

RhoG Signals in Parallel with Rac1 and Cdc42*

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RhoG is a member of the Rho family of small GTPases and shares high sequence identity with Rac1 and Cdc42. Previous studies suggested that RhoG mediates its effects through activation of Rac1 and Cdc42. To further understand the mechanism of RhoG signaling, we studied its potential activation pathways, downstream signaling properties, and functional relationship to Rac1 and Cdc42 *in vivo*. First, we determined that RhoG was regulated by guanine nucleotide exchange factors that also activate Rac and/or Cdc42. Vav2 (which activates RhoA, Rac1, and Cdc42) and to a lesser degree Dbs (which activates RhoA and Cdc42) activated RhoG *in vitro*. Thus, RhoG may be activated concurrently with Rac1 and Cdc42. Second, some effectors of Rac/Cdc42 (IQGAP2, MLK-3, PLD1), but not others (e.g. PAKs, POSH, WASP, Par-6, IRSp53), interacted with RhoG in a GTP-dependent manner. Third, consistent with this differential interaction with effectors, activated RhoG stimulated some (JNK and Akt) but not other (SRF and NF- κ B) downstream signaling targets of activated Rac1 and Cdc42. Finally, transient transduction of a tagged Rac1(17N) dominant-negative fusion protein inhibited the induction of lamellipodia by the Rac-specific activator, Tiam1, but not by activated RhoG. Together, these data argue that RhoG function is mediated by signals independent of Rac1 and Cdc42 activation and instead by direct utilization of a subset of common effectors.

The Rho family of small GTPases constitutes a major branch of the Ras superfamily of proteins, and like the other Ras-like GTPases, they function as GDP/GTP-regulated molecular switches where the GTP bound form is active and the GDP bound is inactive (1, 2). Proteins in the Rho family are activated by guanine nucleotide exchange factors (GEFs)¹ and inactivated by GTPase-activating proteins (GAPs). GEFs stimulate

the exchange of GDP for GTP on the GTPase. Rho GEFs are also referred to as Dbl family proteins, and all members possess a tandem Dbl homology (DH) catalytic domain and a pleckstrin homology (PH) regulatory domain structure (3, 4). GAPs inactivate Rho proteins via stimulation of their intrinsic GTPase activity (5). Rho family proteins have an additional group of negative regulatory proteins, guanine nucleotide dissociation inhibitors, that both inhibit nucleotide exchange and regulate Rho protein association with membranes (6).

Currently, at least 18 mammalian Rho family GTPases have been identified, and Cdc42, Rac1, and RhoA have been the most extensively studied and characterized. Perhaps the best characterized function of Rho proteins is their ability to regulate the actin cytoskeleton and thereby regulate cell morphology, adhesion, and migration (7, 8). Cdc42 induces actin polymerization and the formation of filopodia in conjunction with its regulation of cell polarity (9–11). Rac1 typically controls cell protrusion through actin polymerization, formation of lamellipodia, and membrane ruffles (12). The characteristic property of RhoA is its stimulation of myosin-based contractility, which in turn controls focal adhesion and stress-fiber formation as well as cellular adhesion and motility (13, 14). In addition, Rho GTPases also regulate gene transcription and cell proliferation and are required for the transforming activity of Ras and other oncoproteins (15, 16).

RhoG is most similar to Rac1 and Cdc42 in sequence identity (72 and 62%, respectively) and function. Earlier studies suggested that RhoG stimulates pathways distinct from those activated by Cdc42 and Rac1. This conclusion was based on the finding that co-expression of activated RhoG together with activated Rac1 and Cdc42 caused a 4-fold enhancement of the transforming activity that was seen with only Rac1 and Cdc42 (17). However, more recent studies support a model in which RhoG mediates similar functions as Rac and Cdc42 by causing the downstream activation of Rac and Cdc42. For example, activated RhoG caused actin cytoskeletal changes in NIH 3T3 cells consistent with simultaneous activation of Rac and Cdc42 (18). Additionally, it was demonstrated that dominant-negative mutants of Cdc42 and Rac1 could block RhoG-induced neurite outgrowth in PC12 cells (19). Finally, transient expression of activated RhoG increased the activation of endogenous Cdc42 and Rac1 (19), suggesting that RhoG signals through Cdc42 and Rac1. The manner in which RhoG causes the activation of Cdc42 and Rac1 was not determined.

Presently, little is known regarding the upstream signals that cause RhoG activation and the effectors of RhoG function. Extracellular stimuli cause activation of Rho GTPase primarily through the activation of Dbl-family GEFs. Several Dbl family members (Vav2, Vav3, and Trio) have been found to activate RhoG *in vitro* (20–22). Because these GEFs also activate Rac, Rac and RhoG may be activated concurrently rather than sequentially. Downstream, little is known about RhoG effector

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¹ The abbreviations used are: GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, Pleckstrin homology; GAP, GTPase activating protein; PCR, polymerase chain reaction; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione S-transferase; GFP, green fluorescent protein; RBD, RhoA-GTP binding domain; PBD, p21 binding domain; GBD, GTPase binding domain; JNK, c-Jun NH₂-terminal kinase; SRF, serum response factor.

binding. In yeast two-hybrid binding analyses, it was determined that RhoG did not interact with Pak1, POR-1, or WASP (18), three binding partners of Rac and/or Cdc42, arguing that RhoG cannot mediate Rac/Cdc42-associated events via the utilization of effectors shared with Rac or Cdc42.

To further evaluate the relationship between RhoG and Cdc42/Rac signaling, we tested the ability of RhoG to bind and become activated by Rac or Cdc42 GEFs, to bind to effectors of Rac and Cdc42, to activate downstream signaling pathways stimulated by Rac and Cdc42, and to determine whether RhoG specifically activates Cdc42 and Rac1. Our results strongly indicate that in our cell system, RhoG mediates its effects independent of an activation of Rac1 and Cdc42.

MATERIALS AND METHODS

DNA Constructs—We isolated cDNA sequences encoding RhoG by polymerase chain reaction (PCR)-mediated DNA amplification from two different human cDNA libraries. Both cDNA sequences were identical to those described previously for wild-type RhoG (Genbank accession no. XM006153). We then utilized this sequence to generate mutant sequences encoding dominant-negative (G15A and T17N) and dominant-activated (Q61L) by using the QuikChange mutagenesis kit (Stratagene). Wild-type and mutated RhoG cDNAs were subsequently subcloned into the pGEX 4T-1 (Amersham Biosciences) bacterial, or pEGFP-C3 (Clontech), and pCDNA3 (Invitrogen) (with an addition of an NH₂-terminal hemagglutinin (HA) tag) mammalian expression vectors. Similarly, bacterial expression vectors for wild-type and mutant (G15/17A and Q61/63L) human Cdc42, Rac1, and RhoA were made by subcloning cDNA sequences encoding these proteins into pGEX 4T-1 for expression of glutathione *S*-transferase (GST)-tagged fusion proteins. cDNA sequences encoding green fluorescent protein (GFP)-tagged activated (Q61L) and dominant-negative (T17N) Rac1 were made by mutagenesis of human wild-type Rac1 using the QuikChange mutagenesis kit (Stratagene) and were then subcloned into pEGFP-C3. cDNA sequences encoding a GFP-tagged fragment of the tandem DH-PH domains of Tiam1 were excised from the Tiam1 DH-PH fragment from the pCGN-Tiam1 DH-PH plasmid vector (23) and subcloned into pEGFP-C1 (Clontech). cDNA sequences encoding a GFP-tagged fragment of the RhoA-GTP binding domain (RBD) of rhotekin, a RhoA-specific effector, was made by PCR-mediated DNA amplification from a pGEX-rhotekin RBD plasmid construct (24) and subcloned into pEGFP-C1. cDNA sequences encoding an HA epitope-tagged fragment of the Cdc42-specific, partial CRIB domain-containing effector IRSp53 ΔSH3 was made by PCR-mediated DNA amplification of the cDNA sequence encoding amino acids 1–364 from a human cDNA library and then subcloned into the pCGN-hygro mammalian expression vector. Mammalian expression vectors for expression of Vav1, Vav2, Dbl, Dbs, Ect2, Lfc, LARG, MLK3, and Rac1(17N) have been described previously by our laboratories (25–33). The following DNA constructs were kindly provided by others: pEGFP ITSN DH-PH (Dr. Wendy Morse-Pruitt, University of North Carolina, Chapel Hill, NC), pCMY-122 (Myc-tagged RhoGIP122) (Dr. Anne Blangy, Centre de Recherches en Biochimie Macromoléculaire, France), pJ3Ω IQGAP2 (Dr. André Bernard, Harvard Medical School, Cambridge, MA), pCGN PLD1 (Dr. Andrew J. Morris, University of North Carolina, Chapel Hill, NC), pCMV6 M Pak1, pCDNA His3(T7) Pak5, pCMV6 M Pak6 (Dr. Jonathan Chernoff, Fox Chase Cancer Center, PA), pYDF30 WASP GBD (Dr. Mark Symons, The Picower Institute for Medical Research, NY), pRK5 POSH RBD and pBabe T7 PAR6 (Dr. Antoine Karnoub, University of North Carolina, Chapel Hill, NC), pCMV-Myc p50RhoGAP (Dr. Jian Zhong, University of North Carolina, Chapel Hill, NC), pTat-HA Rac1(17N) (Dr. Steven F. Dowdy, Washington University, St. Louis, MO), pEGFP Cdc42(61L) (Dr. Mark Philips, New York University, NY), and Myc epitope-tagged Tiam1 C1199 (a constitutively activated truncated variant), pAC 90 m-1 (Gideon Bollag, Onyx Pharmaceuticals).

Cell Culture, Transfections, and Protein Transduction—NIH 3T3 cells were grown in Dulbecco's modified Eagles medium (Sigma) supplemented with 10% bovine calf serum (Sigma) and penicillin/streptomycin/fungizone (Invitrogen). DNA transfections were done by using LipofectAMINE Plus (Invitrogen) by the protocol recommended by the manufacturer. Microinjections were done by plating cells on 35-mm culture dishes with 1.2 mm glass bottoms (MatTek Corp.) 24 h before injection. On the day of injection, normal growth medium was replaced by microinjection medium (50% normal growth medium and 50% of Hank's balanced saline solution buffered with 10 mM HEPES, pH 7.2), and the cultures were placed under phase contrast at 400× on a Nikon

Eclipse TE300 inverted fluorescent microscope, and expression vector plasmids diluted to 25 ng/μl in double distilled H₂O were injected. Nuclear injections were performed using an Eppendorf Microinjection System (InjectMan 5179 and Femtojet 5246, Eppendorf) at a pressure of 20 hPa. To control the injection process and identify microinjected cells, rhodamine-labeled dextran (Sigma) was mixed with the DNA at final a concentration of 10 mg/ml. Approximately 25 cells were injected for each condition. After microinjection, cells were changed back to normal growth medium and incubated at 37 °C in a 10% CO₂ incubator for 3 h before fixing and immunofluorescent staining. For transduction of tat-tagged bacterial fusion proteins, cells were plated on glass coverslips on day 0, LipofectAMINE-mediated transfection with pEGFP RhoG(61L), Rac1(61L), or Tiam1 DH-PH day 1, and tat-Rac1(17N) was added at a concentration of 50 μg/ml in serum-free medium at day 2 for 3 h before cells were fixed and stained. Bacterially expressed tat-tagged protein was purified and delivered to cells (50 μg/ml) as described previously (34). For LipofectAMINE-mediated transduction of GST or GST-Pak1 PBD, the cells were plated on coverslips in 24-well plates and transfected as described above with expression plasmid DNAs encoding the indicated proteins. The day after the transfection, 5 μg of GST or GST-Pak1 PBD protein was mixed with 5 μl of LipofectAMINE and then added to each well in serum-free medium. Two h after the addition of the protein-LipofectAMINE mix, the cells were fixed and stained. Immunofluorescent labeling for the transduced protein showed that at least 90% of the cells had taken up the protein.

GEF and Effector Binding to Rho Proteins—*In vitro* protein interaction analyses between cellularly expressed GEFs or effectors to recombinant Rho proteins was performed as described previously (31). Briefly, epitope-tagged GEFs or effectors were transiently overexpressed in NIH 3T3 cells by LipofectAMINE Plus transfection. Twenty-four h post-transfection, cells were lysed and GST or GST-tagged versions of Rho proteins were bound to glutathione Sepharose beads (Amersham Biosciences) were added to the lysates and rotated for 30 min. The beads were washed three times with lysis buffer, and the bound material was subjected to SDS-PAGE and Western blot analyses with antibodies that recognize the epitope tag associated with each expressed protein (anti-Myc 9E10, Sigma; anti-HA HA11, Covance; anti-GFP, Clontech; anti-T7, Novagen). For GEF binding assays, bacterially expressed protein for nucleotide-free G15A (G17A in RhoA) mutants of the Rho proteins were used. For effector binding assays, the GTPase-deficient, constitutively GTP-bound Q61L (Q63L in RhoA) mutants were used.

In Vitro Exchange Assays—Fluorescence spectroscopic analysis of *N*-methylanthraniloyl-GTP incorporation into GDP-preloaded GST-RhoG was carried out using a FLUOstar fluorescence microplate reader (BMG Lab Technologies) at 25 °C using procedures similar to those described previously (35). Exchange reaction assay mixtures containing 20 mM Tris, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 50 mg/ml bovine serum albumin, 1% glycerol, 500 nM *N*-methylanthraniloyl-GTP (Biomol), and 2 μM GTPase were prepared and allowed to equilibrate by shaking. At the indicated time, bacterially expressed Vav2 DH-PH-CRD (100 nM), Dbs DH-PH (150 nM), Tiam-1 DH-PH (150 nM), or Dbl DH-PH (150 nM) was added and the relative *N*-methylanthraniloyl fluorescence (excitation = 360 nm, emission = 455 nm) was monitored. Experiments were performed in triplicate. The DH-PH proteins were kind gifts from Drs. Michelle Booden (Vav2) and John Sondek (Dbs, Dbl, and Tiam1) (University of North Carolina, Chapel Hill, NC).

Immunofluorescence—NIH 3T3 cells were transiently transfected with expression constructs encoding the indicated protein 24 h before they were fixed with paraformaldehyde, permeabilized with Triton X-100, stained with Texas Red-labeled phalloidin (Molecular Probes), and mounted on slides. Fluorescence microscopy was performed with a Zeiss Axioscope equipped with a MicroMAX 5-MHz cooled charge-coupled device camera (Princeton Instruments) and analyzed using Meta-morph software (Universal Imaging Corp.).

Cdc42/Rac1/RhoA Activity Assays—Assays for the nucleotide bound status of Cdc42, Rac1, and RhoA was performed as described previously (36, 37) by LipofectAMINE transfection of NIH 3T3 cells 24 h before they were lysed and subjected to pull-down assays with either GST-Pak1 PBD (for GTP-bound Cdc42 and Rac1) or GST-rhotekin RBD (for GTP-bound RhoA) bound to glutathione Sepharose beads, run on SDS-PAGE, gels and the subject to Western blot analysis with antibodies specific for Cdc42, Rac1, or RhoA (Transduction Laboratories). To control for equal cellular protein being used in each sample, total cell lysates were also run on gels and subjected to Western blot analysis for the Rho protein tested.

Downstream Signaling Pathway Activation Assays—Assays for acti-

vation of JNK, Akt, and Pak1 were done by transiently transfecting NIH 3T3 cells with the empty vector alone, or vectors encoding activated Rho proteins or GEFs, followed by 20 h of serum starvation in medium supplemented with 0.5% serum prior to lysis in SDS-PAGE sample buffer, separation by SDS-PAGE, and immunoblot analysis for phosphorylated and activated JNK or Akt with phospho-specific antibodies (Cell Signaling catalog number 9271 and 9255, respectively) and total JNK and Akt (Cell Signaling). For Pak1 activation assays, an expression vector for a Myc epitope-tagged Pak1 was co-transfected with the constructs to be tested, to increase the sensitivity of the assay. The remainder of the assay was done as described for JNK and Akt but with a phospho-specific Pak1 antibody (a kind gift from Dr. Jonathan Chernoff, Fox Chase Cancer Center, Philadelphia, PA) and a Myc epitope antibody (9E10, Sigma). Activation of serum response factor (SRF) and NF- κ B was determined by co-transfecting the expression construct to be tested with reporter plasmids in which the luciferase gene is under the control of an SRF- or an NF- κ B-responsive minimal promoter sequence 24 h before lysis of the cells and luciferase activity was analyzed using enhanced chemiluminescence reagents and a Monolight 2010 luminometer (Analytical Luminescence) as described previously (38).

RESULTS

RhoG Can Be Activated by GEFs That Also Activate RhoA, Rac1, and Cdc42—Extracellular stimuli cause activation of Rho GTPases most commonly through activation of Dbl-family proteins. Thus, to better understand the stimuli by which RhoG could be activated in cells, we evaluated the ability of a variety of Dbl family proteins to activate RhoG. For these analyses, we determined the ability of the isolated DH/PH domains of various Dbl family proteins to bind nucleotide-free RhoG or to stimulate the exchange of guanine nucleotide on wild-type RhoG *in vitro* (Fig. 1). Vav1 and Vav2 are two highly related GEFs for Rac and Cdc42 (26, 32), and both bound strongly to RhoG and a DH-PH fragment of Vav2 efficiently stimulated guanine nucleotide exchange *in vitro*. The related RhoA and Cdc42 GEFs, Dbl and Dbs (28, 39), both bound weakly to nucleotide-free RhoG, but only Dbs stimulated guanine nucleotide exchange activity on RhoG *in vitro*. Ect2 has been described as a RhoA and Rac-specific GEF (40) and it also bound weakly to nucleotide-free RhoG. In contrast, the Cdc42-specific GEF intersectin-L (41, 42) did not bind RhoG and the Rac-specific GEF Tiam1 (43, 44) did not bind or stimulate the exchange of nucleotide on RhoG. Finally, the RhoA-specific exchange factors LARG (31) and Lfc (45) did not bind to RhoG. In the cases in which binding and exchange of nucleotide were both tested, we observed a direct correlation between the two assays for all the Rho proteins described. However, one exception involved Dbl, which did bind nucleotide-free RhoG in our assays, although surprisingly did not stimulate the exchange of nucleotide on wild-type RhoG. From these data, we concluded that RhoG can be activated by a variety of GEFs known to activate Rac and/or Cdc42.

RhoG Interacts with Some, but Not All, Rac And Cdc42 Effectors—RhoG has been found previously not to interact with the Rac1 and/or Cdc42-specific effectors Pak1, WASP, and POR-1 when analyzed by two-hybrid binding assays (18). To further evaluate the ability of RhoG to interact with effectors of RhoA, Rac, and/or Cdc42, we performed pull-down analyses using bacterially expressed GST-tagged fusion proteins of activated mutants of Cdc42, Rac1, RhoG, and RhoA with lysates of cells in which Rho protein effectors had been transiently expressed (Fig. 2). Similar to previous observations, we found that RhoG did not bind Pak1 and WASP. In addition, it did not bind to Pak5, Pak6, PAR6, IRSp53, or POSH. Activated RhoG did, on the other hand, bind to the previously reported RhoG-binding fragment RhoGIP122, as well as the Rac/Cdc42-specific effectors MLK3, PLD1, and IQGAP2 (Fig. 2). All four of these interactions were shown to depend on GTP loading of the GTPase, because the binding to wild-type (GDP-loaded) RhoG

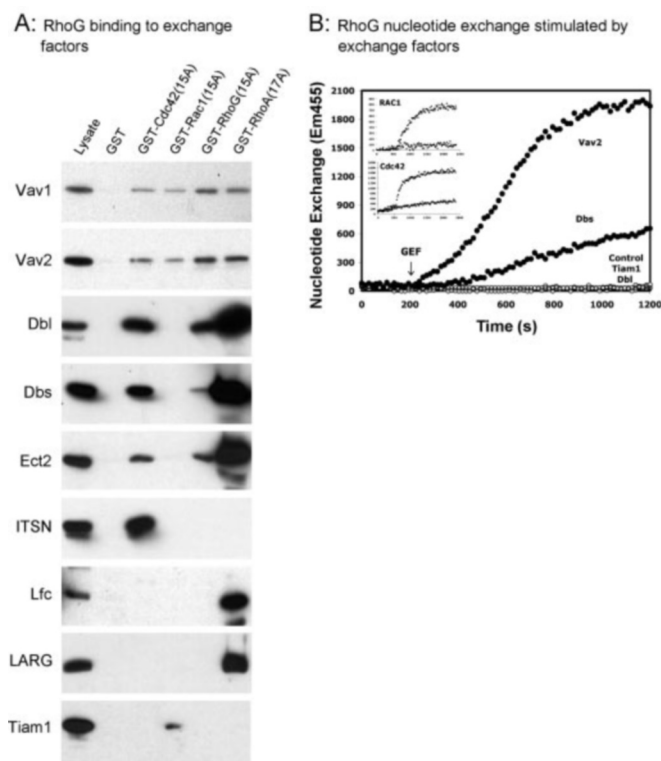


FIG. 1. Dbl family protein interaction with and activation of RhoG. *A*, RhoG association with Dbl family proteins *in vitro*. The indicated Dbl-family proteins were expressed transiently in NIH 3T3 cells as HA- or GFP-epitope-tagged fusion proteins. The cells were then lysed and used to evaluate the ability of bacterially expressed dominant-negative Rho GTPases to form stable complexes. The association of each Dbl family protein was assessed by SDS-PAGE and Western blot analysis using anti-HA or -GFP monoclonal antibody. *B*, Dbl family protein stimulation of guanine nucleotide exchange on RhoG. Fluorescence spectroscopic analysis of *N*-methylanthraniloyl-GTP incorporation into GDP-preloaded recombinant GST-RhoG by bacterially expressed DH/PH domain-containing fragments of Vav2, Dbl, Dbs, and Tiam1 was done as described under "Materials and Methods." Inserts represent nucleotide exchange activity of Tiam1 (Rac1) and Dbl (Cdc42) used in these experiments. Data shown are representative of three independent experiments, with each performed in duplicate.

was strongly diminished. These new potential RhoG effectors include both CRIB-domain containing (MLK3) and non-CRIB (IQGAP2 and PLD1) effectors. Furthermore, we could not detect RhoG binding to the RhoA effector rhotekin or the two RhoGAPs, p50 and p190 (data not shown). These data indicate that RhoG signals, in part, through some of the same downstream effectors as Cdc42 and Rac1.

Activated RhoG and Rac1 Cause Similar Changes in Cell Morphology and Actin Organization—When transiently expressed as a GFP-tagged (Fig. 3) or an HA epitope-tagged (data not shown) protein in NIH 3T3 cells, an activated Q61L mutant (RhoG(61L)) (Fig. 3, *C* and *D*) gave a change in cell shape and actin organization similar to what was seen in cells expressing Rac1(61L) (Fig. 3, *E* and *F*). However, with Rac1(61L), lamellipodia formed in all directions around the periphery of the cell, whereas with RhoG(61L), lamellipodia often developed at several regions but were absent from one end of the cell, resulting in a more polarized appearance. In contrast, expression of Cdc42(61L) caused a limited formation of filopodia (Fig. 3, *G* and *H*), and when co-expressed with Rac1(61L) the two together were unable to reconstitute the polarized morphology of RhoG(61L)-expressing cells (Fig. 3, *I* and *J*). Interestingly, the RhoG(61L)-induced morphology is similar to what was seen when an activated mutant of Vav2, an activator of Cdc42, Rac1, and RhoG, was expressed in these cells (Fig. 3, *K* and *L*),

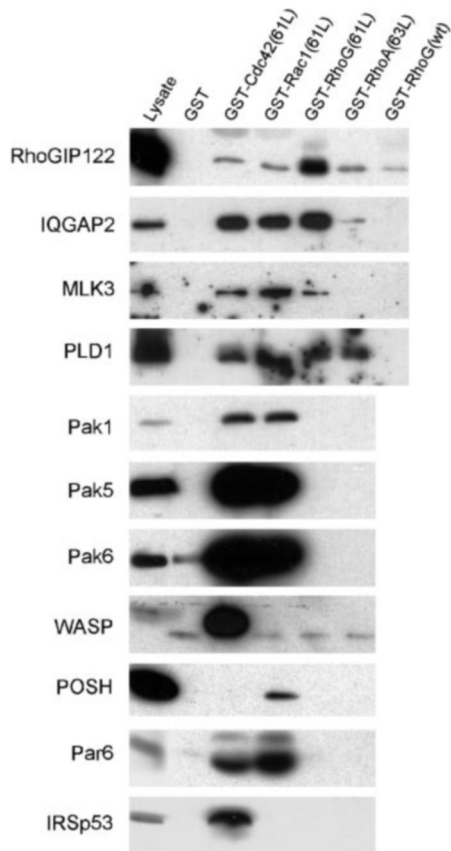


FIG. 2. RhoG binds to effectors of Rac and Cdc42. Expression vectors encoding epitope-tagged full-length or truncated (containing the Rho-binding fragments) of Rho family effectors or GAPs were transiently transfected into NIH 3T3 cells. Cell lysates containing the ectopically expressed proteins were then subjected to pull-down analyses using bacterially expressed GST-tagged constitutively activated mutants of Cdc42, Rac1, RhoG, or RhoA bound to glutathione Sepharose beads. Binding was detected by SDS-PAGE and immunoblot analyses using monoclonal antibodies to detect the epitope-tagged proteins. Both wild-type and GTPase-deficient RhoG were used to evaluate the nucleotide dependence of binding.

indicating that Vav2 might mediate its morphological effects partly through activation of RhoG. These results suggest that RhoG, in part, might regulate cell protrusions by unique mechanisms that do not involve the activation of Rac1 and Cdc42.

Overexpression of Activated RhoG, As Well As Activated Rac1 and Cdc42, Leads to Activation of Endogenous Rac1 and Cdc42—Pull-down analyses showed previously that transient overexpression of activated RhoG in PC12 cells caused activation of endogenous Rac1 and Cdc42, suggesting that in PC12 cells, these two GTPases are targets of downstream signaling from RhoG (19). We extended these analyses to a different cell type and also found that activated RhoG caused activation of endogenous Rac1 and Cdc42 (Fig. 4). To verify the specificity of these activities, we also evaluated the consequences of activated GFP-tagged Rac1(61L) and Cdc42(61L). Surprisingly, these also promoted the apparent activation of endogenous Rac and Cdc42 (Fig. 4), indicating that the effect seen by exogenously expressed activated RhoG in our cells is either a non-specific artifact of overexpression or possibly reflects a physiological feedback loop in which several Rac/Cdc42-like GTPases activate each other. In no case did RhoG(61L), Rac1(61L), or Cdc42(61L) have any effect on the GTP loading of endogenous RhoA (data not shown), indicating that overexpression of activated GTPases may only affect the GTP levels of closely related Rho proteins. Nevertheless, these observations question

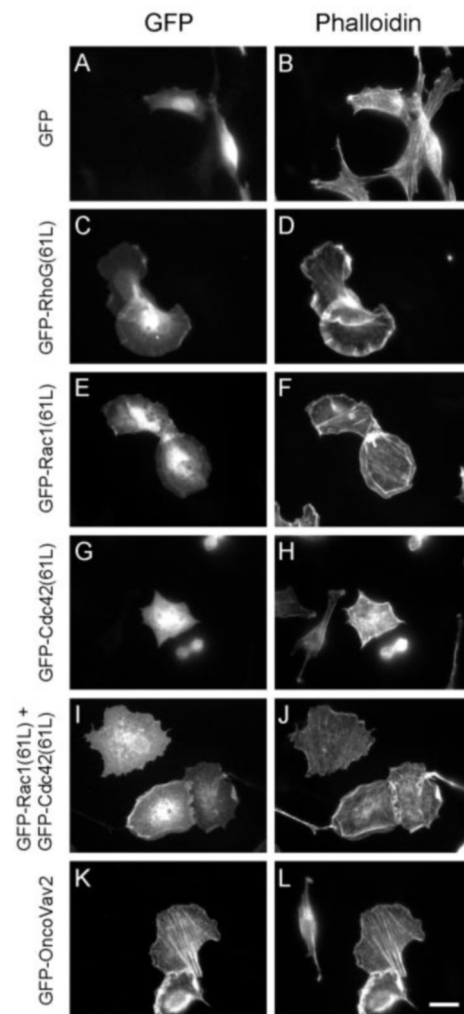


FIG. 3. Morphological effects of GFP-tagged activated RhoG and Rac1 in NIH3T3 cells. NIH 3T3 cells were grown on glass coverslips and were transfected with expression vectors encoding either EGFP alone (A and B), or EGFP fusion proteins of RhoG(61L) (C and D), Rac1(61L) (E and F), Cdc42(61L) (G and H), Rac1(61L) and Cdc42(61L) (I and J), or oncoVav2 (K and L). After 24 h, the cells were fixed and stained with Texas Red-labeled phalloidin to visualize filamentous actin. The coverslips were mounted and analyzed by immunofluorescence for GFP (A, C, E, G, I, and K) or phalloidin (B, D, F, H, J, and L). Bar = 20 μ m.

whether overexpression of activated mutants of GTPases followed by pull-down analysis is a reliable approach to determine whether one Rho GTPase activates another in a cascade.

RhoG 61L Activates JNK and Akt but Does Not Activate SRF and NF- κ B—To further compare the downstream effector interactions of RhoG with those of Rac and Cdc42, we evaluated the ability of RhoG to activate the JNK mitogen-activated protein kinase, the Akt serine/threonine kinase, and the SRF and NF- κ B nuclear transcription factors. NIH 3T3 cells were transiently transfected with either empty vector or expression vector plasmid DNAs encoding RhoG(61L), Rac1(61L), or the DH-PH domain fragment of the Rac-specific GEF Tiam1 and then assayed for the activation of the signaling pathways described above. We included Tiam1 because RhoG activation of Rac would likely involve activation of a Dbl-family protein. Whereas RhoG(61L) activated JNK and Akt to similar levels as Rac1(61L) (Fig 5, A and B), only Rac1 significantly activated SRF- or NF- κ B-dependent gene transcription (Fig. 5, C and D). Tiam1 DH-PH promoted a weak activation of all four of the studied pathways. In addition, whereas Rac1(61L) could activate Pak1, as determined by an activation-specific phospho-

Pak antibody (46), RhoG(61L) did not (data not shown). These data indicate, like the effector binding studies, that RhoG activates some, but not other, signals that are downstream of Rac1.

RhoG Morphology Is Not Blocked by Short Term Introduction of Dominant-Negative Rac1 or the Rac/Cdc42-binding Domain

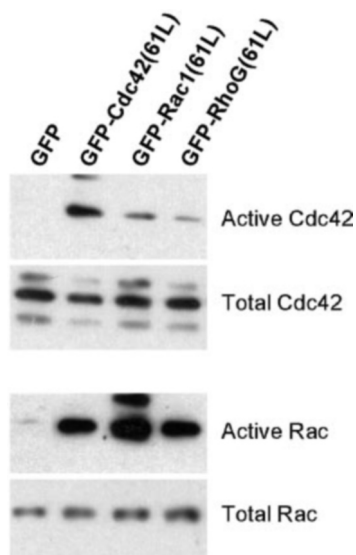
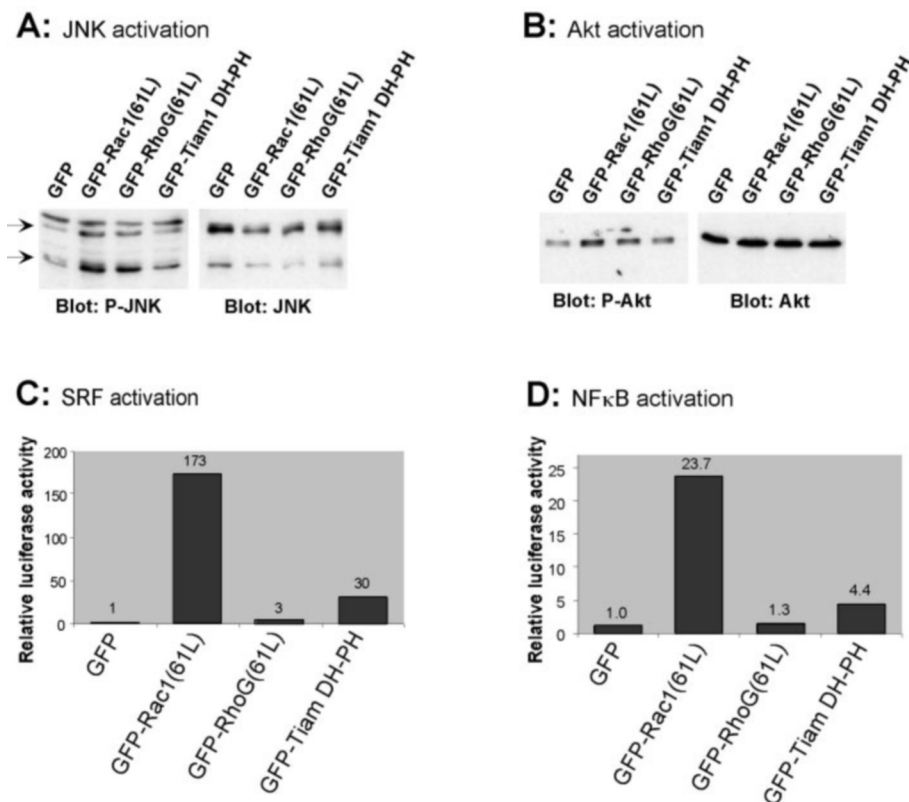


FIG. 4. Activation of endogenous Cdc42 and Rac1 by overexpression of activated Rho-family mutants. NIH 3T3 cells were transiently transfected with the control pEGFP vector alone or with pEGFP vectors encoding Cdc42(61L), Rac1(61L), or RhoG(61L). Twenty-four h post-transfection, cells were lysed and the amount of active, GTP-bound Cdc42 and Rac1 was determined by pull-down analyses using bacterially expressed GST-Pak1 PBD. The relative amounts of GTP-bound and total (from total cell lysates) Cdc42 and Rac1 was determined by immunoblot analyses using anti-Cdc42 and anti-Rac1 antibodies. Data shown are representative of four independent experiments.

of *Pak1*—The morphologic changes caused by activated RhoG were reported previously to be blocked by co-expression of either dominant-negative Rac1 or Cdc42 (18). To test this in our cells, we co-transfected expression vectors for a GFP-tagged RhoG(61L) mutant and an HA-tagged Rac1(17N) mutant, and the cells were studied by immunofluorescence analyses after 18 h. Similar to what has been described previously, we also found that the co-expression of Rac1(17N) inhibited RhoG(61L)-mediated induction of membrane ruffling and lamellipodia (data not shown). As a negative control to evaluate the specificity of these dominant-negative proteins, we tested the effect of RhoG(17N) when co-expressed with RhoG(61L). Because Q61L mutants of Rho proteins are activated constitutively in a GEF-independent manner as a consequence of their impaired intrinsic and GAP-stimulated GTPase activity, their GTP loading should be insensitive to inhibition by dominant-negative T17N mutants. Surprisingly, RhoG(17N) also inhibited RhoG(61L)-induced changes in cell morphology (data not shown). In addition, we saw that the morphological effects of Rac1(61L) could be inhibited by co-expression of either RhoG(17N) or Rac1(17N) (data not shown). To test whether the effects of the dominant mutants were nonspecifically affecting the GTP loading of the co-expressed activated mutants, we performed pull-down analyses for the GTP loading of Rac1(61L) when expressed alone or when co-expressed with Rac1(17N) or RhoG(17N). As expected, the dominant-negative mutants did not lower the GTP loading of Rac1(61L) (data not shown). Therefore, we concluded that the ability of Rac1(17N) to block the actions of RhoG(61L) may not reliably demonstrate that RhoG causes downstream activation of Rac1.

We speculated that the effects of transient overexpression of this dominant-negative protein may cause nonspecific, and possibly cytotoxic effects as a consequence of prolonged overexpression. Therefore, we utilized short-term approaches to evaluate the role of Rac1 activation in RhoG function. For these analyses, we introduced the dominant-negative Rac1(17N) protein for shorter periods of time (2–3 h) by either using a mem-

FIG. 5. RhoG(61L) activates some, but not all, Rac/Cdc42-mediated downstream signaling pathways. NIH 3T3 cells were transiently transfected with expression plasmids encoding GFP, GFP-Rac1(61L), GFP-RhoG(61L), or GFP-Tiam1 DH-PH. Twenty-four h post-transfection, the cells were lysed and assayed for JNK (A) and Akt (B) activation by immunoblot analyses using antibodies that recognize only the phosphorylated and activated forms of JNK (A) or Akt (B). The phospho-specific JNK antibody reacted nonspecifically with an additional protein migrating slower than the two isoforms of JNK (both marked with arrows). Additionally, cells were co-transfected with reporter plasmids where the luciferase gene is under the control of an SRF-responsive (C) or an NF- κ B-responsive (D) minimal promoter sequence. Luciferase activity was determined using a luminometer as described under “Materials and Methods.” The luciferase data presented are the means of triplicate samples from a representative experiment. The expression levels of the GFP-tagged proteins were similar in all cases as determined by immunoblot analyses for GFP (data not shown).



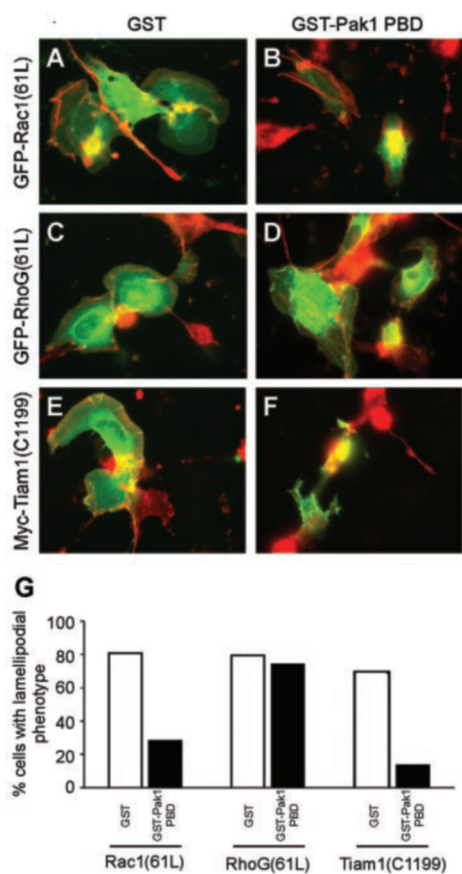


FIG. 6. Tiam1- and Rac1(61L) but not RhoG(61L)-dependent morphology is inhibited by transient transduction of the Rac/Cdc42 binding fragment of Pak1. NIH 3T3 cells were grown on coverslips and were then transfected with expression vectors encoding GFP-tagged Rac1(61L), RhoG(61L), or Myc epitope-tagged Tiam1(C1199). After 24 h, the growth medium was replaced with serum-free medium. Five μ g of either GST (A, C, and E) or GST-Pak1 PBD (B, D, and F) mixed with LipofectAMINE was added to each well. After 2 h, the cells were fixed, stained, and analyzed by immunofluorescence for expression of the GFP- or Myc-tagged proteins (green) and the presence of filamentous actin (red). Bar = 20 μ m. Approximately 200 cells from each condition were scored for expression of a lamellipodial phenotype, and the percentages of cells with this phenotype were calculated (G).

brane-permeable tat fusion-tagged Rac1(17N) protein (data not shown) or by microinjection of a Rac1(17N) expression vector (data not shown), or alternatively, we introduced GST fusion protein containing the isolated Rac/Cdc42-binding fragment of Pak1 (Pak1-PBD) to block Rac and Cdc42-dependent downstream signaling (Fig. 6). When dominant-negative Rac1 was introduced for a short period of time, we did not see loss of either Rac1(61L)- or RhoG(61L)-mediated induction of morphological effects (data not shown). In contrast, the induction of membrane ruffling and lamellipodia caused by expression of GFP-tagged DH-PH fragment of Tiam1 was effectively blocked, indicating that the dominant-negative protein was functional under these assay conditions (data not shown). When we introduced GST-Pak1 PBD, we potently inhibited the morphological effects of both Rac(61L) (Fig. 6, A, B, and G) and constitutively activated Tiam1(C1199) activation of endogenous Rac (Fig. 6, E, F, and G). The RhoG-dependent morphological effects, on the other hand, were largely unaffected by the addition of the Rac/Cdc42-inhibitory fragment (Fig. 6, C, D, and G). These results argue that under our assay conditions, the morphological effects induced by activated RhoG are not dependent on a downstream activation of Rac1.

DISCUSSION

RhoG shares strongest sequence similarity with Rac and Cdc42. Therefore, it is not surprising that RhoG exhibits functions similar to those regulated by Rac and Cdc42. However, previous studies suggested that this overlap in function is not a consequence of RhoG utilization of downstream effector targets shared with Rac and Cdc42. Instead, a model has been described whereby RhoG regulates changes in actin organization and stimulates signal transduction by causing downstream activation of Rac and Cdc42 (18). In the present study, we have evaluated the ability of upstream and downstream regulators of Rac and Cdc42 to regulate RhoG function. First, we determined that some Dbl-family proteins that activate RhoA, Rac1, and Cdc42 also activate RhoG, indicating that RhoG can be activated concurrently with other Rho GTPases. Second, we found that RhoG can interact with some, but not all, effectors of Rac and/or Cdc42. Finally, we determined that dominant-negative Rac1 or the Rac/Cdc42-binding fragment of Pak1 failed to block RhoG signaling under conditions in which Rac activation by Tiam1 was blocked effectively. Taken together, these observations argue that RhoG may also mediate functions shared with Rac and Cdc42 by utilization of common effectors. To date, no extracellular stimuli have been described that cause activation of RhoG via stimulation of GTP binding. Instead, RhoG mRNA expression was shown to be stimulated by serum and peptide growth factors (47). Previous studies had determined that Dbl-family proteins that can cause activation of Rac (e.g. Vav and Trio) also cause activation of RhoG. We extended these observations and found that some (Vav2), but not all (Tiam1), Rac GEFs can activate RhoG. Additionally, we found that the Cdc42 and/or RhoA GEFs, Dbl, Dbs, and Ect, bind to RhoG *in vitro* and could therefore possibly function as RhoG exchange factors *in vivo*. In summary, we have extended the repertoire of Dbl family proteins that can cause activation of RhoG, providing further evidence for RhoG activation by diverse extracellular stimuli.

Consistent with the ability of RhoG to signal in parallel with Cdc42 and Rac1, we found that RhoG showed GTP-dependent binding to some Cdc42/Rac1 effectors (IQGAP2, MLK3, and PLD1). It could therefore be expected for RhoG to signal by direct association with these proteins and trigger their downstream signals. For example, MLK3 mediates Rac1 activation of JNK as well as p70 S6 kinase (33, 48). Hence, RhoG is likely to activate these kinase cascades directly, rather than via indirect activation of Rac or Cdc42. On the other hand, RhoG did not bind to a number of other Rac1/Cdc42 effectors that were tested (Pak1, Pak5, Pak6, WASP, Par6, POSH, and IRSp53). IRSp53, which was originally described to link Rac1 to WAVE/Arp2/3 and lamellipodia formation (49), only bound Cdc42, in agreement with two recent reports (50, 51). Our observation that RhoG can activate some Cdc42/Rac1 downstream pathways, but not others, provides further evidence that RhoG does not mediate its cellular effects solely through activation of Cdc42 and Rac1. If this had been the case, one would have expected that RhoG would cause activation of the same signaling pathways activated by Cdc42/Rac1. For example, RhoA, Rac1, and Cdc42 have all been shown to activate NF- κ B and SRF (52, 53), yet we found that RhoG failed to activate these transcription factors.

One key observation that supported a model in which RhoG mediates its functions by downstream activation of Rac and Cdc42 was that activated RhoG causes increases in the levels of GTP-bound Rac1 and Cdc42. In agreement with these previous studies (19), we also found that transient overexpression of activated RhoG caused increased GTP loading of endogenous Cdc42 and Rac1. However, we also unexpectedly detected a

similar activation of endogenous Cdc42 and Rac1 by overexpressing activated Rac1 or Cdc42. One possible explanation for the apparent increase of GTP-loaded endogenous Rac1 and Cdc42 could be due to the possibility that the overexpressed activated mutants have sequestered GAPs that would normally inactivate the endogenous proteins. An alternative explanation could be that activation of phosphatidylinositol 3-kinase downstream of all three GTPases leads to an activating feedback loop by activating GEFs (54, 55). Either way, these results argue that the ability of RhoG to activate Rac and Cdc42 may not simply reflect the most straightforward interpretation, that RhoG specifically causes downstream activation of Rac and Cdc42. Thus, whether activation of endogenous RhoG can in turn activate Rac and Cdc42 remains unanswered from these studies using constitutively activated and overexpressed Rho GTPases. Clearly, caution must be exercised when using these mutants to define the downstream signaling pathways normally stimulated by the endogenously activated protein.

A second key observation that implicated Rac and Cdc42 activation downstream of RhoG was the demonstrated ability of dominant-negative Rac1 and Cdc42 to block the ability of RhoG to cause actin reorganization. Consistent with these previous studies (18, 19), we also saw that co-expression of dominant-negative Rac1 inhibited the morphology caused by activated RhoG. However, we found that dominant-negative Rac1 blocked the activity of activated Rac1(61L) as well. Because this mutant is activated by a defect in GAP responsiveness and is activated independent of GEF activity, the inhibition caused by Rac1(17N) may be an artifact of overexpression and hence prevent a straightforward interpretation of Rac1 function downstream of RhoG. Although dominant-negative Rho GTPases have been utilized extensively and have shown remarkable specificity of action in many studies, our results here emphasize the potential nonspecific actions of these mutants when expressed at high levels for prolonged time periods. Instead, when we expressed the same dominant-negative of Rac1 for a short period of time and under conditions in which the level of expression can be better controlled, we did not see an effect of Rac1(17N) on the morphology induced by activated forms of RhoG or Rac1, even though it efficiently inhibited the morphology caused by a DH-PH fragment of Tiam1, a Rac-specific GEF. Furthermore, when we did short time period transduction of the Rac/Cdc42-binding fragment of Pak1, which binds to GTP-bound Rac1 and Cdc42, but not RhoG, we observed strong inhibition of lamellipodia formation caused by activated Rac1 and activated Tiam1. RhoG-dependent lamellipodia formation, on the other hand, was unaffected by the addition of this fragment, strongly arguing that the morphological effects caused by activated mutants of RhoG are not mediated by activation of Rac1.

In summary, we conclude that RhoG mediates functions independent of causing activation of Rac and Cdc42. Although it certainly remains possible that such a GTPase cascade may still mediate some functions of RhoG, our findings indicate that overexpression of constitutively activated or dominant-negative Rho GTPases may cause artifactual signaling events that do not accurately reflect the signaling functions of endogenously activated Rho GTPases. Together, our data along with the previous observations that RhoG cooperates with Rac1 and Cdc42 in cellular transformation (17) emphasize that RhoG function can be mediated by its direct interaction with effectors. This raises the question of what effectors for RhoG exist in the cell. In addition to the three potential RhoG effectors that we have described here, IQGAP2, MLK3, and PLD1, two additional effectors have been described. The first one is the fragment RhoGIP122 (22). The full-length protein from which this

fragment is derived has not yet been characterized, so the function of this potential effector is still unknown. More recently, kinectin was identified as a RhoG effector. Kinectin binds RhoG in a GTP-dependent manner (56), possibly coupling RhoG to kinesin and the microtubule cytoskeleton, an observation that is consistent with earlier reports that RhoG localizes to microtubules (18). As suggested by Vignal and coworkers, this could also contribute to the polarized phenotype of cells expressing activated RhoG. Future studies in our laboratories will be aimed at defining the individual roles of the known RhoG effectors as well as searching for new, additional RhoG-specific effectors.

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