Fig. 6, B); in contrast, p68RacGAP and p50RhoGAP are largely localized to the cytoplasm (Fig. 6, A and B, *arrow*). However, when the expression of Vezf1 and p68RacGAP was examined simultaneously, we found that a significant fraction of p68RacGAP co-localized with Vezf1 in the nucleus and that

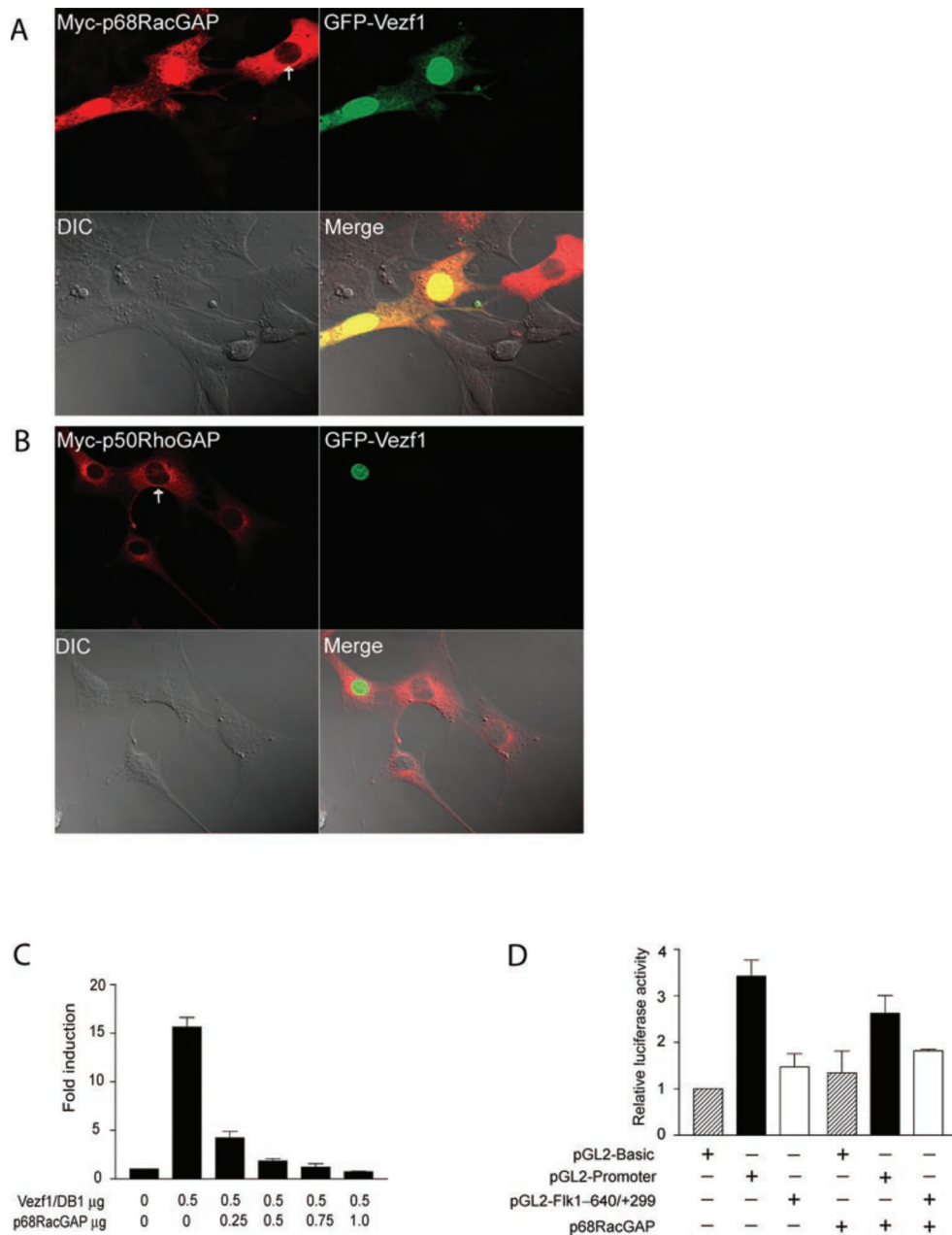


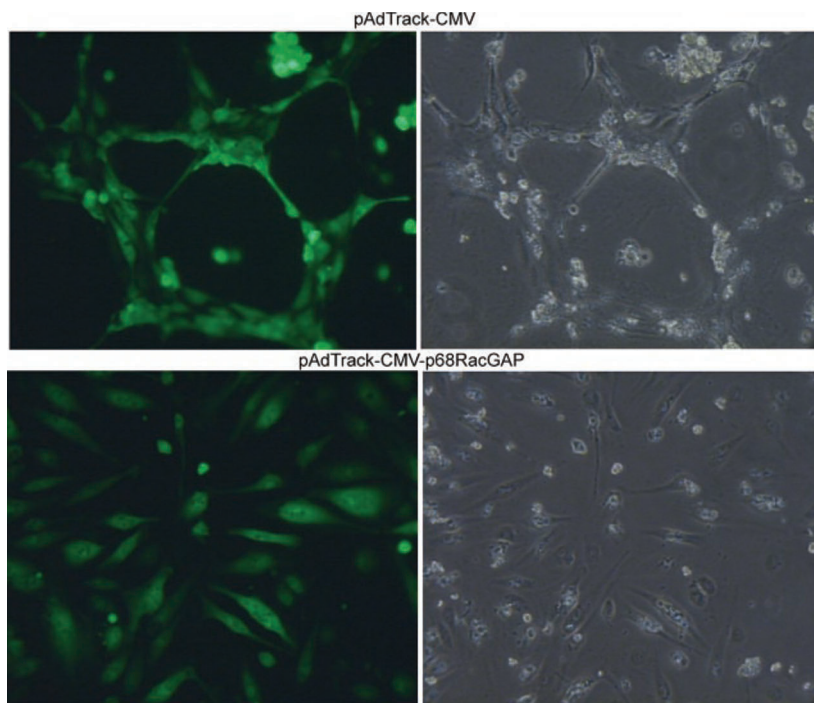
FIG. 6. Subcellular and functional interactions of p68RacGAP and Vezf1. *A* and *B*, NIH/3T3 cells were transfected with GFP-Vezf1 and Myc-p68RacGAP (*A*) or Myc-p50RhoGAP (*B*). Cells were visualized by confocal microscopy. GFP-Vezf1 localizes to the nucleus, while p50RhoGAP and p68RacGAP localize predominantly to the cytoplasm (*arrow* in *A* and *B*). However, co-expression of both Vezf1 and p68RacGAP proteins results in co-localization of p68RacGAP and Vezf1 to the nucleus (indicated by *yellow color* in merged image) and cytoplasmic relocation of a portion of Vezf1 (*A*); however, expression of both Vezf1 and p50RhoGAP did not result in co-localization or translocation of either protein outside of their normal location (*B*). Morphology of cells is indicated by differential interference contrast (*DIC*). *C*, to test whether p68RacGAP affects Vezf1-dependent transactivation of the endothelin-1 promoter activity, MECs were transfected with the indicated amounts of Vezf1 and Myc-p68RacGAP and 1.0 μ g of pGL2-204/+170 (an endothelin-1 promoter-luciferase reporter vector). The vector pCMV- β Gal was used to correct for differences in transfection efficiency. Increasing concentrations p68RacGAP resulted in a dose-dependent transcriptional inhibition of endothelin-1 activity. *D*, to test whether p68RacGAP is a nonspecific transcriptional inhibitor, MECs were transfected with pGL2-Basic, pGL2-Promoter, and pGL2-Flk1 with and without p68RacGAP. As shown, p68RacGAP did not affect their basal transcriptional activity.

a fraction of Vezf1 was localized in the cytoplasm (Fig. 6A). Co-expression of p50RhoGAP and Vezf1 did not affect the intrinsic localization of either protein (Fig. 6B). The results of this experiment indicate that the interaction of p68RacGAP and Vezf1 may affect protein trafficking, suggesting a potential mechanism for interaction of the two regulatory proteins p68RacGAP and Vezf1, and that this interaction may modulate Rac signaling and/or the transcriptional activity of Vezf1.

Since we have previously shown that Vezf1 potently transactivates the endothelin-1 promoter (13), we utilized this transcriptional activity as a model to test the effect of p68RacGAP on

Vezf1-dependent gene regulation. Vezf1 was co-transfected with increasing concentrations of p68RacGAP along with pGL2-204/+170 (an endothelin-1 promoter-reporter vector) into MECs, and the transcriptional activity of the endothelin-1 promoter was determined by measuring normalized luciferase activity. As shown in Fig. 6C, p68RacGAP potently and dose-dependently inhibited the Vezf1-dependent transactivation of the endothelin-1 promoter activity. However, p68RacGAP did not have any effect on the basal activity of pGL2-Basic, pGL2-Promoter, or pGL2-Flk-1 (Fig. 6D), indicating that p68RacGAP is not a nonspecific inhibitor of transcription. This experiment supports our

FIG. 7. p68RacGAP modulates endothelial cells capillary tube formation. HUVECs were infected with an adenovirus that bicistronically expresses GFP and p68RacGAP (*lower panels*) or GFP alone (*upper panels*). Forty-eight hours later, cells were plated in 6-well plates precoated with Matrigel and allowed to form capillary tubes for 12 h. Cells were fixed and examined by fluorescence (*left panels*) and light (*right panels*) microscopy. Cells infected with p68RacGAP are resistant to differentiation and formation of capillary tubes compared with the GFP-only infected cells.



previous observation that p68RacGAP interacts with Vezf1 (Fig. 2, A, B, and C) and indicates that p68RacGAP specifically modulates Vezf1-dependent transcriptional activity.

p68RacGAP Modulates Endothelial Cell Capillary Tube Formation—One of the first steps during vascular development is the differentiation of endothelial cells from pluripotent stem cells, the formation of primitive tubes, and the recruitment of surrounding mesenchymal cells that eventually differentiate into vascular smooth muscle cells. The migration of these cells in the correct spatial and temporal organization ultimately leads to the formation of mature vascular tissues and organs. This complex assembly and patterning of the capillary plexus is regulated by a wide array of developmental cues that lead to morphological and Rho-mediated cytoskeletal change involving disassembly and reassembly of actin structures that provide the structural framework that ultimately defines cell shape and polarity. It has been shown previously that Rac1 regulates endothelial capillary tube assembly (8). Based on our expression analysis and functional characterization, we sought to determine whether p68RacGAP might modulate Rac1-dependent endothelial cell assembly into capillary tubes. We infected HUVECs with adenovirus co-expressing p68RacGAP with GFP or GFP alone. Forty-eight hours later, cells were trypsinized and plated on 6-well plates precoated with Matrigel (Fig. 7). GFP-infected cells formed a network of capillary tube-like structures that recapitulates branching morphogenesis of angiogenesis (*upper panels*); however, the p68RacGAP-infected HUVECs thrived but were resistant to formation of capillary tube-like structures under the same conditions that stimulated endothelial cell differentiation in GFP-infected cells (*lower panels*). This experiment suggests that p68RacGAP inhibits Rac1 and prevents the formation of protrusive lamellipodia formation necessary for the coordinated migration of endothelial cells in the presence of the appropriate extracellular signal.

DISCUSSION

We have taken a molecular and cellular approach to understand the function of Vezf1, a developmentally regulated endothelial cell-specific transcription factor, as part of a larger effort to understand the molecular steps that determine pattern-forming events that result in creation of blood vessels. In ad-

dition to defining *cis*-acting elements and downstream target genes (13), we have searched for interaction networks that may explain how Vezf1 contributes to angiogenesis. In the course of these studies, we have discovered p68RacGAP, a Rac-specific GTPase that interacts with Vezf1 *in vitro* and *in vivo* and that also inhibits endothelial cell migration and tube formation.

The RhoGAP proteins, like Rho GTPases, are found in eukaryotes ranging from yeast to humans, suggesting an evolutionarily conserved role in eukaryotic cell regulation. Since the identification of the Breakpoint cluster region protein, BCR, as a RhoGAP in 1991 (20), the number of RhoGAPs (about 80) outnumbers the 25 Rho GTPase substrates (3). This suggests that multiple GAP proteins have a specific role in regulating distinct functions of each GTPase. The importance of the regulatory function of GAP proteins is underscored by the loss of GAP function due to mutation of the tumor suppressor neurofibromin 1, a GAP that is a negative regulator of the Ras signal transduction pathway. Patients with mutations in neurofibromin 1 are at increased risk of developing nervous system neoplasms seen in type 1 neurofibromatosis (21). In addition, mutations in oligophrenin-1, a RhoGAP protein that affects cell migration and morphogenesis, leads to X-linked mental retardation (22). Finally recent studies of p190 RhoGAP showing that RhoGAPs are required for axon outgrowth, guidance, and fasciculation further underscore the necessary role for these proteins in cellular physiology (23). By analogy, it is likely that p68RacGAP regulates a subset of endothelial events regulated by Rac activity or that it couples Rac activation to other events within the vasculature. Given our observation that increasing p68RacGAP levels inhibit endothelial cell tube formation in cultured cells (Fig. 7), endothelial cell migration and angiogenesis are among the promising key cellular and physiologic processes regulated by this new GAP.

Although the present studies focused on cell migration in the context of angiogenesis as a function of Rac within the endothelium, it is important to recognize that Rac has multiple roles within the endothelium, any of which may be specifically modulated by RacGAPs such as p68RacGAP. Rac1 and Rac2 are necessary components of the vascular NADPH oxidase that produces an oxidative burst in response to mitogenic stimuli

and that is required for signaling downstream of thrombin and other vascular growth factors (24). Integrin-specific cell cycle progression in endothelial cells is controlled by activation of Rac (25), and expression of endothelial chemoattractant proteins is also Rac-dependent (26). It is likely that individual RacGAPs function in distinct Rac-dependent cellular events in the endothelial compartment. Remarkably such a model has not yet been tested extensively for any RacGAP family member, and almost nothing is known about the role of specific RacGAPs in phenotypic modulation of endothelial cells. Analysis of p68RacGAP may therefore provide an important method to understand how Rac-dependent signaling contributes to diverse but tightly regulated events within the endothelium.

Our studies indicate that p68RacGAP is both a GAP for Rac family members and a Vezf1 interaction partner; these disparate activities allow us to build a model that can form the basis for additional studies to refine the function of this new protein. It is tempting to speculate that p68RacGAP provides a tool for integrating the regulation of several levels of signaling events within the endothelium. In particular, our data suggest that p68RacGAP can dually inhibit Rac signaling and Vezf1-dependent transcriptional events. Although the function of Vezf1 within the endothelium is incompletely understood, studies to date suggest a role for this transcription factor in regulation of developmental angiogenesis and perhaps vasomotor tone in adulthood (11, 13). Thus, p68RacGAP may allow endothelial cells to arrest angiogenic responses at multiple key steps and also to coordinate signaling and transcription during phenotypic modulation of blood vessels. Interactions between Rho signaling and transcription during vascular smooth muscle cell differentiation have recently been demonstrated in studies examining the influence of Rho-dependent cytoskeletal rearrangements on serum response factor activation (27), providing a precedent for such cross-talk in vascular cells. However, despite the fact that proteins with GAP activity are frequently dependent on protein-protein interactions to regulate their activity, interactions with transcription factors have not previously been recognized in any biological context (6). To our knowledge, the association of Vezf1 and p68RacGAP represents the first example.

One puzzle in such a model is where and when p68RacGAP interacts with a binding partner such as Vezf1. Our confocal microscopy data indicate that p68RacGAP is predominantly distributed within the cytoplasm (Fig. 6A), which would be consistent with its role in regulating Rac activity. We have previously shown that Vezf1 is predominantly nuclear localized (13); however, our current data show that interaction of both regulatory proteins may lead to trafficking of both proteins outside of their usual cellular compartment. It is possible that p68RacGAP and/or Vezf1 undergo nucleocytoplasmic shuttling under appropriate conditions to maximize the possibility of interaction. However, we also observed a highly reproducible overlap in expression of Vezf1 and p68RacGAP in the nuclear space even in the absence of specific stimulation (Fig. 6A). This expression pattern is reminiscent of the interaction between Vezf1 and another protein in the Rho signaling family, RhoB,

that was recently demonstrated by Prendergrast and colleagues (28). RhoB interacts with Vezf1 only in the perinuclear space, and RhoB inhibits Vezf1-dependent transcriptional regulation. Taken together with the experiments presented here, these data suggest that Vezf1 is particularly receptive to regulation that occurs in and adjacent to the nuclear membrane; perhaps the nuclear and perinuclear space represents a previously unsuspected locus for enriched activity of Rho family members particularly when they are required to modulate transcriptional activity. Although we did not test the reverse possibility in our present studies, it is equally plausible that reciprocal interactions also exist and that activation of Vezf1 also affects Rac activity. These studies will be possible and potentially informative when more is known about how Vezf1 is regulated at the cellular level.

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