A Novel Strategy for Specifically Down-regulating Individual Rho GTPase Activity in Tumor Cells*

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The Ras-related Rho family of small GTPases regulates the actin cytoskeleton, cell movement, and cell growth. Unlike Ras, up-regulation or overexpression of these GDP/GTP binding molecular switches, but not activating point mutations, has been associated with human cancer. Although they share over 85% sequence identity, RhoA, RhoB, and RhoC appear to play distinct roles in cell transformation and metastasis. In NIH 3T3 cells, RhoA or RhoB overexpression causes transformation whereas RhoC increases the cell migration rate. To specifically target RhoA, RhoB, or RhoC function, we have generated a set of chimeric molecules by fusing the RhoGAP domain of p190, a GTPase-activating protein that accelerates the intrinsic GTPase activity of all three Rho GTPases, with the C-terminal hypervariable sequences of RhoA, RhoB, or RhoC. The p190-Rho chimeras were active as GTPase-activating proteins toward RhoA in vitro, co-localized with the respective active Rho proteins, and specifically down-regulated Rho protein activities in cells depending on which Rho GTPase sequences were included in the chimeras. In particular, the p190-RhoA-C chimera specifically inhibited RhoA-induced transformation whereas p190-RhoB-C specifically reversed the migration phenotype induced by the active RhoC. In human mammary epithelial-RhoC breast cancer cells, p190-RhoC-C, but not p190-RhoA-C or p190-RhoB-C, reversed the anchorage-independent growth and invasion phenotypes caused by RhoC overexpression. In the A375-M human melanoma cells, p190-RhoC-C specifically reversed migration, and invasion phenotypes attributed to RhoC up-regulation. Thus, we have developed a novel strategy utilizing RhoGAP-Rho chimeras to specifically down-regulate individual Rho activity and demonstrate that this approach may be applied to multiple human tumor cells to reverse the growth and/or invasion phenotypes associated with disregulation of a distinct subtype of Rho GTPase.

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The Ras-related Rho family of small GTPases regulates the actin cytoskeleton organization, cell to cell or cell to extracellular matrix adhesion, intracellular membrane trafficking, gene transcription, apoptosis, and cell cycle progression (1–3). Like Ras, they exist in an inactive, GDP-bound and an active, GTP-bound conformations. Rho GTPases are activated by a class of positive regulators, the Db1 family guanine nucleotide exchange factors (GEFs)¹ that catalyze the release of bound GDP and facilitate the binding of GTP (4), whereas deactivation of Rho proteins is achieved through their intrinsic GTP-hydrolytic activities that are further stimulated by a class of negative regulators, the GTP-activating proteins (GAPs) (5). A third class of regulators of Rho GTPases, the Rho GDP-dissociation inhibitors, can negatively impact Rho protein activities by sequestering them in the GDP-bound state and preventing effective cycling between the two conformational states (6). Upon binding to GTP, Rho GTPases may further interact with an array of potential effector molecules to elicit cellular responses (7).

It has become increasingly clear that Rho proteins play important roles in many aspects of cancer development, and each member of the Rho family may be involved to a different extent at different tumor progression stages (8–10). For example, it was shown that constitutively active RhoA has oncogenic potential (11), and RhoA acts as a critical signaling component in Ras-induced transformation (12). Furthermore, RhoB promotes the invasiveness of rat hepatoma cells (13) and induces metastasis of NIH 3T3 fibroblasts (14), whereas RhoC was revealed as a key regulator of migration and metastasis in a human melanoma cell line (15). Upon introduction into normal mamalian epithelial cells, RhoC readily caused transformation and invasion, leading to an inflammatory breast cancer cell phenotype (16). On the other hand, RhoB is required for the apoptotic responses induced by farnesyltransferase inhibitors or DNA damaging agents and may have a suppressor or negative modifier function in cancer progression (17). Unlike Ras, there are no reports of mutation-caused constitutive activation of Rho proteins in tumors. Recent studies of primary human tumors (18–21) revealed that many Rho GTPases, including RhoA and RhoC, are highly expressed in a variety of cancer types such as colon, lung, testicular germ cell, head and neck squamous cell carcinoma, pancreatic ductal adenocarcinoma, and inflammatory breast cancer, and in some cases, the Rho protein up-regulation and/or overexpression correlates with poor prognosis (22). These observations help put Rho proteins in a lineup of potential molecular targets for anti-cancer therapy (8).

Currently available molecular tools to target Rho GTPase pathways at the small G-protein level include the dominant negative mutants of Rho proteins (23), the p21-binding domain of effectors (24) and a class of bacterial toxins that can modify

¹ The abbreviations used are: GEF, guanine nucleotide exchange factor; CS, calf serum; FBS, fetal bovine serum; GAP, GTPase-activating protein; GST, glutathione S-transferase; HME, human mammary epithelial; GFP, green fluorescent protein; EGFP, enhanced GFP; DMEM, Dulbecco's modified Eagle's medium; Ni-NTA, nickel-nitrilotriacetic acid; HA, hemagglutinin.
Rho GTPase functions (25). Although they have been widely used in cell biological studies, each of these reagents has its own drawbacks. The dominant negative forms of Rho proteins act by sequestering the upstream GEF activators of endogenous Rho GTPases and tend to be nonspecific among closely related Rho family members (23). Further, because of their non-catalytic nature, a 3–5-fold overexpression versus the endogenous Rho protein level is typically needed for effective blockage of the endogenous activity, and this may not be desirable in many in vivo situations. Similarly, the limited specificity of effector p21-binding domains and bacterial toxins such as Clostridium botulinum C3-transferase is worrisome in terms of differentiating the roles of highly homologous isofoms such as RhoA, RhoB, and RhoC. Furthermore, very little is known about the anti-cancer potential of these Rho GTPases inhibitors.

Because RhoGAPs have been established as the major class of negative regulators of Rho GTPase signaling (5), and some of them are actually known for their tumor suppressor function (26, 27), another possible approach for inhibiting Rho GTPase activities in tumor cells is to employ RhoGAPs as antagonists for Rho activities. The conserved GAP domain in RhoGAPs contains the necessary and sufficient structural determinants for Rho protein recognition and GTPase catalysis but displays limited substrate specificity (5). For example, the RhoGAP domain of p190 RhoGAP can catalyze GTP hydrolysis of RhoA, RhoB, and RhoC equally well but works weakly on Rac or Cdc42 (28, 29). It depends on other regulatory domains in the RhoGAP to direct and regulate substrate selection and catalysis in vivo (30, 31). Conversely, although Rho proteins may share up to 90% sequence identity, each Rho subtype appears to play distinct roles in cellular transformation and metastasis. The differences in Rho GTPase functions may come in part from their distinct subcellular localization patterns that are mostly determined by the unique C-terminal hypervariable sequences in each case (32, 33).

In the present study, we have developed a novel strategy combining the RhoGAP domain of p190 RhoGAP with the C-terminal hypervariable sequences of Rho proteins to specifically target RhoA, RhoB, or RhoC activity in human tumor cells. We show that although RhoA, RhoB, and RhoC are equally effective in stimulating actin stress fiber formation and focal complex assembly in NIH 3T3 cells, RhoA and RhoB are capable of transforming cells whereas RhoC induces cell migration. The chimera made of p190 and the C terminus of RhoC specifically inhibited RhoA-induced cell transformation whereas the p190-RhoC chimera specifically reversed RhoC-mediated migration phenotype by down-regulating RhoC activity. Moreover, when applied to the HME-RhoC human inflammatory breast cancer cells or to the A375-M human melanoma cells, the p190-RhoC chimera was effective in reversing the anchorage-independent growth and/or the invasion phenotypes that were attributed to RhoC up-regulation. Our results suggest that such a RhoGAP-based approach targeting specific Rho GTPase activity could be useful in reversing tumor cell phenotypes associated with disregulation of a distinct subtype of Rho GTPase.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Site-directed mutants of human RhoA, RhoB, and RhoC were generated by polymerase chain reactions using the *Pfu* polymerase, with primers that contained the desired mutations following the published protocols (34). The mutant cDNAs were subcloned into the BamHI and EcoRI sites of mammalian expression vector pCEFL-GST, and the mutants were expressed as glutathione S-transferase (GST) fusions (35). The sequences of mutagenized DNA inserts were confirmed by automated sequencing. All point mutants used are described by single-letter amino acid designations.

Construction of p190-Rho Chimeras—p190-Rho chimeric cDNAs were generated by fusing the cDNAs encoding the RhoGAP domain of rat p190 and the cDNAs encoding the C terminus of human RhoA, RhoB, or RhoC after PCR amplification of the respective sequences. The primer sequences are as follows for RhoA C terminus, 5′ CGGATT-CTTACAGGAGGCTGGAGATTTTCTTCACGCAGTTCTTCGCTC 3′; for RhoB C terminus, 5′ CGCAGGCTCTCAAGACATCGGGACCTGGAGGTCAGATGCTG- TGCCGCGTTAGCTGTCGACAGAGAAGCCTTTCCCTCGC 3′; and for RhoC C terminus, 5′ CGGAATTCTTGGAGGACCTGGGACTTGATTTCTCCTTGAGGTGGCGTACCATTCTCAGAGAATGGGACAGCCCTCCTCGACGTGTTCCTTGCGGACCTGGAGGTCAGATGCTGCAGTTCTTCGCTC 3′. The ligation products of cDNAs encoding p190RhoGAP domain and the respective Rho sequences were cloned into the MIEG-MCS expression vector (6) to generate the chimeras as (HA)- or FLAG-tagged fusions, together with the enhanced green fluorescent protein (EGFP) bicistronically (36, 57, 58), as well as the pCEFL-GST vector for transient expression in mammalian cells.

**Cell Culture and Transfection**—NH 3T3 cells were cultured in DMEM supplemented with 10% calf serum (CS) in a 5% CO2 incubator at 37 °C. COS-7 cells, and the retroviral packaging Phoenix cells were cultured in DMEM with 10% fetal bovine serum (FBS). The A375 and HME tumor cell lines were maintained as described (15, 16). To generate stable transfectants, NIH 3T3 cells were seeded in 6-well plate at a density of 1.5 × 10⁵ cells in DMEM medium supplemented with 10% FBS. The cells were transfected with the pCEFL-GST-Rho constructs in the next day using LipofectAMINE Plus (Invitrogen) following the manufacturer’s instructions. Selection of stable transfectants was carried out by adding 0.35 mg/ml of G418 to the culture medium 48 h after transfection. After ~2 weeks culturing in the selection medium, the surviving cells including over 100 colonies were examined for the expression of GST fusion proteins by anti-GST immunoblotting.

**GTPase Activity Assay**—The intrinsic and p190GAP-stimulated GTPase activities of RhoA were measured as described (34) by the nitrocellulose filter-binding method. Briefly, recombinant RhoA were preloaded with [γ-³²P]GTP (10 μCi, 6000Ci/mmol) (PerkinElmer Life Sciences) in a 100-μl buffer containing 50 mMK Hepes, pH 7.6, 0.2 mM bovine serum albumin, 0.5 mM EDTA, and 0.5 mM MgCl2 at ambient temperature before the addition of MgCl2 to a final concentration of 5 mM. An aliquot of the [γ-³²P]GTP-loaded RhoA was mixed with GAP assay buffer containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 0.2 mM mg bovine serum albumin, and 5 mM MgCl2 in the presence or absence of various GAPs. At different time points the reaction was terminated by filtering the reaction mixture through nitrocellulose filters. The radioactivity retained on the filters was then subjected to quantitation by scintillation counting.

**Retroviral Gene Transfer**—Various p190-Rho chimeras were expressed in NIH 3T3 cells or tumor cells by the retroviral infection method. Production of recombinant retrovirus in the retroviral packaging Phoenix cells and subsequent host cell infection were carried out according to the described protocols (36, 37). The infected cells were harvested 72 h post-infection. EGFP-positive cells (typically 10–50%) were isolated by fluorescence-activated cell sorting and were used for further analysis.

**Rho Effector Pull-down Assay**—To determine the RhoA, RhoB, or RhoC activity in cells, the effector pull-down assay (38) was carried out in the respective cells that have been serum-starved for 20 h. The effector probe for Rho-GTP, recombinant (His)₅-Rhotekin, was expressed and purified from *Escherichia coli* and immobilized on the Ni-NTA-agarose beads. The bead-associated (His)₅-Rhotekin (about 1 μg/sample) was incubated with the respective cell lysates expressing various Rho-GTP proteins in the presence of 4°C, and the co-precipitates were analyzed by Western blotting with anti-GST or anti-His monoclonal antibody.

**Transformation assay**—Measurements of the anchorage-independent growth of mutant Rho protein expressing cells or tumor cells were carried out as described (38, 39). Briefly, 2 × 10⁴ cells were suspended in 10% FBS-supplemented Ham’s F-12 medium containing 100 μg/ml with growth factors and 0.3% agarose and were plated on top of a solidified, 0.6% agarose. The cells were fed weekly by the addition of 1 ml of medium. Three weeks after plating, the colonies grown larger than 50 μm in diameter were scored under a microscope. To measure cell transforming activity, 5 × 10⁴ cells transfected with various Rho constructs were mixed with 5 × 10⁴ wild type, and the colonies were plated in 100-mm dishes in a medium containing DMEM supplemented with 10% CS. The cells were fed every other day, and the viable foci were scored 14 days post-plating.

**Wound Healing and Migration Assays**—For wound healing assays,
cells were plated at 2 × 10^4/dish density in 60-mm diameter dishes. A plastic pipette tip was drawn across the center of the plate to produce a clean 1-mm-wide wound area after the cells have reached confluency. After a 12-h culturing in DMEM supplemented with 0.5% CS, cell movement into the wound area was examined at different time points using a phase-contrast microscope. The distances between the leading edge of the migrating cells and the edge of the wound were compared (40).

Cell migration was also measured by using a Transwell plate inserted with a 6.5-mm polycarbonate membrane (8.0-μm pore size; Costar Corp.). Briefly, 5 × 10^4 cells were suspended in 0.2 ml of culture medium and were added to the upper chamber. 10% FBS in culture medium was used as chemotactant in the lower chamber. The cells were incubated for 18 h in a humidified CO2 incubator at 37 °C. The cells that traversed the 8.0-μm membrane pores and spread to the lower surface of the membrane were stained with 5% Giemsa solution, and the cells retained in the membrane were counted in at least six different fields. Each experiment was carried out in triplicate, and the error bars represent the mean standard error.

**Invasion Assay**—Cell invasion assays were performed using the 6.4-mm Biocoat Matrigel invasion chambers equipped with the 8.0-μm pore sized PET membrane filters (BD Biosciences) according to the manufacturer's instructions. Briefly, 2.5 × 10^4 cells were suspended in 0.5 ml of culture medium and were added to the upper chamber. 10% FBS in the culture medium was plated in the lower chamber as chemotactant. Cells in the invasion chambers were incubated in a humidified incubator. The cells that traversed the Matrigel matrix and the 8-μm membrane pores and spread to the lower surface of the filters were stained with 5% Giemsa solution for visualization. Each data point of the invasion test was derived from triplicate chambers, and error bars represent the mean standard error.

**Immunofluorescence**—Cells grown on coverglasses were fixed with 3.7% formaldehyde in phosphate-buffered saline for 15 min and washed with phosphate-buffered saline once followed by permeabilization with 0.1% Triton X-100 for 20 min. The cells were then blocked with 2% bovine serum albumin for 20 min. For actin staining, the cells were incubated with rhodamine-phalloidin. For vinculin staining, the cells were labeled with anti-vinculin monoclonal antibody (Sigma) followed by incubation with a rhodamine-conjugated goat anti-mouse secondary antibody. To determine the intracellular localization of various Rho proteins and p190-Rho chimeras, the fluorescence-activated cell sorting-isolated, EGFP-positive cells were labeled with anti-HA monoclonal antibody or with anti-GST polyclonal antibody followed by rhodamine- or cy5-conjugated secondary antibody staining. The stained cells were mounted onto slides in Aqua-mount and viewed with a Zeiss LSM510 confocal microscope or a Leica fluorescence microscopy equipped with the deconvolution software (Improvision, Inc.).

**RESULTS**

**Distinct Roles of RhoA, RhoB, and RhoC in Cell Transformation and Migration**—Among the three closely related Rho proteins, RhoA, RhoB, and RhoC, RhoA is the best characterized one and has been shown to regulate actin stress fiber and focal complex formation (41), to promote cell growth (39) and to transform NIH 3T3 fibroblasts (42). By contrast, the function of RhoB or RhoC in fibroblasts has not been examined in detail and has not been directly compared with that of RhoA. To make comparisons of the cellular roles of RhoA, RhoB, and RhoC, we have generated two sets of activating mutants for each of the Rho proteins: the fast-cycling Rho-F30L and the GTPase-defective Rho-Q63L. Both types of the mutants result in the net enhancement of the active Rho-GTP species in cells but involve distinct mechanisms; Rho-F30L proteins contain significantly increased intrinsic GDP/GTP exchange activity and remain responsive to RhoGAP stimulation to cycle between the GDP- and GTP-bound states (39), whereas the Rho-Q63L mutants cannot hydrolyze bound GTP and are locked into the GTP-bound active conformation (42). We introduced these mutants into NIH 3T3 cells and generated the mutant expressing stable clones. Western blots show that the two mutant forms of all three Rho GTPases were expressed in the cells but at a lesser level than the endogenous Rho (Fig. 1A).

Staining of the cells with fluorescently labeled phalloidin or anti-vinculin antibody revealed that under the serum-free conditions, both active forms of RhoB and RhoC stimulated the actin stress fiber and focal adhesion plaque formation, effects similar to that induced by the active RhoA mutants or by 10% CS stimulation (Fig. 1, B and C). Because the amino acid sequences of the three Rho proteins are over 85% identical overall and are almost 100% identical in the switch I effector domain, it is likely that they utilize the same set of effector targets to mediate actin structural changes and focal adhesion assembly.

Although RhoB has been suggested to be involved in Ras-mediated oncogenic transformation (43), RhoA is the only one among the three that has been shown to possess transforming activity (42). When the transformation ability of the RhoA, RhoB, and RhoC mutants were directly compared in a foci-forming assay, we found that RhoB displayed a potent transforming activity similar to RhoA in both RhoB-F30L and RhoB-Q63L forms. In contrast, neither active forms of RhoC were able to transform cells (Fig. 2A). RhoA was shown previously (44) to be involved in fibroblast cell movement by mediating cell body contraction. When the migration rates of the cells expressing the active mutants of RhoA, RhoB, or RhoC were compared in a wound healing assay, we found that RhoC, but not RhoA or RhoB, was able to significantly increase migration of the cells.
from the edge of the wound to the open space in the middle of the wound (Fig. 2B). These results indicate that individual Rho proteins have unique functions in the two aspects related to tumorigenesis, transformation and migration. RhoA, in particular, is important for cell growth control and transformation, whereas RhoC might be a key player in mediating cell movement. Although RhoB is capable of transforming cells under the overexpression conditions, it may serve as a sensor for DNA damage signals and mediate apoptotic responses of the cells based on the genetic evidence (17). The functional differences of these Rho proteins might be attributed to the sequence divergence in their C-terminal hypervariable region, an area that is known to be critical for their distinct intracellular distribution (32, 33).

**Targeting Individual Rho GTPase Activities by p190-Rho Chimeras**—The commonly used biochemical tools to implicate the involvement of a Rho protein in a particular signaling pathway include the dominant negative mutant of the Rho protein and certain bacterial toxins that can modify the Rho protein function. These reagents are limited by their nonspecific nature in interfering with Rho GTPase functions (23, 25) and may have limited therapeutic value in targeting specific Rho proteins. To specifically inhibit individual Rho protein function, we hypothesize that the negative regulatory role of RhoGAPs, the RhoGAP domain in particular, could be exploited to down-regulate Rho protein activity if it is directed to where the active Rho GTPase substrates reside in cells. The RhoGAP domain of p190 has been demonstrated previously (28, 29) as a catalyst to specifically stimulate GTP-hydrolysis of Rho, not Rac or Cdc42, but it cannot distinguish among the RhoA, RhoB, and RhoC subtypes. When introduced into cells by microinjection, it is mostly cytosolic and readily disassembled the actin stress fiber structure (45). In cells the RhoGAP domain is likely to be tightly regulated by other structural motifs in full-length p190, and both of its catalytic activity and intracellular location may be altered by phosphorylation and protein-protein interaction in response to extracellular stimuli (30, 31). On the other hand, the C-terminal polybasic hypervariable region of Rho proteins, including the CAAX isoprenylation motif, appears to determine the subcellular distribution of respective Rho proteins, including the CAAX isoprenylation motif, appears to determine the subcellular distribution of respective Rho GTPases (32, 33). It seemed therefore logical that by fusing the RhoGAP domain with the C-terminal region of individual Rho protein we would provide the catalytic, GTP-hydrolyzing domain with a specific Rho targeting signal. When expressed at a low level in a controlled manner, such a GAP-Rho chimera might constitute an effective and specific inhibitor of individual Rho activity. Thus, we have generated a set of p190 GAP domain and the C terminus of RhoA, RhoB, or RhoC chimeric constructs to test this hypothesis (Fig. 3).

To ensure that the C-terminal sequences of various Rho proteins fused to the GAP domain of p190 do not interfere with the GAP function of p190, we expressed the GST-tagged p190 and p190-Rho chimeras in COS-7 cells, purified them by glu-
activities as p190 GAP domain alone on the RhoA substrate to p190-RhoB-C, and p190-RhoC-C displayed comparable GAP activity in vitro. Closed circles, GST; open triangles, p190-RhoA-C; open squares, p190-RhoB-C; closed squares, p190-RhoC-C. Co-localization of the p190-Rho chimeras with that of the F30L mutant form of RhoA, RhoB, or RhoC. The GST-Rho-F30L expressing cells were infected with the respective retrovirus expressing HA-tagged p190-Rho chimeras, and the EGFP-positive cells were isolated and imaged after anti-GST and anti-HA immunofluorescence staining. The anti-GST and anti-HA staining patterns, as well as the overlay of the images, are shown.

Fig. 4. The p190-Rho chimeras are active GAPs in vitro and target to the intracellular compartments where respective active Rho proteins reside. A, various p190GAP and p190-Rho chimeras were expressed in COS-7 cells by using the pCEFL-GST vector and were purified from the transfected cells by glutathione-agarose affinity chromatography. The GST-p190 and chimera proteins were detected by anti-GST Western blotting. B, the GAP activities of the respective proteins were assayed on recombinant RhoA-ly-32P-GTP by a filter binding assay. Closed circles, GST; open squares, p190 GAP domain; open triangles, p190-RhoA-C; closed triangles, p190-RhoB-C; closed squares, p190-RhoC-C. C, co-localization of the p190-Rho chimeras with that of the F30L mutant form of RhoA, RhoB, or RhoC. The GST-Rho-F30L expressing cells were infected with the respective retrovirus expressing HA-tagged p190-Rho chimeras, and the EGFP-positive cells were isolated and imaged after anti-GST and anti-HA immunofluorescence staining. The anti-GST and anti-HA staining patterns, as well as the overlay of the images, are shown.

Likewise, p190-RhoC-C displayed a similar localization pattern to RhoC-F30L and co-localized with anti-GST signals with a uniquely diffused but punctuated pattern around the perinuclear region (Fig. 4C). These results suggest that the different C-terminal sequences of the Rho proteins can direct the p190-Rho chimeras to distinct locations similar to the Rho GTPases themselves.

To evaluate the efficacy and specificity of the chimeric constructs in cells, we employed the above described fast-cycling mutant RhoA-F30L and GTPase-defective mutant Rho-Q63L expressing cells as testing systems, taking advantage of the facts that the F30L mutant form of the Rho GTPases is fully responsive to GAP stimulation whereas the GTPase-defective Rho-Q61L mutants would not be affected by the RhoGAP treatment, but both mutant forms behave similarly in eliciting cellular effects such as induction of transformation or migration. We chose two retroviral expression vectors, SF91-EMCV-ires-GFP and MIEG3, for delivery of the constructs, because the former one allows high copy number insertion of the chimeras into the host cell genome whereas the latter typically introduces a single or low copy number of gene of interests, and both contain a bicistronically expressed EGFP as a marker that permits quick and semi-quantitative isolation of the p190 expressing cells base on the EGFP fluorescence (36, 37, 57, 58). When the fluorescence-activated cell sorting-purified EGFP-positive cells were examined for the expression of Flag (by SF91-EMCV-ires-GFP) or HA (by MIEG3)-tagged p190-Rho chimeras by Western blotting, all constructs were found to be expressed at a similar level (data not shown).

To examine whether the p190-Rho chimeras can be used to specifically target individual Rho GTPase activities, the GTP-bound form of RhoA-F30L or RhoC-F30L in cells co-expressing RhoA-F30L or RhoC-F30L, together with p190, p190-RhoA-C, p190-RhoB-C or p190-RhoC-C, were probed by affinity precipitation with immobilized His-Routekin. As shown in Fig. 5A and Fig. 6A, p190-RhoA-C and p190-RhoC-C specifically down-regulated the RhoA-F30L and RhoC-F30L activity by ~50 and ~60%, respectively. Moreover, in cells expressing the GTPase-defective form of RhoA or RhoC, i.e. RhoA-Q63L or RhoC-Q63L, p190-RhoA-C and p190-RhoC-C did not affect the amount of RhoA-Q63L-GTP and RhoC-Q63L-GTP precipitated from the cell lysates by Routekin (data not shown). Thus, the p190-Rho chimeras, p190-RhoA-C and p190-RhoC-C in particular, can specifically down-regulate individual Rho protein activity depending on the C-terminal sequences of the Rho protein fused to the RhoGAP domain.

To demonstrate the functional outcome of p190-Rho chimera application, the transformation and migration phenotypes caused by expression of RhoA-F30L or RhoC-F30L (Fig. 2) were examined in cells co-expressing RhoA-F30L or RhoC-F30L and various chimeras. Fig. 5B and Fig. 6B show that the RhoA-F30L-induced transformation can be specifically inhibited by expression of p190-RhoA-C using the high copy number retroviral vector, SF91-EMCV-ires-GFP, whereas the RhoC-F30L-stimulated cell migration can be specifically reverted by p190-RhoC-C but not by p190, p190-RhoA-C, or p190-RhoB-C, by using the low copy number vector, MIEG3. Moreover, the p190-Rho chimeras had no effect on the RhoA-Q63L- or RhoC-Q63L-induced cell transformation or migration (data not shown). These results further suggest that the p190-Rho chimeras are useful tools to functionally revert cellular phenotypes caused by elevation of individual Rho activity.

Reversal of Tumor Cell Transformation and/or Invasion by p190-RhoC-C Chimera—We next asked whether the p190-Rho chimeras could be applied to human cancer cells that show characteristics of transformation and/or invasion because of overexpression or up-regulation of specific Rho GTPase. It has
been demonstrated previously (16) that the HME acquire the transformation and invasion activities by overexpression of RhoC, resulting in phenotypes similar to that of inflammatory breast cancer cells. In the soft agar-based growth assay, very few colonies were seen grown in the control retrovirus-treated HME cells (Fig. 7A). Upon introduction of RhoC, the colony-forming activity of the cells (HME-RhoC) increased dramatically. As shown in Fig. 7A, the colony-forming activity of HME-RhoC cells was inhibited by ~5-fold upon treatment with the retrovirus expressing p190-Rho-C whereas it was not affected by the control EGFP expressing virus or virus expressing p190, p190-RhoA-C, or p190-RhoB-C. Moreover, the invasive activity of HME-RhoC was also specifically inhibited by p190-Rho-C-C but not by EGFP alone or by p190, p190-RhoA-C, or p190-RhoB-C (Fig. 7B).

In a previous gene array screening, the mRNA level of RhoC was found to be significantly elevated in the highly metastatic human melanoma A375-M cells compared with the non-invasive parental A375 cells (15). The invasive and metastatic properties of A375-M were attributed in part to the increased RhoC activity (15). When the set of p190-Rho chimeras were applied to the A375-M tumor cells, the p190-RhoC-C chimera was found to reduce both the migration and the invasion activities of A375-M significantly (Fig. 8, A and B), similar to the extent caused by forced expression of excess amount of dominant negative RhoA (15). Again, p190, p190-RhoA-C, or p190-RhoB-C, as well as the retrovirus-expressed EGFP, had no detectable effect on the migration or invasion property of the tumor cells (Fig. 8, A and B). Thus, it appears that the p190-Rho chimeras can be applied to human tumor cells to specifically down-regulate individual Rho GTPase activities and to reverse the growth and/or invasion phenotypes associated with overexpression of a distinct subtype of Rho GTPase.

**DISCUSSION**

The genes encoding H-, N- and K-Ras GTPases were among the first human oncogenes identified and have been found to be mutated in about 30% of human cancers (46). These mutations typically result in constitutively active Ras proteins that are GTPase-defective and unresponsive to Ras GAP stimulation. To target oncogenic Ras in cancer, several strategies have been developed. Farnesyltransferase inhibitors inhibit Ras function by preventing its post-translational modification by farnesyliosoprenoid (47), and the intracellular antibody capture technology was effective in generating intrabodies that bind to the oncogenic Ras protein (48). Another approach was the generation of a set of peptides derived from mutated Ras to act as antigens for cytotoxic and helper T-cell recognition for immunotherapy (49). Antisense technology has also been applied to specifically inhibit the expression of pathogenic Ras to reverse Ras-induced tumor proliferation (50). Recent progress in small
interference RNA technology may present additional means to specifically target signaling molecules whose overexpression or elevated activity is associated with cancer development (59, 60). However, some of these strategies are still at an infant stage of development or have encountered difficulties at the cell biology stage or when applied to clinical cases, and it remains a challenge to develop novel approaches that may effectively alter the Ras-associated tumorigenic properties by interfering with Ras or Ras-pathway related signaling molecules.

Rho GTPases are key regulators of multiple cell functions related to cancer development (8). They affect cell-to-cell or cell-to-extracellular matrix adhesion, cell movement and morphological transformation, gene induction, apoptosis, and cell cycle progression (1–3). Moreover, RhoA, Rac1, and Cdc42 have been implicated as essential components for Ras-induced transformation (11). Accumulating evidence points to specific roles of individual members of Rho family in different aspects of the tumor development, such as increased proliferation, loss of contact inhibition, transformation, invasion into the adjacent tissues, and metastasis to the distant organs (8–10). Similar to Ras, Rho GTPases and the signaling pathways controlled by them may therefore make attractive targets for therapeutic intervention in cancer.

Unlike Ras, no constitutively activating mutations of Rho
proteins have been found in human tumors so far. Extensive screening has revealed that up-regulation or overexpression, rather than mutation, of specific Rho GTPases often associates with tumorigenesis and sometimes correlates with poor prognosis (18–22). Currently proposed methods of interfering with Rho GTPase function include designing geranylgeranyltransferase inhibitors for Rho proteins similar to the farnesyltransferase inhibitors for Ras, inhibiting the GEF activity or Rho-GEF interaction to suppress endogenous Rho activity, blocking the interaction between a Rho GTPase and its specific downstream effectors, and inactivating effector functions (8). Although these approaches are reasonable and may yield useful reagents for blocking Rho GTPase pathways (e.g. compound Y35674 specifically inhibits Rho kinase activity; see Ref. 51), most have not evolved to a stage where a specific inhibition of selected Rho functions can be achieved. At the Rho activity level, dominant negative mutants of Rho GTPases and Rho GTPase-modifying bacterial toxins remain the predominant reagents of choice for the cell biological studies of Rho protein function (23, 25). However, concern over their specificity and efficacy in many situations limits their value, and whether they can be applied to the study of human diseases or be used for therapeutic purposes remains to be tested.

The RhoGAP family regulatory proteins of Rho GTPases include over 70 mammalian members (5). The GAP activities of RhoGAPs are tightly controlled in cells via complex protein-protein or protein-lipid interactions so that the Rho GTPases would not be in the off-state all the time. Many RhoGAP domains, however, appear to represent a constitutively active structural module and display limited specificity toward the Rho GTPase substrates. Under overexpression or microinjection conditions, RhoGAP domain alone could cause Rho GTPase down-regulation and disruption of certain Rho-mediated cellular functions (45, 52, 53). This likely is because of partial saturation of intracellular compartments by the introduced RhoGAP domain and may not be desirable in studies to extract more specific function of individual members of Rho GTPases. In this context, the concern for specificity when utilizing RhoGAP domain alone for down-regulation of Rho proteins is similar to the use of dominant negative Rho GTPases or bacterial toxins.

In the present studies, we hypothesize that the negative regulatory role of the RhoGAP domain could be exploited to specifically down-regulate individual Rho protein activity, provided that it could be expressed at a level in a controlled manner and could be directed to sites where active Rho GTPase substrates are localized by means of the C-terminal intracellular targeting sequences of the Rho protein. In particular, p190GAP fused with the RhoA, RhoB, or RhoC C-terminal sequences might be useful for specific down-regulation of the respective cellular Rho GTPase activities. This is an attractive model system, because as we demonstrated in NIH 3T3 cells, RhoA, RhoB, and RhoC play different roles in mediating cell transformation and migration (Fig. 2). Previous gene targeting studies of RhoB function have shown that distinct from the positive influence on tumor growth or metastasis by RhoA or RhoC (11, 15), RhoB may act as a negative modifier or suppressor in cancer development, mediating cellular responses to DNA damage or farnesyltransferase inhibitors (17). Thus, achieving specific targeting of different subtypes of Rho proteins would be highly desirable in the treatment of tumor cells to reverse the growth and/or invasion phenotypes caused by overexpression of RhoA or RhoC while preserving the tumor suppressor function of RhoB.

As a demonstration of principle, we showed that the p190-Rho chimera were biochemically active as GAPs for RhoA in vitro and indeed co-localized to the distinct intracellular locations specified by the Rho C-terminal sequences. This differential localization may also partly explain the differential functions displayed by different Rho subtypes. Further cellular assays revealed that p190-Rho chimeras specifically down-regulated Rho protein activities in cells depending on the C-terminal sequences in the chimeras when expressed in a controlled manner by retroviral induction. Moreover, p190-RhoA-C and p190-RhoC-C specifically inhibited the RhoA- or RhoC-stimulated cell transformation or migration. Although it was effective in specifically down-regulating Rho or RhoC activity and in reversing the transformation or migration phenotype induced by the respective fast-cycling mutant, p190-RhoA-C and p190-RhoC-C had no effect on the activity of or phenotype induced by the GTase-defective mutant RhoA-Q63L or RhoC-Q63L. These results validate the RhoGAP-based approach to specifically down-regulate the biochemical and biological activity of individual Rho subtypes in cells.

To examine the efficacy of this approach in tumor cells, we applied the p190-Rho chimera constructs to two different human tumor cells of which the oncogenic and invasive properties have previously been attributed to elevated RhoC expression (15, 16). In both the breast cancer HME-RhoC cells and the melanoma A375-M cells, p190-RhoC-C, but not other p190-Rho constructs, had a potent inhibitory effect on the anchorage-independent growth and/or migration and invasion. Thus, the p190-Rho-based method appears to work exceptionally well at the tumor cell level in specifically down-regulating the activity of an individual Rho subtype and in reversing the tumor cell phenotypes. It remains to be seen whether this approach can be used in other cancer cell types and whether the reversal of phenotypes of the cancer cells will hold in an animal model.

Like other cases of retrovirus-based gene transfer approach, the copy number of the introduced p190-Rho chimeras in the host cells will likely to be critical for the efficacy of their application. Although p190-Rho-C-C was effective in down-regulating the biochemical activity and migration phenotype of RhoC when expressed with the low copy number vector MIEG3 (Fig. 6), we have found that expression of p190-RhoA-C or p190-RhoB-C with MIEG3 is insufficient for down-regulating the biochemical activity or transforming activity of RhoA-F30L or RhoB-F30L (data not shown). However, expression of p190-RhoA-C with the high copy number retroviral vector, SF91-EMCV-ires-GFP, which has been in use for human gene therapy trials (57, 58), can specifically decrease both the biochemical activity of p30RhOA and the RhoA-F30L-induced transformation under conditions in which p190-RhoB-C, p190-RhoC-C, or p190 alone expressed by using the same vector did not affect RhoA-F30L activity or the RhoA-F30L-induced transformation (Fig. 5). These results highlight the importance of dose dependence in effectiveness and specificity when applying this method to future animal and human trials.

In addition to p190, which is active GAP toward RhoA, RhoB, and RhoC, the GAP domain of Bcr has been shown to be specific for Rac but cannot distinguish among Rac1, Rac2, and Rac3 subtypes (54), whereas Cdc42GAP favors catalyzing the GTP hydrolysis of Cdc42 but remains quite active toward other Rho proteins (55). Like that of RhoA, RhoB, and RhoC, the C-terminal sequences of Rac1, 2, and 3 and Cdc42, including the lipid modification CAAX motif, have been known to direct their distinct intracellular localization patterns (33). Given the strong evidence of a role of various Rac subtypes and Cdc42 in tumorigenesis and other human diseases (8, 56), it will be of interest to test whether the BcrGAP fused with the respective Rac C-terminal sequences and the Cdc42GAP fused with the corresponding Cdc42 sequences would work as nicely to specif-
A Novel Strategy to Specifically Down-regulate Rho GTPases

44625

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