Characterization of p190RhoGEF, A RhoA-specific Guanine Nucleotide Exchange Factor That Interacts with Microtubules*

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Rho family GTPases control numerous cellular processes including cytoskeletal reorganization and transcriptional activation. Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) which stimulate the exchange of bound GDP for GTP. We recently isolated a putative GEF, termed p190RhoGEF that binds to RhoA and, when overexpressed in neuronal cells, induces cell rounding and inhibits neurite outgrowth. Here we show that the isolated tandem Dbl homology/pleckstrin homology domain of p190RhoGEF activates RhoA in vitro, but not Rac1 or Cdc42, as determined by GDP release and protein binding assays. In contrast, full-length p190RhoGEF fails to activate RhoA in vitro. When overexpressed in intact cells, however, p190RhoGEF does activate RhoA with subsequent F-actin reorganization and serum response factor-mediated transcription. Immunofluorescence studies show that endogenous p190RhoGEF localizes to distinct RhoA-containing regions at the plasma membrane, to the cytosol and along microtubules. In vitro and in vivo binding experiments show that p190RhoGEF directly interacts with microtubules via its C-terminal region adjacent to the catalytic Dbl homology/pleckstrin homology domain. Our results indicate that p190RhoGEF is a specific activator of RhoA that requires as yet unknown binding partners to unmask its GDP/GTP exchange activity in vitro, and they suggest that p190RhoGEF may provide a link between microtubule dynamics and RhoA signaling.

Some GEFs have been shown to function in specific biological processes: for example, Ect2 regulates cytokinesis (10) while Vav plays a role in adaptive immunity (11). Furthermore, genetic studies have revealed the importance of GEFs in development: DRhoGEF mediates cell shape changes during gastrulation of Drosophila embryos (12), and UNC-73A is required for cell and growth cone migration in Caenorhabditis elegans (13). However, the biological roles of most Dbl family GEFs remain unclear.

In neuronal cells, RhoA mediates neurite retraction and cell rounding in response to G protein-coupled receptor agonists such as lysophosphatidic acid and thrombin (14). Receptor stimulation induces translocation of RhoA from the cytosol to the plasma membrane (15) and subsequent activation of RhoA. RhoA activation and neurite retraction are mediated by the G12/13 subfamily of trimeric G proteins (17) and subsequent activation of RhoA. RhoA activation and neurite retraction are mediated by the G12/13 subfamily of trimeric G proteins (16) whose (17) activated subunits can bind to and activate at least two distinct RhoGEFs, notably p115RhoGEF (18) and PDZ-RhoGEF (19).

We recently isolated a novel RhoA-binding protein of 190 kDa that contains a DH/PH domain and, hence, is a putative GEF for Rho family GTPases (20). We called this protein p190RhoGEF, and showed that it is ubiquitously expressed. When overexpressed in neuronal cells, p190RhoGEF mimics activated RhoA in stimulating cytoskeletal contraction and preventing neurite outgrowth. It remains unclear, however, to what extent the DH/PH catalytic domain of p190RhoGEF is a bona fide activator of RhoA and/or other Rho family members.

p190RhoGEF contains several potential regulatory motifs, in-
including an N-terminal leucine-rich region, a cysteine-rich zinc-finger domain and a C-terminal region that may form an α-helical coiled-coil (Fig. 1).

In the present study, we have characterized p190RhoGEF in further biochemical and cell biological detail. We show that p190RhoGEF is a specific activator of RhoA both further biochemical and cell biological detail. We show that p190RhoGEF may provide a link between microtubule dynamics and RhoA signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 and N1E-115 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics and NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and antibiotics. COS-7 cells were transfected using the DEAE-dextran method, whereas NIH3T3 cells were transfected using LipofectAMINE PLUS (Life Technologies) as described by the manufacturer. For luciferase assays, transfection was stopped after 3 h by switching to culture medium containing 0.5% newborn calf serum.

Plasmids—Cloning of full-length p190RhoGEF (bp 1–5400) and ΔNp190RhoGEF (bp 2156–5400) has been described (20). pcDNA3-HA-p190RhoGEF was generated in two steps. First, a polymerase chain reaction was performed on the full-length p190RhoGEF cDNA, followed by digestion with XbaI-ApaI and subcloning into pcDNA3-HA, resulting in pcDNA3-HA-ΔRhoGEF. Subsequently, an Apal-ApaI fragment of full-length p190RhoGEF (bp 625–5400) was cloned into pcDNA3-HA-ΔRhoGEF resulting in the final construct pcDNA3-HA-p190RhoGEF (bp 105–5400). Removal of a BamHI-NotI fragment from pcDNA3-HA-p190RhoGEF generated ΔCp190RhoGEF (bp 105–4135). The p190RhoGEF-DH/PH (bp 2534–3734) deletion mutant was generated by using primers cccagcgca-tagctagccgggctggg and ccaccaagactggggcctttgtgatgcgctggg. The Y1003A, in pcDNA3-HA-p190RhoGEF using the primers cccagcgcatagctagccgggctggg and ccaccaagactggggcctttgtgatgcgctggg. The endogenous kit (stratagene) was used to make a single-point mutation, Y1003A, in pcDNA3-HA-p190RhoGEF using the primers cccagcgca-taaccagggccgctaggct and cccagcgca-taaccagggccgctaggct. Polymerase chain reaction was performed on the full-length p190RhoGEF cDNA, followed by digestion with SalI and NotI and subcloning into pMT2em-HA. The Quick mutagenesis kit (stratagene) was used to make a single-mutation, Y1003A, in pcDNA3-HA-p190RhoGEF using the primers cccagcgca-taaccagggccgctaggct and cccagcgca-taaccagggccgctaggct. The GST-C terminus (bp 4189–5400) construct was prepared by subcloning a BamHI-EcoRI fragment from pcDNA3-HA-p190RhoGEF into pRP-259, a derivative of pGEX-1N. The construction of GST-C21 (GST-C21), GST-PK-CD (GST-PBD), pMT2em-HA-C1199Tiam1 and SRE. L-luciferase plasmids are described elsewhere (21–24).

Antibodies—A polyclonal anti-Rho-GEF serum, termed antibody 40, was made by immunizing rabbits with a GST fusion protein containing the DH/PH domain of p190RhoGEF (bp 2534–3734). This plasmid was generated by using a SalI-EcoRI fragment from pMT2emHA-DH/PH cDNA into pRPl62, a derivative of pGEX-3X. Rho and Rac proteins were detected with 26C4 (Santa Cruz Biotechnology) and 23A8 (Upstate Biotechnology) monoclonal antibodies. GST was detected with the 2F3 monoclonal antibody. Cytoskeletal structures were analyzed using rhodamine-conjugated phalloidin (Molecular probes) and monoclonal anti-tubulin antibodies (Sigma). A polyclonal anti-tubulin antibody (Sigma) was used for immunoprecipitation.

GDP/GTP Exchange Assay—Recombinant Rho was prepared from Escherichia coli using a bacterial expression system as described previously (25). Single 9-cm dishes of COS-7 cells were transiently transfected with 5 μg of expression plasmid encoding HA-tagged DH/PH domain. In case full-length p190RhoGEF was assayed, 20 dishes were transfected. After 48 h cells were lysed in 300 μl of 0.1 M NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM diethiothreitol, 0.2% Triton X-100 and protease inhibitors. After clearance (13,000 rpm, 10 min), cell lysates were precluded with nonspecific mouse immunoglobulins pre-coupled to protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) and subsequently incubated with 12CA5 monoclonal antibodies coupled to protein A-Sepharose. GDP dissociation from RhoA was assayed exactly as described (26) using 30 μl of slurry (50% beads/buffer) per assay. Control immune complexes were prepared from mock-transfected COS-7 cells.

Fluorescence Measurements—Recombinant proteins of DH/PH domain, truncated RhoA (amino acid residues 1–181), Rac1 and Cdc42 were produced as GST fusion proteins in E. coli strain BL21 (DE3) and purified by glutathione-Sepharose affinity chromatography (Amersham Pharmacia Biotech, Upsalla, Sweden) as described (27). The fluorescent derivative of GDP, mGDP (2',3'-O-(N-methylanthraniloyl)-guanosine 5'-diphosphate), in complex with the respective GTPases was prepared (28). The nucleotide exchange activity of the DH/PH domain was determined on an LS50B Perkin-Elmer spectrofluorometer (Nork-\-walk, CT) using 0.1 μM mGDP-bound GTPase, 20 μM GDP, and different concentration of DH/PH domain in 30 mM Tris-HCl, 5 mM MgCl2, 10 mM KPO4, 3 mM dithioerythritol, pH 7.4, at 25 °C as described for Cdc25 (28). Exponential fits to the data were done using the program Grafit (Eirihcask software).

GTPase Pull-down Assay—Preparation of GST-C21 and GST-PBD and analysis of cellular activation of Rho and Rac was performed as described previously (21, 22). In brief, transfected COS-7 cells were lysed in Nonidet P-40 fish buffer. Lysates were centrifuged to remove debris, and incubated for 45 min at 4 °C with 20 μl of GST-Sepharose loaded with 20 μg of either GST-C21 or GST-PBD. The beads were washed three times with the Nonidet P-40 buffer and the bound proteins were separated by SDS-PAGE and Western blotting.

Complex Formation Assay—To determine interaction between p190RhoGEF and GST-GTPases, transfected COS-7 cells were lysed in a buffer containing 0.1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, and protease inhibitors. Lysates were incubated for 2 h at 4 °C with E. coli expressed, GST-RhoA, GST-Rac, or GST-Cdc42 bound to GST-Sepharose. Samples were washed three times in the 0.1% Triton X-100 buffer and the bound proteins were subjected to SDS-PAGE and Western blotting using monoclonal antibody 12CA5. Experiments testing the nucleotide dependence of the interactions were performed similarly, except that GST-RhoA was preloaded in a buffer containing 20 mM Tris-HCl, 1 mM dithiothreitol, 10 mM EDTA, 5 mM MgCl2, and 50 μM of either GDP or GTPγS for 1 h at 4 °C.

Immunofluorescence—N1E-115 and NIH3T3 cells were grown on glass coverslips. After overnight culturing in serum-free medium, the cells were fixed in 3.7% formaldehyde and were processed for immunofluorescence as described (15) using 12CA5, antibody 40, or anti-RhoA antibodies as indicated. Rhodamine-conjugated phalloidin and anti-tubulin antibody were used to stain F-actin and tubulin, respectively.

Luciferase Assay—Luciferase activities were measured 24 h after the start of the transfection by using the Dual-Luciferase Reporter Assay.
System (Promega) as described by the manufacturer. In the Dual-Luciferase Reporter assay the activities of firefly and Renilla luciferases are measured sequentially from a single sample. Luminescence intensities were measured using the TD 20/20 luminometer (Turner Designs model, Promega) counting the ratio of luminescence between the firefly and the Renilla luciferase reactions.

Microtubule Binding Assay—Transfected COS-7 cells were lysed in tubulin buffer (Cytoskeleton) containing 0.1% Triton X-100. Lysates were centrifuged at 100,000 \( \times g \) for 1 h in a Beckman airfuge. Supernatant fractions were incubated for 30 min at room temperature with purified, Taxol-stabilized microtubules, which were generated using the Microtubule/Tubulin Biochem Kit from Cytoskeleton. Alternatively, purified GST fusion proteins were prepared and incubated with microtubules. Microtubules were pelleted by high-speed centrifugation (100,000 \( \times g \) for 1 h) and supernatant and pellet fractions were subjected to SDS-PAGE and immunoblotting using anti-HA or anti-tubulin antibodies.

Metabolic Labeling—p190RhoGEF was expressed in COS-7 cells as described above. Two days after transfection cells were serum-starved for 1 h, labeled with \( ^{35} \)S-methionine/cysteine for 4 h, and then lysed in a buffer containing 0.2% Triton X-100, 100 mM NaCl, 20 mM Tris, pH 7.4, 1 mM dithiothreitol, 1 mM EGTA, and 5 mM MgCl\(_2\). Lysates were clarified and precipitated with 12CA5 or tubulin antibodies precoated to protein A-Sepharose. Reprecipitation was performed by boiling the immunoprecipitates for 5 min in lysis buffer containing 0.1% SDS, followed by adding 9 volumes of lysis buffer and antibodies precoated to protein A-Sepharose. Precipitates were washed extensively, subjected to SDS-PAGE, and analyzed by autoradiography.

RESULTS

p190RhoGEF: Homology to Rho-Specific GEFs—Fig. 1 shows the structural features of p190RhoGEF in comparison to other Dbl family GEFs. The DH/PH domain of p190RhoGEF is most closely related to the DH/PH domains in the Rho-specific GEFs Brx and proto-Lbc (52% identity), Lfc (50%), GEF-H1 (48%), and p115RhoGEF (25%) (22–26). Aside from its DH/PH domain, p190RhoGEF contains a region with high propensity to form coiled coils, as is also observed in GEF-H1. We note that p190RhoGEF lacks an apparent LH domain that can interact directly with G protein \( \alpha \) subunits, as in p115RhoGEF (18).

p190RhoGEF Activates RhoA, but neither Rac nor Cdc42—We examined whether p190RhoGEF can stimulate GDP/GTP ex-
RhoA was loaded in a buffer containing 10 mM EDTA, 5 mM MgCl₂, and but not RhoA in COS-7 cells (Fig. 2).

Conversely, the Rac-specific GEF Tiam-1 (32) activates Rac-1.

P190RhoGEF in COS-7 cells activates RhoA but not Rac1. PAK (GST-PBD) was used to pull-down activated Rac (31). Fig. 3 shows that p190RhoGEF binds to RhoA but not to Rac1 or Cdc42. Alternatively, GST-RhoA was loaded with either GDP or GTP and RhoA, GST-Rac, and GST-Cdc42 were added to the lysates. After washing, the pull-downs were analyzed for the presence of p190RhoGEF by SDS-PAGE and Western blotting using anti-HA antibody. Blots were reprobed with anti-GST antibody to assess that equal amounts of the GST-GTPases were used. Full-length p190RhoGEF binds to RhoA, but not to Rac or Cdc42. Binding of full-length p190RhoGEF to GST-RhoA was tested as in A. GST-RhoA was depleted from nucleotide by preincubation in a buffer containing 10 mM EDTA (ND). Alternatively, GST-RhoA was loaded in a buffer containing 10 mM EDTA, 5 mM MgCl₂, and 50 μM of either GDP or GTP (GTP). It is seen that full-length p190RhoGEF has a slight preference for interaction with the nucleotide-depleted form of RhoA.

Since p190RhoGEF shows in vitro and in vivo exchange activity toward RhoA, we examined whether the two proteins can form a complex. To this end, we precipitated full-length p190RhoGEF (HA-tagged) from COS-7 cell lysates using immobilized GST fusion proteins of RhoA, Rac1, or Cdc42. As shown in Fig. 3A, we find that p190RhoGEF binds to RhoA but not to Rac1 or Cdc42.

GEFs stimulate GDP/GTP exchange by inducing a conformational change in the GTPase, resulting in the release of bound GDP. GEFs stabilize a transition state, in which interaction occurs with nucleotide-free GTPase. GEFs specific for each of the Rho family GTPases (24). NIH-3T3 cells were transiently cotransfected with pSRE.L and full-length p190RhoGEF. As shown in Fig. 5A, p190RhoGEF mimics active RhoA in inducing the formation of membrane ruffles or filopodia. These findings in 3T3 cells are consistent with the observation that p190RhoGEF induces cytoskeletal contraction and inhibits neurite outgrowth in N1E-115 neuroblastoma cells (20).

In Vitro Activity of Full-length Versus Truncated p190RhoGEF—Aside from the tandem DH/PH domain, p190RhoGEF contains several structural elements that may play a role in regulating its activity and/or intracellular localization. We compared the in vitro exchange activity of the full-length protein to that of the isolated catalytic DH/PH domain. Fig. 4A shows that, while the DH/PH domain promotes GDP/GTP exchange on RhoA, the full-length protein is completely inactive. About equal amounts of proteins were precipitated from the cell lysates (Fig. 4A, lower panel). Even under conditions where the full-length protein was used in excess of the DH/PH domain, exchange activity was only detected with the isolated DH/PH domain (Fig. 4B). Although the Rho-specific GEFs Ect-2 and GEF-H1 are active as full-length proteins (10, 36), the activities of most GEFs have only been tested with the isolated DH/PH domains. Of note, in fibroblast transformation assays, Lbc, Dbl, Ost, and p115RhoGEF are only active when truncated (5, 29, 37–39). The striking difference in in vitro activity between full-length and truncated p190RhoGEF suggests that the full-length protein contains an intrinsic autoinhibitory domain and requires cellular factors to be activated.

p190RhoGEF Induces Actin Stress Fibers and Activates SRF-mediated Transcription—When activated, RhoA, Rac, and Cdc42 induce specific rearrangements of the actin cytoskeleton. In fibroblasts, RhoA induces stress fiber formation, whereas lamellipodia and filopodia are formed in response to activated Rac and Cdc42, respectively. We examined the cytoskeletal changes induced by full-length p190RhoGEF in NIH-3T3 fibroblasts. As shown in Fig. 5A, p190RhoGEF mimics active RhoA in inducing the formation of actin stress fibers, with no sign of the formation of membrane ruffles or filopodia. These findings in 3T3 cells are consistent with the observation that p190RhoGEF induces cytoskeletal contraction and inhibits neurite outgrowth in N1E-115 neuroblastoma cells (20).

In addition to their effects on the actin cytoskeleton, Rho GTPases can modulate gene transcription. In particular, activated forms of RhoA, Rac1, and Cdc42 can activate the serum response factor (SRF) (40). We examined whether p190RhoGEF could activate SRF-mediated transcription by using a reporter plasmid in which the luciferase gene is under the control of a c-Fos serum-responsive promoter element (pSRE.L). This modified SRE element depends on SRF, but not on Elk-1 activity, making it a specific reporter for signaling by Rho family GTPases (24). NIH-3T3 cells were transiently cotransfected with pSRE.L and full-length p190RhoGEF. As

![Characterization of p190RhoGEF binding to RhoA. A, binding of full-length p190RhoGEF to GST-RhoA, GST-Rac, and GST-Cdc42. COS-7 cells were transiently transfected with HA-tagged full-length p190RhoGEF. Cells were lysed 48 h after transfection and GST-RhoA, GST-Rac, and GST-Cdc42 were added to the lysates. After washing, the pull-downs were analyzed for the presence of p190RhoGEF by SDS-PAGE and Western blotting using anti-HA antibody. Blots were reprobed with anti-GST antibody to assess that equal amounts of the GST-GTPases were used. Full-length p190RhoGEF binds to RhoA, but not to Rac or Cdc42. B, binding of full-length p190RhoGEF to nucleotide-depleted (ND) GDP- or GTPγS (GTP) loaded GST-RhoA. Binding of full-length p190RhoGEF to GST-RhoA was tested as in A. GST-RhoA was depleted from nucleotide by preincubation in a buffer containing 10 mM EDTA (ND). Alternatively, GST-RhoA was loaded in a buffer containing 10 mM EDTA, 5 mM MgCl₂, and 50 μM of either GDP or GTP (GTP). It is seen that full-length p190RhoGEF has a slight preference for interaction with the nucleotide-depleted form of RhoA.](image-url)
shown in Fig. 5B, expression of p190RhoGEF results in a 5-fold activation of SRF, similar to what is observed after serum stimulation (Fig. 5B).

We also used a point mutant of p190RhoGEF in which residue Tyr1003 is replaced by an alanine (Y1003A mutant). Tyr1003 is a conserved residue in the catalytic DH domain of all known GEFs; its mutation in Lbc renders this GEF biologically inactive (37). Similarly, mutant p190RhoGEF(Y1003A) is biologically inactive as it fails to promote cytoskeletal contraction when expressed in N1E-115 cells.2 As shown in Fig. 5B, cotransfection of the inactive Y1003A mutant with pSRE.L does not lead to enhanced SRF-mediated transcription, indicating that p190RhoGEF exchange activity (i.e. activation of RhoA) is required for the transcriptional response. Taken together, our findings demonstrate that full-length p190RhoGEF is active in vivo, and acts as a Rho-specific GEF that promotes stress fiber formation, cytoskeletal contraction, and activation of SRF-mediated gene transcription.

Intracellular Localization of p190RhoGEF—To obtain possible clues to the biological role(s) of p190RhoGEF, we analyzed its intracellular localization in neuronal N1E-115 cells by immunofluorescence using polyclonal antibodies (number 40).

2 F. P. G. van Horck, W. H. Moolenaar, and O. Kraneburg, manuscript in preparation.
p190RhoGEF staining, it is seen that both proteins localize to the same regions at the plasma membrane (Fig. 6B). It seems likely that these are the sites where RhoA is activated by p190RhoGEF. Through double labeling experiments, we find that these regions are distinct from CD44- or caveolin-containing plasma membrane subdomains. C, containing of N1E-115 cells with antibody 40 (left panel) and rhodamine-conjugated phalloidin (right panel) shows that the p190RhoGEF positive filaments do not colocalize with actin filaments. D, double labeling with antibody 40 (left panels) and anti-tubulin antibody (right panels) shows that a pool of p190RhoGEF is localized to the microtubular network. In B, C, and D, the left panels show p190RhoGEF staining, middle panels RhoA, F-actin, or tubulin staining, as indicated; the merged staining pattern is shown in the right panels.

The filamentous structures that are stained with anti-p190RhoGEF antibody in a subpopulation of the cells (Fig. 6A, C, and D) do not colocalize with actin filaments, as visualized by rhodamine-conjugated phalloidin (Fig. 6C). When the cells were double-stained with anti-p190RhoGEF and anti-tubulin antibodies, p190RhoGEF is detected at the radial and cortical microtubule systems (Fig. 6D).

**p190RhoGEF Interacts with Microtubules via Its C Termi-nums**—We next examined, by biochemical means, whether p190RhoGEF associates with microtubules. To this end, we used an in vitro assay system using purified, Taxol-stabilized microtubules and cytosolic COS-7 cell extracts containing either full-length p190RhoGEF or deletion mutants lacking either the N- or C-terminal regions. Since microtubules can be pelleted by high-speed centrifugation, binding of p190RhoGEF to microtubules can be tested in co-sedimentation assays. Fig. 7A (left panel) shows that, in the absence of microtubules, full-length p190RhoGEF and the ΔN and ΔC truncation mutants remain in the high-speed supernatant. In the presence of microtubules, however, we find that both full-length p190RhoGEF and the ΔN mutant, but not the ΔC mutant, cosediment with microtubules during high-speed centrifugation (Fig. 7A). These results suggest that p190RhoGEF binds to microtubules, either directly or indirectly, and that the C-terminal region is essential for this interaction.

To determine whether the interaction of p190RhoGEF with microtubules is direct or indirect, GST fusion proteins were made that encompass either the complete C-terminal part (deleted in ΔC; residues 1343–1693) or the catalytic DH/PH domain (residues 811–1210). Using the purified fusion proteins in a microtubule cosedimentation assay, we find that the C- minus of p190RhoGEF cosediments with microtubules, whereas the DH/PH domain does not (Fig. 7B). Taken together, these experiments indicate that a pool of p190RhoGEF directly interacts with microtubules in vivo, and that microtubule interaction is mediated by the C-terminal domain of p190RhoGEF.

**In Vivo Interaction of p190RhoGEF and Tubulin**—To determine whether p190RhoGEF interacts with microtubules in intact cells, we immunoprecipitated tubulin from metabolically labeled COS-7 cells, overexpressing either full-length p190RhoGEF or the deletion mutant which lacks the C-terminal domain (RhoGEFΔC). Fig. 8 shows that the tubulin antibody coprecipitates p190RhoGEF, but not the ΔC deletion mu-
p190RhoGEF and anti-tubulin. It is seen that full-length and PAGE and immunoblotting using anti-HA antibodies to detect tion. Supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE and immunoblotting using anti-HA antibodies to detect p190RhoGEF (mutants) were allowed to bind for 30 min at room tempera-ture. Microtubules were then pelleted by high-speed centrifuga-

FIG. 7. Direct binding of p190RhoGEF to microtubules. A, cosedimentation of p190RhoGEF (mutants) and microtubules. COS-7 cells were transiently transfected with HA-tagged full-length, ΔC-, or ΔN-RhoGEF. Cells were lysed 48 h after transfection and cytosolic extracts were prepared by high-speed clearance of the lysates. Purified, Taxol stabilized microtubules were added where indicated and p190RhoGEF (mutants) were allowed to bind for 30 min at room temperature. Microtubules were then pelleted by high-speed centrifugation. Supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE and immunoblotting using anti-HA antibodies to detect p190RhoGEF and anti-tubulin. It is seen that full-length and ΔN-, but not ΔC-, RhoGEF cosediment with microtubules. B, GST fusion proteins encompassing either the complete C-terminal region or the isolated DH/PY domain of p190RhoGEF (see schematic representation) were incubated with purified microtubules and used in a cosedimentation assay as in A. The C terminus of p190RhoGEF cosediments with microtubules.

tant. To verify that the 190-kDa protein is indeed HA-tagged RhoGEF, the immunoprecipitates were denatured and re-precipitated using anti-HA antibodies. Fig. 8 shows that anti-HA antibodies indeed re-precipitate full-length p190RhoGEF from tubulin immunoprecipitates. Thus, in agreement with the immunofluorescence studies and the in vitro assays, p190RhoGEF interacts with tubulin in intact cells via its C-terminal domain.

Microtubule Dynamics and p190RhoGEF Activity—Microtu-
bule dynamics and F-actin reorganization are interconnected via Rho family GTPases (41). For example, microtubule growth activates Rac1 (42), whereas microtubule disruption leads to RhoA activation (43). Thus, the observed interaction of p190RhoGEF with microtubules may serve to link microtubule dynamics to RhoA activation. To test this, we employed the RhoA activation pull-down assay. Full-length p190RhoGEF was expressed in COS-7 cells and the cells were treated with nocodazole to disrupt microtubules or were left untreated. As shown in Fig. 9, both nocodazole treatment and expression of p190RhoGEF lead to activation of endogenous RhoA. However, we find that microtubule disruption does not affect the ability of overexpressed p190RhoGEF to activate RhoA. This is not due to maximal RhoA activation in these cells since overexpression of the isolated DH-PH domain leads to a more prominent activation of RhoA. Similarly, the addition of Taxol-stabilized microtubules to either full-length p190RhoGEF or the isolated DH/PY domain in in vitro exchange reactions did not affect RhoGEF exchange activity (not shown). Further work is needed to establish how microtubule binding may influence p190RhoGEF action or vice versa.

DISCUSSION

We have shown that p190RhoGEF is a RhoA-specific GEF and thus belongs to the same subclass of Dbl family GEFs as Lbc, Lfc, GEFH-1, and p115RhoGEF. This conclusion is based upon various lines of evidence. First, the catalytic core domain, RhoGEF-DH/PY, promotes GDP release from RhoA, but not from Rac or Cdc42, in vitro. Second, binding assays show that p190RhoGEF interacts with RhoA, but not with Rac or Cdc42. Third, we find that RhoGEF-DH/PY activates endogenous RhoA but not Rac1 in intact cells, as measured by pull-down assays. Finally, p190RhoGEF mimics activated RhoA in stimulating stress fiber formation and SRF-mediated transcription in 3T3 cells.

Dbl family GEFs show tissue-specific distribution patterns and distinct substrate specificities (30). In addition, preserved structural motifs in distinct GEF family members have presumably evolved to couple specific upstream signals to activation of small GTPases. Since full-length p190RhoGEF is inactive in vitro, p190RhoGEF activity must be regulated by cellular factors. We and others have shown that RhoA activation by G protein-coupled receptors is mediated via the G12/13 family of heterotrimeric G proteins (16, 44, 45). Indeed, G12(12) and G13 can directly bind to both p115RhoGEF and PDZ-RhoGEF via the N-terminal LH domain present in both GEFs. The interaction of p115RhoGEF with the G13 subunit results in an activation of p115RhoGEF exchange activity (18, 46). The p190RhoGEF sequence, however, does not contain an LH domain. Moreover, we have not been able to detect an interaction between p190RhoGEF and either G12 or G13 in transfected COS-7 cells. It thus seems unlikely that p190RhoGEF is activated by G12/13 subunits.

Our findings further indicate that a pool of p190RhoGEF may be regulated through direct interaction with microtubules. We detect interaction with microtubules in intact cells and find that it is mediated via a C-terminal region in p190RhoGEF. Three other GEFs, notably GEFH-1, Lfc, and Ect2, have also been reported to localize to microtubules (10, 36, 47). Changes in microtubule stability are known to impinge on Rho signaling (41); microtubule growth activates Rac (42), whereas microtu-
bule destabilization promotes RhoA activation (43). It has been suggested that an unidentified RhoA activator resides on mi-crotubules from which it may be released upon microtubule depolymerization (41). One obvious candidate is p190RhoGEF, although our preliminary studies do not show enhanced p190RhoGEF-mediated activation of RhoA in microtubule-disrupted cells. However, these experiments should be interpreted with care. Subtle changes in microtubule stability may locally regulate microtubule-bound p190RhoGEF and such effects could easily be missed in nocodazole-treated cells that overexpress p190RhoGEF. Moreover, nocodazole affects a number of signaling pathways that may directly or indirectly influence p190RhoGEF or RhoA activity (48). An alternative possibility is that microtubule binding activates p190RhoGEF, although our preliminary experiments to test this possibility have remained inconclusive. Interestingly, it has recently been re-

3 F. P. G. van Horck, W. H. Moolenaar, and O. Kranenburg, unpublished observations.
ported that activation of RhoA causes rapid stabilization of a subset of microtubules via an as-yet-unknown mechanism (49). One possibility that remains to be examined is that p190RhoGEF may function as a "scaffold" to bring microtubules into proximity with activated RhoA and thereby regulates RhoA-mediated stabilization of microtubules.

Although we do not know which upstream signals regulate p190RhoGEF activity, we find that in neuronal cells p190RhoGEF and RhoA localize to the same regions at the plasma membrane. Presumably, these are the sites of RhoA activation by p190RhoGEF. One possibility is that these regions are equivalent to "puncta," as described in epithelial cells (50), which are plasma membrane regions where microtubules terminate.

The localization of p190RhoGEF to microtubules could also allow it to regulate microtubule-bound effectors distinct from RhoA. Such an additional signaling mode has been reported for Lfc, which can activate Jun N-terminal kinase (JNK) in a Rac-dependent manner without promoting GDP/GTP exchange on Rac (47). A possible link between p190RhoGEF and JNK comes from a recent study showing that p190RhoGEF can directly bind to a JNK-interacting protein, termed JIP-1 (51). JIP-1 serves as a scaffold protein and selectively mediates signaling by the "mixed-lineage" kinase to JNK activation (52).

Like p190RhoGEF and Lfc, mixed-lineage kinase and JNK both localize to microtubules (47, 53). However, we have not been able to detect changes in JNK activity following overexpression of p190RhoGEF in COS-7 cells. Therefore, it remains unclear whether the localization of p190RhoGEF at microtubules and its binding to JIP are functionally coupled. In any case, the possibility that p190RhoGEF may control a wider range of cellular processes than those predicted by its GDP/GTP exchange activity should be taken into account.

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FIG. 8. Coimmunoprecipitation of p190RhoGEF and tubulin. COS-7 cells were transiently transfected with empty vector, HA-tagged full-length p190RhoGEF, or deletion mutant RhoGEFΔC. Cells were metabolically labeled with [35S]methionine/cysteine and lysed 48 h after transfection. Tubulin and HA-RhoGEF were immunoprecipitated (IP) using anti-tubulin (T) and anti-HA (HA) antibodies, respectively, and either subjected to SDS-PAGE or denatured and then re-precipitated using anti-HA antibodies, as described under "Experimental Procedures." It is seen that full-length p190RhoGEF, but not RhoGEFΔC, coimmunoprecipitates with tubulin.

FIG. 9. Effect of microtubule disruption on p190RhoGEF-mediated RhoA activation. COS-7 cells were transiently transfected with empty vector, HA-tagged full-length p190RhoGEF, or DH/PH domain as indicated. Cells were cultured overnight in serum-free medium and were treated with nocodazole where indicated (1 h, 10 µM). Immunofluorescence analysis using anti-tubulin antibodies showed that this results in a complete disappearance of the microtubular network (not shown). The activity state of RhoA was determined as in Fig. 2B. Both p190RhoGEF as well as nocodazole induce activation of RhoA but p190RhoGEF-mediated activation of RhoA is not potentiated in cells lacking microtubules.
Characterization of p190RhoGEF

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