p200 RhoGAP Promotes Cell Proliferation by Mediating Cross-talk between Ras and Rho Signaling Pathways*

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p200 RhoGAP, a member of the Rho GTPase-activating protein (RhoGAP) family, was previously implicated in the regulation of neurite outgrowth through its RhoGAP activity. Here we show that ectopic expression of p200 RhoGAP stimulates fibroblast cell proliferation and cell cycle progression, leading to transformation. The morphology of the foci induced by p200 RhoGAP is distinct from that formed by Rac or Rho activation but similar to that induced by oncogenic Ras, raising the possibility that p200 RhoGAP may engage Ras signaling. Expression of p200 RhoGAP results in a significant increase of Ras–GTP and the activation of two downstream signaling pathways of Ras, ERK1/2 and phosphatidylinositol 3-kinase. Inhibition of Ras or ERK1/2, but not phosphatidylinositol 3-kinase, effectively suppresses the foci formation induced by p200 RhoGAP, suggesting that the Ras-ERK pathway is required for p200 RhoGAP-mediated cell transformation. p200 RhoGAP co-localizes with p120 RasGAP in cells and forms a complex with p120 RasGAP, and this interaction is mediated by the C-terminal region and the Src homology 3 domain of p200 RhoGAP and p120 RasGAP, respectively. Mutations of p200 RhoGAP that disrupt interaction with p120 RasGAP abolish its Ras activation and cell transforming activities. Interestingly, the RhoGAP activity of the N-terminal RhoGAP domain in p200 RhoGAP is also required for its full transforming activity, and expression of a dominant negative RhoA mutant that blocks RhoA cycling between the GDP- and GTP-bound states suppresses p200 RhoGAP transformation. These results suggest that a Rho GTPase-activating protein may have a positive input to cell proliferation and provide evidence that p200 RhoGAP can mediate cross-talks between Ras- and Rho-regulated signaling pathways in cell growth regulation.

Rho GTPases are intracellular binary molecular switches cycling between the active, GTP-bound and the inactive, GDP-bound states (1–3). Upon activation, Rho GTPases interact with a large number of downstream effectors that regulate a variety of cellular processes, including actin cytoskeleton rearrangement, transcriptional activation, cell growth, cell survival, and vesicle trafficking (4, 5). The cycling between the GDP- and GTP-bound states of Rho GTPases is thought to be regulated by at least three distinct families of regulatory proteins; the Rho guanine nucleotide exchange factors (GEFs) promote GDP release and GTP binding (6), the Rho guanine nucleotide dissociation inhibitors sequester Rho GTPases in the GDP bound states and interfere with their membrane association (7), and Rho GTPase-activating proteins (GAPs) accelerate the intrinsic GTP-hydrolytic activity to produce the GDP-bound, inactive Rho GTPases (8).

RhoGAPs are defined by the presence of a conserved, ~150-amino acid sequence termed the RhoGAP domain. The RhoGAP domain is necessary and sufficient for binding to the GTP-bound Rho GTPases and stimulating their GTPase activity (8). In addition to the RhoGAP domain, multiple functional domains are found in RhoGAPs that are expected to participate in the spatiotemporal regulation of RhoGAP function. In particularly, SH3 domains and proline-rich motifs are frequently present in RhoGAP family members and may recruit intracellular protein partners to modulate RhoGAP domain activity (9). So far most studies of RhoGAPs have heavily relied on the expression of the RhoGAP domain in assessing their cellular functions. This approach can produce misleading results, since it is possible that full-length RhoGAP may display distinct cellular phenotypes from the RhoGAP domain, as is shown in the case for a Rac1-specific RhoGAP, n-chimaerin. Microinjection of the GAP domain of n-chimaerin inhibited Rac-stimulated lamellipodia formation, whereas full-length n-chimaerin cooperated with Rac1 to mediate lamellipodia and filopodia formation (10). It is thus likely that additional functional motifs in individual RhoGAPs are important in mediating multiple protein-protein or protein-lipid interactions to elicit physiologically relevant cell activities.

RhoGAPs are involved in various biological processes, such as neuronal morphogenesis, cell growth and transformation, and polarity establishment by down-regulation of Rho GTPases (8). Given their role in GTP-hydrolysis of Rho GTPases, RhoGAPs are intuitively expected to play a negative, or “tumor suppressor,” role in cell proliferation. Several lines of observation support this possibility. First, RhoGAP-insensitive Rho GTPase mutants that are constitutively GTP-bound can induce trans-

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forming phenotype in NIH 3T3 cells. Subsequent transplantation of these cells into nude mice can cause tumor formation (11, 12). Second, Ras and other oncogenes, such as Bcr-abl and Src, in part depend on Rho GTPase activities for transformation, and elevated Rho-GTP species have been associated with several human tumors, suggesting that increased Rho activities promote tumor cell proliferation, whereas decreased Rho activities may have a negative impact on cell growth (13, 14). Third, several reports have suggested that RhoGAPs may act as potential tumor suppressors by down-regulating Rho GTPase activities (15–17). For example, overexpression of the RhoGAP domain of p190 RhoGAP inhibited Ras-induced transformation in fibroblasts (18), whereas in a mouse model p190 RhoGAP suppressed platelet-derived growth factor-induced glioma formation (19).

We have previously identified a brain-specific RhoGAP, p200 RhoGAP, which was also independently identified and termed Grit by Nakamura et al. (20), GC-GAP by Zhao et al. (21), RICS by Okabe et al. (22), and p250 GAP by Nakazawa et al. (23). We have shown that p200 RhoGAP is a Rho-specific GAP that is capable of inducing differentiation of neuronal cells through its GTPase-activating activity (24). In the present study, we report a surprising finding that expression of full-length p200 RhoGAP in NIH 3T3 cells induces focus formation and anchorage-independent growth and enhances cell proliferation under low serum conditions. The cell growth-promoting activity by p200 RhoGAP is in part mediated by Ras and ERK1/2 pathway activation, and this is associated with its ability to sequesterate p120 RasGAP through an interaction between their C-terminal region and SH3 domain, respectively. Beside the C terminus Ras activation effect, p200 RhoGAP also utilizes its N-terminal RhoGAP domain to down-regulate endogenous RhoA activity, thereby facilitating the GDP/GTP cycling of RhoA under serum stimulation to promote cell proliferation. Our results present a novel concept that a RhoGAP molecule could promote, rather than suppress, cell proliferation. Moreover, they raise an interesting possibility that RhoGAPs like p200 RhoGAP may functionally coordinate Ras and Rho signaling pathways in cell growth regulation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The cDNA encoding the RhoGAP domain of p200 that bears a mutation of arginine to lysine at the predicted catalytic arginine residue (residue 58) was generated by introducing a point mutation using PCR-based site-directed mutagenesis (25). The cDNAs encoding the p200 RhoGAP domain (residues 1–251), the GAP domain containing the arginine mutation (GAP(R58K)), full-length p200 RhoGAP (residues 1–1738), the full-length p200 RhoGAP containing the arginine mutation (p200(R58K)), and the C-terminal region of p200 RhoGAP (p200-C; residues 224–1738) were subcloned into the mammalian expression vector pCEFL-GST using EcoRI and NotI sites as GST fusion proteins and into the pKH3 vector using the EcoRI site to be expressed as HA fusion proteins. In addition, serial deletion mutants of the proline-rich motifs in p200 RhoGAP, del1 (residues 1–842), del2 (residues 1–947), del3 (residues 1–989), del4 (residues 1–1052), and del5 (residues 1–1498), were also cloned into the pCEFL or pKH3 vectors. A GST fusion construct of the SH3 domain of Abl was kindly provided by Dr. Steve Taylor (University of California). The GST fusion construct encoding SH2-SH3-SH2 of p120 RasGAP was from Dr. Jeffrey Settleman (Harvard Medical School). The CDNA encoding the SH3 domain of p120 RasGAP was subcloned to pGEX-2T vector to be expressed as a GST fusion.

**Cell Transfection and Secondary Focus Formation Assay**—NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (CS) in 5% CO2 at 37 °C. Stable transfection in NIH 3T3 cells was carried out with Lipofectamine (Invitrogen) according to the manufacturer’s protocol. 48 h after transfection, the medium was changed to DMEM containing 10% calf serum and G418 (400 μg/ml) (Cellgrow). The cells were grown for 14 days in the selection medium, and the resulting single colonies were isolated for passage as clonal lines. The cell lines were maintained in the presence of G418 (250 μg/ml). For the secondary focus-forming assay, 50 × 103 wild-type NIH 3T3 fibroblasts were mixed with 5 × 103 cells from the cloned cell lines and plated on a 100-mm cell culture dish. The cultures were sustained for 2 weeks with medium changes every other day. The cells were washed once with phosphate-buffered saline, fixed with methanol, and stained with Giemsa staining solution (1:20 dilution in H2O) (Sigma). A Canon imaging system was used to take the photos of the stained dishes. Transient transfection of NIH 3T3 cells was carried out with FuGENE 6 reagent (Roche Applied Science) according the manufacturer’s protocol. The transfected cells were changed into fresh medium 48 h after transfection and were used directly for the indicated assays. The efficiency of the transient transfection reaction routinely reached 50–70% of the cell populations as estimated by anti-HA or anti-GST immunofluorescent staining of the transfected cells.

**Anchorage-independent Growth Assay**—Stably transfected NIH 3T3 cells with pCEFL-GST p200 RhoGAP clones were mixed in 0.3% agarose with 10% calf serum-DMEM and plated on top of 0.6% agarose-coated 35-mm dishes. Fresh agarose media were added every week for 2–3 weeks. Images of foci grown in the soft agar were obtained under a phase-contrast microscope with ×40 magnification. The quantifications of foci were from three independent experiments.

**Cell Proliferation and DNA Content Analysis**—The growth rates of the cloned cell lines were determined as described by Qiu et al. (26). Briefly, 105 cells were plated in 6-well plates in the presence of 10% calf serum and changed to 0.5% calf serum on the next day. Cell numbers at different time points were determined using a hemocytometer. Data were gathered from three independent experiments. Cell saturation densities were determined as described before (29, 30).

The DNA content analysis was carried out as previously described (27). Briefly, 1 × 105 cells were resuspended in buffer containing 0.1% sodium citrate, 0.3% Nonidet P-40, 0.05 mg/ml propidium iodide, 0.02 mg/ml RNase and incubated for 30 min on ice. The cells were collected by a brief centrifugation and were resuspended in fresh buffer. The cells were then passed through a 27-gauge needle several times and filtered through 37-μm nylon mesh immediately before flow analysis. The flow
analysis was performed using FACScalibur (BD Biosciences) according to the DNA analysis template provided by the manufacturer, and the results were analyzed by the CellQuest program.

**Ras, Cdc42, RhoA, and Rac1 Activity Assays—**Various HA-tagged p200 RhoGAP constructs were transiently transfected into NIH 3T3 cells. After 48 h, cells were lysed in a buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, 2 mM NaF, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin) for 15 min at 4 °C. The extracts were sonicated followed by centrifugation (14,000 rpm, 10 min). Active Ras, RhoA, Cdc42, and Rac1 levels were determined by performing the effector domain pull-down assays using the GST-Ras binding domain (RBD) domain of c-Raf1 for Ras-GTP, the GST-RBD domain of Rhotekin for RhoA-GTP, and the GST-p21 binding domain of PAK1 for Rac1-GTP and Cdc42-GTP. The levels of active Ras, Cdc42, Rac1, and RhoA were detected by Western blotting with monoclonal anti-Ras, anti-Cdc42, and anti-Rac1 antibodies (BD Transduction Laboratories) and a monoclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

**Immunoprecipitation—**Transiently transfected cells were incubated in a buffer containing 50 mM Hepes, pH 7.3, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂, 2 mM NaF, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin) for 15 min at 4 °C. The extracts were clarified by centrifugation (14,000 rpm, 10 min). To precipitate GST fusion proteins expressed in NIH 3T3 cells, glutathione-agarose or anti-GST beads (Sigma) were incubated with the cell extracts for 2 h at 4 °C on a tumbler. To immunoprecipitate HA-tagged proteins in the cell lysates, monoclonal anti-HA antibody (Roche Applied Science) and protein A/G agarose beads (Santa Cruz Biotechnology) were incubated with the cell extracts for 2 h at 4 °C on a tumbler. The precipitates were washed three times with the lysis buffer and once with the buffer without Triton X-100.

GST-fused SH3 domain or the SH2-SH3-SH2 module from p120 RasGAP, the SH3 domain from Abl, and GST alone were expressed in *Escherichia coli* using the pGEX-2T vector. These proteins were purified by using glutathione-agarose beads from the bacterial extracts. When necessary, the GST fusion proteins were eluted with 20 mM glutathione in 100 mM Tris base (pH 8.5–9.0) and 100 mM NaCl. Protein concentrations were measured using the Bradford method (Bio-Rad).

**Western Blot Analysis—**For Western blot analysis, cell lysates or co-precipitates were separated in 4–15% gradient or 12% gels and transferred onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked with 1% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature and probed with primary antibodies followed by horseradish peroxidase-coupled secondary antibody for enhanced chemiluminescence analysis (Amersham Biosciences). The anti-GST antibody was purchased from Sigma, the anti-HA antibody was from Roche Applied Science, and the anti-p120 antibody was from BD Transduction Laboratories. Anti-ERK, anti-phospho-ERK, anti-Akt, and anti-phospho-Akt antibodies were purchased from Cell Signaling (New England Biolabs). All primary antibodies were diluted (1:1000) in TBS-T buffer containing 1% bovine serum albumin. Peroxidase-conjugated donkey antimouse IgG (Jackson ImmunoResearch Laboratories) or horseradish peroxidase-linked anti-rabbit IgG (Amersham Biosciences) were used as secondary antibodies.

**Immunocytochemistry—**p200 RhoGAP-expressing NIH 3T3 cells were cultured in either DMEM with 10% calf serum or serum-free DMEM overnight. The cells were fixed with 2% paraformaldehyde in phosphate-buffered saline for 10 min, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and incubated with 1% bovine serum albumin in phosphate-buffered saline for 30 min. The cells were then incubated with polyclonal anti-p120 RasGAP (BD Transduction Laboratories) and monoclonal anti-GST antibodies (Sigma), washed with phosphate-buffered saline, and stained with TRITC-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-mouse secondary antibodies, respectively (Molecular Probes, Inc., Eugene, OR). Cells subject to the transient transfection of pKH3-p200 RhoGAP and pCEFL-p200 RhoGAP were incubated with monoclonal anti- HA and anti-GST antibodies, respectively, followed by TRITC-conjugated anti-mouse secondary antibodies (Molecular Probes). The fluorescent images were captured by using a Zeiss fluorescence microscope.

**Adenoviral and Retroviral Transductions—**p200 RhoGAP-stably expressed NIH 3T3 cells were cultured in DMEM containing 10% calf serum. Recombinant retroviruses were produced using the Phoenix cell packaging system by transient expression of relevant cDNAs cloned in the MIEG3 retroviral vector containing bicistronically expressed EGFP. The p200 RhoGAP-expressing NIH 3T3 cells were transduced with N19RhoA-expressing retrovirus and harvested 48–72 h postinfection. The EGFP-positive cells were isolated by fluorescence-activated cell sorting. In parallel, the p200 RhoGAP-expressed NIH 3T3 cells were infected with N17Ras and GFP-expressing adenovirus (a kind gift from Dr. Nancy Ratner, Cincinnati Children’s Hospital Medical Center) and harvested 24 h postinfection. The efficiency of the adenoviral infection was over 90%.

**RESULTS**

**Ectopic Expression of p200 RhoGAP in NIH 3T3 Cells Induces Transformation—**p200 RhoGAP was previously shown to be involved in the regulation of neurite outgrowth by exerting its RhoGAP activity (24). In the course of subsequent functional characterizations of p200 RhoGAP, we repeatedly found that ectopic expression of p200 RhoGAP in NIH 3T3 cells induced transforming foci (data not shown), and cell clones expressing p200 RhoGAP readily formed secondary foci among NIH 3T3 cells (Fig. 1, A and B) and grew anchorage-independently in soft agar medium (Fig. 1, C and D). These observations suggest that p200 RhoGAP could cause cell transformation at appropriate expression levels.

In addition to focus induction and anchorage-independent colony formation, the full-length p200 RhoGAP-expressing cells grew with significant advantages in low serum (0.5% CS) conditions (Fig. 2A), reached a saturation density almost double
that of wild-type cells (Fig. 2B), and displayed significantly increased S-phase (2–3-fold) and G2/M (1.5-fold) phase populations during cell cycle progression (Fig. 2C). These observations were verified in multiple independently produced clones (two such clones are shown in Figs. 1 and 2). These results indicate that full-length p200 RhoGAP promotes, rather than suppresses, cell growth and that signaling pathways mediated by p200 RhoGAP can modulate cell contact inhibition, growth factor dependence, and cell cycle progression to favor cell growth transformation.

p200 RhoGAP Induces "Ras-like" Focus Morphology—Previous studies have shown that the morphologies of foci induced by activated Rho GTPases or oncogenic RhoGEFs are highly compact with refractile cells on the periphery, which are distinct from the more flat, spreading, and swirling morphology induced by activated Ras (28–30). We observed that full-length p200 RhoGAP induces, rather than suppresses, cell growth and that signaling pathways mediated by p200 RhoGAP can modulate cell contact inhibition, growth factor dependence, and cell cycle progression to favor cell growth transformation.

p200 RhoGAP Increases Ras Activity and Requires ERK1/2 Activation for Transformation—To examine if p200 RhoGAP-induced cell transformation might involve Ras signaling pathways, Ras activity was measured in p200 RhoGAP-expressing cells and relevant control cells. The HA-tagged p200 RhoGAP expression in NIH 3T3 cells was detected by anti-HA blotting of the cell lysates, whereas the GTP-bound, active Ras was probed by using the immobilized RBD of cRaf-1 to form a stable complex with Ras-GTP followed by anti-Ras immunoblotting. As shown in Fig. 4A, the amount of Ras-GTP was significantly increased when p200 RhoGAP was expressed under both serum-free and serum-stimulated conditions, suggesting that p200 RhoGAP regulates Ras activity independently of serum. Concomitant with elevated Ras-GTP in the p200 RhoGAP-expressing cells, two downstream effectors of Ras, AKT and ERK1/2, were activated by p200 as revealed by anti-phospho-
AKT and -ERK1/2 blotting (Fig. 4B). These results suggest that p200 RhoGAP expression causes Ras and Ras-regulated AKT and ERK1/2 pathway activation.

To further determine if the PI3K and/or ERK1/2 signaling pathway is required for p200 RhoGAP-induced cell transformation, pharmacologic inhibitors for PI3K (Ly294002) and MEKK (U0126) at the concentrations known to specifically block the PI3K-AKT or MEKK-ERK1/2 activities, respectively, were applied to the p200 RhoGAP-expressing cells. As shown in Fig. 5A, 7.5 μM Ly294002 or 20 μM U0126 could specifically suppress AKT or ERK1/2 phosphorylation induced by p200 expression to levels comparable with that of control cells. Inhibition of the ERK1/2 pathway by U0126 was effective in suppressing the focus formation induced by p200 RhoGAP in a dose-dependent manner, whereas inhibition of PI3K activity by Ly294002 at up to 7.5 μM concentration had little effect on the focus-forming activity (Fig. 5B). However, at 20 μM concentra-
RhoGAP was able to interact with p120 RasGAP by forming an immunoprecipitation. Fig. 7

p200 RhoGAP Interacts with p120 RasGAP—Previous studies have shown that p120 RasGAP may serve as a cross-talk bridge between Ras and a RhoGAP member, p190 RhoGAP. p190 RhoGAP is one of the major interacting partners for p120 RasGAP by binding to the SH2 domain of p120 RasGAP through a phospho-tyrosine motif after Src or related tyrosine kinase phosphorylation (31–34). To examine the possibility that p200 RhoGAP may interact with p120 RasGAP to affect Ras activity, indirect immunoprecipitation was performed to determine the subcellular localization patterns of p200 RhoGAP and p120 RasGAP. We found that the stably expressed p200 RhoGAP and endogenous p120 RasGAP were colocalized in cytoplasm and intracellular vesicles under both serum stimulation and serum-free conditions (data not shown), suggesting that they may form a constitutive complex in cells. This possibility is confirmed by immunoprecipitation experiments using GST-tagged p200 RhoGAP or p200 mutants, p200 R58K with a key arginine residue in the RhoGAP domain involved in GAP-catalysis mutated to lysine that is defective in the RhoGAP catalytic activity, p200 C that lacks the N-terminal RhoGAP domain, or p200 GAP that contains only the N-terminal RhoGAP domain (Fig. 6A), to complex with endogenous p120 RasGAP. In the presence or absence of serum stimulation, full-length p200 RhoGAP, but not the RhoGAP domain, interacted with p120 RasGAP in an immune complex (Fig. 6B). p200 R58K or p200 C retained p120 RasGAP binding ability (Fig. 6B). When wild-type, constitutively active mutant (Y527F) or dominant negative mutant (K295R/Y527F) of c-Src was co-expressed with p200 RhoGAP, no effect on the interaction between p200 RhoGAP and p120 RasGAP was detected (data not shown). These results suggest that distinct from p190 RhoGAP, p200 RhoGAP interacts with p120 RasGAP in a serum- and Src phosphorylation-independent manner, and the interaction is mediated through a region of p200 independent from the RhoGAP domain.

p120 RasGAP Interacts with C Terminus of p200 RhoGAP via Its SH3 Domain—To examine the mode of interaction between p200 RhoGAP and p120 RasGAP, a series of C-terminal truncated p200 mutants that retained different proline-rich motifs(s), as well as the SH2-SH3-SH2 and SH3 domains of p120 RasGAP, were generated (Fig. 7A). The p200 constructs were expressed in NIH 3T3 cells as GST-fusions (Fig. 7B), and their ability to bind to endogenous p120 RasGAP was determined by Western blotting for p120 RasGAP after anti-GST immunoprecipitation. Fig. 7C shows that wild-type, full-length p200 RhoGAP was able to interact with p120 RasGAP by forming an immunocomplex, but none of the deletion mutants, including the p200-del 5 that retained residues 1–1498, were able to co-immunoprecipitate with p120 RasGAP. These results suggest that the C-terminal region of p200 that includes the last proline-rich motif is required for complex formation with p120 RasGAP.

To determine the sequences of p120 RasGAP that are important for interacting with p200 RhoGAP, we focused on the SH3 domain that may specifically recognize proline-rich motifs in p200. For this purpose, bacterially expressed GST fusion proteins containing the SH3 domain or the SH2-SH3-SH2 module of p120 RasGAP were expressed and immobilized on glutathione-agarose beads, and they were incubated with cell lysates derived from NIH 3T3 cells expressing HA-tagged p200 RhoGAP. Fig. 7D shows that the GST-fused p120 RasGAP SH3 domain or SH2-SH3-SH2 domain tandem, but not the SH3 domain of Abl kinase, was sufficient to bind to p200 RhoGAP from the cell lysates. To further examine if the interaction is mediated by a direct protein-protein binding between the SH3 domain of p120 RasGAP and p200 RhoGAP, purified GST, GST-SH3 of Abl, GST-SH3, or GST-SH2-SH3-SH2 of p120 RasGAP was incubated with the immunopurified HA-p200 RhoGAP for a complex formation assay. As shown in Fig. 7E, HA-p200 RhoGAP was able to pull-down GST-SH3 or GST-SH2-SH3-SH2 of p120 RasGAP but was unable to complex with GST or GST-SH3 of Abl. Together these results indicate that the interaction between p200 RhoGAP
and p120 RasGAP is mediated through their C-terminal region containing a proline-rich motif and SH3 domain, respectively.

Disruption of the Interaction between p200 RhoGAP and p120 RasGAP Abolishes Ras Activation and Cell Transforming Activities—To further confirm if the interaction between p200 RhoGAP and p120 RasGAP directly correlates with the activation of Ras and cell transformation, Ras activities were measured in p200 RhoGAP wild type, its deletion mutants, and control vector-expressed cells. The HA-tagged p200 RhoGAP constructs were expressed in NIH 3T3 cells, and the GTP-bound activated Ras was isolated by using the RBD domain of c-Raf-1. Compared with wild-type p200 RhoGAP, none of the five deletion mutants that have lost p120 RasGAP binding abilities were able to affect the amount of Ras-GTP in cells (Fig. 8A), suggesting that disruption of the binding between p200 RhoGAP and p120 RasGAP leads to the loss of Ras activation. We analyzed p200-del4 and p200-del5 mutants further in focus-forming assays. The results showed that, consistent with their diminished Ras activating potential (Fig. 8A), neither mutants were able to induce focus formation like wild-type p200 RhoGAP (Fig. 8B). Both p200-del4 and p200-del5 appeared to retain the RhoGAP activity when expressed in cells (Fig. 8C). Thus, the mutants of p200 RhoGAP that disrupt interaction with p120 RasGAP also lose Ras activation and cell transforming activities.

p200 RhoGAP-induced Cellular Transformation Requires Both the RhoGAP Domain and C-terminal Region—Since the C terminus of p200 RhoGAP is responsible for interaction with p120 RasGAP and Ras activation, one relevant question is whether the C terminus is sufficient for the full transforming activity of p200 RhoGAP. To address this issue, we tested a number of p200 mutant constructs, including p200 R58K, p200 GAP domain (p200 GAP), the RhoGAP domain with R58K mutation (p200 GAP R58K), and the C-terminal region of p200 RhoGAP (p200-C) (Fig. 6A). All constructs were expressed at a comparable level in NIH 3T3 cells (Fig. 9A). Recent gene targeting
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Dominant Negative Mutants of Ras and RhoA Inhibit p200 RhoGAP-induced Cell Transformation — To determine the involvement of Ras and Rho activities in p200 RhoGAP-mediated growth transformation, we expressed a dominant negative mutant of Ras, N17Ras, or mutant of RhoA, N19RhoA, into the p200 RhoGAP transformed cells by adenovirus or retrovirus-mediated transduction. Fig. 10 shows that the expression of N17Ras or N19RhoA significantly reduced the transforming potential of p200 RhoGAP, indicating that p200 RhoGAP-induced cellular transformation requires both Ras and Rho activities. Interestingly, neither N17Ras nor N19RhoA was able to completely inhibit the p200-mediated transformation, possibly because of incomplete blockade of Ras or Rho signaling due to the mutant expression dosages or to dual requirement of both Ras and Rho pathways for the full transforming activity. Since the dominant negative mutant RhoA not only reduces the GTP-bound form of Rho species but also inhibits the cycling of Rho between the GTP-bound and GDP-bound states, these results are consistent with that effective cycling of Rho promoted by p200 RhoGAP in collaboration with serum stimulation contributes to its transforming potential.

DISCUSSION

In a series of simultaneous papers, p200 RhoGAP (also termed Grit, GC-GAP, RICS, and p250GAP) was reported as a Rho GTPase-activating protein mainly expressed in the brain that interacts with multiple SH3 domain-containing signaling molecules. In addition to an N-terminal RhoGAP domain that appears to be specific for Rho GTPase-activating activity, p200 RhoGAP harbors multiple proline-rich motifs in its C-terminal half, suggesting potential interactions with SH3 domain-containing proteins (24). In the present studies, we found that full-length p200 RhoGAP expression could promote NIH 3T3 cell cycle progression, allow serum- and anchorage-independent growth, and induce transformation. These data are somewhat surprising, given the conventional knowledge that RhoGAPs function as negative regulators of Rho GTPases and thus are expected to suppress, rather than enhance, cell growth transformation (4). Our subsequent characterizations of p200 RhoGAP identified its C terminus-mediated Ras activation and N terminus-mediated RhoGAP activity as important contributing mechanisms to the cell transforming activity. In particular, the Ras-activating and transforming potentials of p200 RhoGAP were correlated with its complex formation with p120 RasGAP through proline-rich motif-SH3 domain interaction. The p200 RhoGAP-stimulated Ras-ERK pathway activation and efficient cycling of Rho between the GTP-bound and GDP-bound states may cooperatively provide proliferative signals, leading to a full transforming activity (Fig. 11). To our knowledge, this is the first evidence that a RhoGAP protein can coordinate the cross-talk between the Rho and Ras pathways to impact cell growth transformation.

One well characterized example of a RhoGAP cross-talk with the Ras signaling pathway is the interaction between p190 RhoGAP and p120 RasGAP. The interaction between p190 RhoGAP and p120 RasGAP is phosphorylation-dependent and is mediated by the SH3 and SH2 domains of p120 RasGAP and phosphotyrosine motifs of p190 RhoGAP (31). Despite inten-
sive effort to understand the mechanism of their interaction, it remains incompletely understood how p190 RhoGAP affects Ras signaling pathway in a cell functional readout, such that the substrate of p190 RhoGAP, RhoA, and the p120 RasGAP-regulated Ras signal might be coordinated by their interaction to influence cell growth.

Unlike the interaction between p190 RhoGAP and p120 RasGAP, p200 RhoGAP appears to interact with p120 RasGAP in a serum-independent manner. They can form a stable immunocomplex and co-localize in cells with or without serum stimulation, suggesting that their interaction is constitutive. The binding mode between p120 RasGAP and p200 RhoGAP is probably mediated through the SH3 domain and C-terminal proline-rich motif in the molecules, respectively. Significantly, the interaction of p200 RhoGAP with the SH3 domain of p120 RasGAP is through direct binding, and mutants of p200 RhoGAP that lack p120 RasGAP binding activity are defective in Ras activation and transformation. Since we have not ruled out the possibility that p200 RhoGAP binding to p120 RasGAP results in an inhibition of its RasGAP catalytic activity, it is possible that sequestration of p120 RasGAP at an intracellular location distinct from that of Ras could serve as the mechanism for p200 RhoGAP-mediated Ras activation. It is important to note that although the elevated Ras-GTP induced by p200 RhoGAP expression is required for the transforming phenotype, the mutant studies of p200 RhoGAP (Figs. 7–9) indicate that binding to p120 RasGAP or the quantitatively increased Ras activity is not sufficient to cause full transformation. Other structural motifs in p200 RhoGAP, including the RasGAP domain, are also involved in the process.

One novel aspect of our present finding is that a RhoGAP, as a down-regulator of Rho-GTP species in cells, can confer cell growth-promoting activity. This is intriguing, especially considering the observation that the loss of RasGAP catalytic activity mutant, p200 R58K, is significantly dampened in transforming potential (Fig. 9), which implicates the RasGAP activity of p200 RhoGAP in promoting cell growth. Analysis of the GTP-bound RhoA, Rac1, and Cdc42 in p200 RhoGAP- or p200 R58K-expressing cells indicates that wild-type p200 RhoGAP causes a down-regulation of RhoA and Cdc42 activities, whereas p200 R58K does not affect RhoA or Cdc42 activity (Fig. 9C). Furthermore, dominant negative Ras expression suppresses, rather than enhances, p200 RhoGAP transforming activity (Fig. 10). These results can be reconciled with our current knowledge of Ras GTPase signaling by the following rationales.

First, the RasGAP activity of p200 RhoGAP may accelerate the return of Rho-GTP to Rho-GDP, facilitating the effective GTP-hydrolysis/GTP-binding cycle of Rho in the presence of
saturating serum stimulation that may keep RhoGEFs in a constitutively “on” state (Fig. 11). In a sense, p200 RhoGAP expression under high serum conditions may produce a similar effect of cell growth stimulation as the fast cycling mutants of Rho, which retains GTP-hydrolytic ability and causes cell transformation more effectively than the constitutively GTP-bound Rho mutants (29, 36). This explanation is consistent with the postulation that Rho GTPases require constant cycling between the GTP- and GDP-bound states to elicit biological effects (37). Thus, a relatively lower Rho-GTP level at the steady state (Fig. 9) may not be disadvantageous to cell growth as the slowed GTPase cycling of Rho. This rationale is also consistent with previous observations that in Ras-transformed cells, the steady-state level of RhoA-GTP and Cdc42-GTP was actually lower than wild-type cells, albeit Rho activity is apparently required for Ras transformation (38). Second, the cellular function of full-length RhoGAPs may be distinct from that manifested by RhoGAP domains, as a number of studies have shown (8, 39). In the case of p200 RhoGAP, the RhoGAP domain alone (and RhoGAP activity) is not sufficient to cause cell transformation. The RhoGAP domain (and RhoGAP activity) only contributes to the transforming potential in the context of full-length p200 RhoGAP, together with its C terminus Ras-activating ability. Consistent with a combined role of the RhoGAP activity of p200 RhoGAP with other functional domains to mediate cell transformation, we could not detect any cell growth-promoting activity of the RhoGAP domain of p200 RhoGAP when it was expressed in cells cultured in high serum conditions or co-expressed with RhoGEFs such as Lbc. Since the C terminus of p200 RhoGAP could cause the activation of the Ras-signaling cascade, including the ERK1/2 and PI3K-AKT pathways, it is possible that the accelerated Rho GTPase cycle could synergize with the Ras pathway to favor cell growth, as has recently been shown for a fast cycling Cdc42 mutant that synergizes with oncogenic Ras to promote cell transformation (40). Third, since the majority of primary sequences of p200 RhoGAP do not show a clear homology with known protein

3 X. Shang, S. Y. Moon, and Y. Zheng, unpublished observations.
domains, there might be unknown protein-protein or protein-lipid interactions mediated by p200 RhoGAP that could coordinate with RhoGAP activity and Ras activation to favor cell growth. To this end, the closest relative of p200 RhoGAP in the RhoGAP family, CdcGAP, has been found to interact with a Cdc42-specific RhoGEF, intersectin, through the middle section of its amino acid sequences (41) and may interact with ERK1/2 directly (42). It remains possible that p200 RhoGAP may interact with and recruit a RhoGEF or Ras downstream signaling molecules to facilitate transformation. These rationales on how the RhoGAP activity of p200 RhoGAP may participate in cellular transformation are currently under investigation.

Whether the observed cell transforming ability of p200 RhoGAP bears pathologic relevance will be an important question to address in future studies. The expression of p200 RhoGAP is enriched in the brain. One area of investigation will thus be focused on examining the expression level and activity of p200 RhoGAP in various brain tumor samples. Another important area of investigation derived from the present findings is to further compare the Rho GTPase activities (i.e., Rho-GTP species levels) in various human tumor specimens, where much emphasis has been on searching for correlations between tumorigenesis/tumor progression and up-regulation of individual Rho GTPase activities. In light of our finding, it is possible that at defined stages of tumor progression or due to distinct tumorigenic mechanisms of certain tumor types, individual Rho-GTP level could be down-regulated as measured by the steady-state, effector domain pull-down method but increased kinetically in the GTPase cycle.

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