Rho Isoform-specific Interaction with IQGAP1 Promotes Breast Cancer Cell Proliferation and Migration*

Received for publication, May 1, 2012, and in revised form, September 14, 2012. Published, JBC Papers in Press, September 19, 2012, DOI 10.1074/jbc.M112.377499

Darren E. Casteel, Stephanie Turner, Raphaela Schwappacher, Hema Rangaswami, Jacqueline Su-Yuo, Shunhui Zhuang, Gerry R. Boss, and Renate B. Pilz

From the Department of Medicine and Cancer Center, University of California, San Diego, La Jolla, California 92093

Background: RhoA/C and RhoB have homologous sequences, but opposing functions.

Results: IQGAP1 binds prenylated, active RhoA/C, but not RhoB; IQGAP1 increases RhoA/C GTP loading and is required for RhoA/C-induced proliferation and motility of breast cancer cells.

Conclusion: IQGAP1 is a regulator and pro-oncogenic effector of RhoA/C.

Significance: Disrupting Rho/IQGAP interactions with prenylation inhibitors may be a useful adjunct for breast cancer treatment.

We performed a proteomics screen for Rho isoform-specific binding proteins to clarify the tumor-promoting effects of RhoA and C that contrast with the tumor-suppressive effects of RhoB. We found that the IQ-motif-containing GTPase-activating protein IQGAP1 interacts directly with GTP-bound, prenylated RhoA and RhoC, but not with RhoB. Co-immunoprecipitation of IQGAP1 with endogenous RhoA/C was enhanced when RhoA/C were activated by epidermal growth factor (EGF) or transfection of a constitutively active guanine nucleotide exchange factor (GEF). Overexpression of IQGAP1 increased GTP-loading of RhoA/C, while siRNA-mediated depletion of IQGAP1 prevented endogenous RhoA/C activation by growth factors. IQGAP1 knockdown also reduced the amount of GTP bound to GTPase-deficient RhoA/C mutants, suggesting that IQGAP1 enhances Rho activation by GEF(s) or stabilizes Rho-GTP. IQGAP1 depletion in MDA-MB-231 breast cancer cells blocked EGF- and RhoA-induced stimulation of DNA synthesis. Infecting cells with adenovirus encoding constitutively active RhoA1.63 and measuring absolute amounts of RhoA-GTP in infected cells demonstrated that the lack of RhoA1.63-induced DNA synthesis in IQGAP1-depleted cells was not due to reduced GTP-bound RhoA. These data suggested that IQGAP1 functions downstream of RhoA. Overexpression of IQGAP1 in MDA-MB-231 cells increased DNA synthesis irrespective of siRNA-mediated RhoA knockdown. Breast cancer cell motility was increased by expressing a constitutively-active RhoCV14 mutant or overexpressing IQGAP1. EGF- or RhoC-induced migration required IQGAP1, but IQGAP1-stimulated migration independently of RhoC, placing IQGAP1 downstream of RhoC. We conclude that IQGAP1 acts both upstream of RhoA/C, regulating their activation state, and downstream of RhoA/C, mediating their effects on breast cancer cell proliferation and migration, respectively.

The Rho family of small GTPases includes Rho, Rac, and Cdc42. Rho family proteins cycle between an active, GTP-bound form and an inactive, GDP-bound form, and regulate multiple cellular processes such as growth, survival, and cellular motility, through interaction with various effector molecules (1). Cycling of Rho family proteins between the GTP- and GDP-bound state is regulated by guanine nucleotide exchange factors (GEFs) promoting GTP loading, and GTPase-activating proteins (GAPs) causing de-activation (1). RhoA and C are oncogenic and linked to increased cancer cell proliferation and invasion/metastasis, whereas RhoB has tumor suppressor properties, inhibiting colony formation and promoting apoptosis (1, 2). Correspondingly, RhoA and C are frequently overexpressed in human cancers, whereas RhoB is often undetectable or lower in cancer cells compared with surrounding normal tissue (1, 3). RhoA is required for cell transformation by oncogenic Ras, and constitutively active RhoA can stimulate DNA synthesis and cell cycle progression (1, 3, 4). Overexpression of RhoC in human mammary epithelial cells promotes anchorage-independent growth, invasiveness, and tumor formation in mice, whereas siRNA-mediated knock-down of RhoC in breast cancer cells inhibits proliferation, invasiveness, and tumor growth in nude mice (3, 5–8). Studies in RhoC-deficient mice demonstrate an important role of RhoC in tumor cell metastasis (9). In contrast, RhoB inhibits Ras transformation, and RhoB-deficient mice show enhanced growth of Ras-transformed tumor cells and increased carcinogen-induced tumorigenesis (10, 11).

Despite the opposing effects of RhoA and C compared with RhoB, the three Rho isoforms have 85% sequence identity. Moreover, RhoB differs in only one amino acid from RhoA and C within the core effector binding domain, but sequences out-

*This work was supported, in whole or in part, by National Institutes of Health Grants K22-CA124517 (to D. C.) and R01-AR051300 (to R. B. P.).
† This article contains supplemental Figs. S1 and S2.
1 Both authors contributed equally to this work.
2 Supported by T32-HL007261.
3 Supported by a research fellowship grant from the Deutsche Forschungsgemeinschaft.
4 To whom correspondence should be addressed: Department of Medicine, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA, 92093-0652. Tel.: 858-534-8805; E-mail: rpilz@ucsd.edu.

The abbreviations used are: GEF, guanine nucleotide exchange factor; BrdU, bromo-deoxyuridine; EGF, epidermal growth factor; GAP, GTPase-activating protein; IQGAP, IQ-motif-containing GTPase-activating protein; RBD, rho binding domain.
IQGAP1 Is a Regulator and Effector of RhoA and C

side this domain contribute to effector protein binding (2, 12). Most Rho effector proteins bind to all three Rho isoforms, e.g. Rho-kinases, protein kinase N, rhoetkin, and mDia (2, 13). Few Rho isoform-specific effector proteins have been identified to date, and none adequately explain the tumor-promoting effects of RhoA/C versus tumor-suppressing effects of RhoB (2, 14–16).

The IQ-motif-containing GTPase-activating protein IQGAP1 is a multi-domain scaffold protein which binds Cdc42 and Rac1, but IQGAP inhibits, rather than activates, GTP hydrolysis by both proteins (17–19). In addition to binding to Cdc42 and Rac1, IQGAP1 interacts with multiple other proteins, and regulates a wide range of cellular processes, such as cell growth and survival, as well as cytoskeletal organization, motility, and cell-cell adhesion (17, 20, 21). Like RhoA and C, IQGAP1 has oncogenic properties and is up-regulated in many cancers, including breast, lung, ovarian, and gastric cancers (17, 20). Forced overexpression of IQGAP1 enhances breast cancer cell proliferation, motility, and invasion, whereas siRNA-mediated knock-down has the opposite effect (22–24). IQGAP1-deficient mice develop normally, but display gastric hyperplasia and polyps (25). Analogous to RhoA and C, IQGAP1 plays an important tumor-promoting role in breast cancer (22), but IQGAP1 has not been observed to bind Rho A or C (18, 19, 26–28).

To better understand the molecular basis for the opposing effects of RhoA/C and RhoB, we searched for RhoA/C- and RhoB-specific interaction proteins using a proteomics screen. We found IQGAP1 is a specific binding partner for GTP-bound RhoA and C, but not RhoB, and is a key modulator of RhoA and C in regulating breast cancer cell proliferation and motility.

EXPERIMENTAL PROCEDURES

Antibodies and DNA Constructs—Murine monoclonal antibodies directed against RhoA (sc-418) and tubulin, and rabbit polyclonal antibodies anti-HA and Myc epitopes were from Santa Cruz Biotechnology (Santa Cruz, CA). The RhoC-specific antibody (#3430) was from Cell Signaling Technology (Danvers, MA), and IQGAP1 antibody was from Millipore (Billerica, MA). Bromo-deoxyuridin (BrdU), Flag-epitope antibody, and eluted with TEV protease prior to analy-

Cells were transiently transfected using 5 μl of Lipofectamine 2000™ (Invitrogen, San Diego, CA) and 1 μg of DNA or 100 pmol of siRNA per ml of FBS-containing DMEM as described previously (32). siRNA oligoribonucleotides were from Qiagen (Valencia, CA), and the sequence of the control siRNA targeting green fluorescent protein (GFP) was described previously (34). The other siRNA target sequences were: 5′-AAGTCTAGGAG-AGTAATGG-3′ for IQGAP1; 5′-AAGTCAAGCATTTCGT-CCAA-3′ for RhoA; and 5′-AAGACCTGGCTCTCATGT-C-3′ for RhoC. The siRNA targeting IQGAP1 was transfected twice 24 h apart; siRNAs targeting RhoA or C were transfected once. Where indicated, cells were infected with adenovirus 6 h after the last siRNA transfection; the multiplicity of infection was titrated to produce amounts of IQGAP1 similar to the
amounts of endogenous proteins. For RhoA<sup>1,63</sup> and RhoC<sup>V14</sup>, cells were infected with two different viral concentrations sufficient to produce a maximal increase in DNA synthesis or migration, respectively.

**Measurement of Rho Activation**—Two different methods were used to measure Rho activation. To quantify absolute amounts of GTP bound to transfected, epitope-tagged RhoA, or RhoC constructs, we employed a coupled enzymatic assay using nucleoside diphosphate kinase and luciferase as described previously (29, 35). To determine the activation state of endogenous RhoA or C separately from the other Rho isoforms, we used the Rho binding domain (RBD) of rhotekin to isolate Rho-GTP, and assessed the amount of GTP-bound RhoA or C by Western blotting as described by Ren et al. (30). In some experiments, we assessed the activation state of endogenous RhoA/C using the RBD pull-down assay combined with enzymatic GTP measurements (29).

**Migration Assays**—For Transwell migration, MDA-MB-231 cells were transfected with the indicated siRNAs as described above, and 6 h later, cells were transferred to 0.1% FBS-containing DMEM. Adenoviral vectors were added as indicated, and 24 h later, 1.5 × 10⁵ cells were plated in DMEM containing 0.2% bovine serum albumin on 8 μm Transwell filters (24 well inserts, BD Biosciences, San Diego, CA) coated with fibronectin (7.5 μg/ml). The wells below the filters were filled with DMEM containing 10% FBS or 20 ng/ml EGF. After incubation for 12–16 h, cells were fixed and stained with Crystal violet. Cells remaining on top of the filter were removed with a sterile pipette tip, and photographs were taken immediately, and 40 h later. Cells that entered the wound, as defined by the borders on the initial photograph, were counted.

**BrdU Incorporation**—MDA-MB-231 cells (1 × 10⁵) were plated on glass cover slips in 24-well dishes; cells were transfected with siRNAs, transferred to medium containing 0.1% FBS, and infected with adenovirus as described for Transwell migration. BrdU (200 μm) was added 24 h later with or without EGF (20 ng/ml) for an additional 16 h. Cells were fixed, stained with anti-BrdU antibody, and counterstained with Hoechst 33342 as described (32). At least 300 cells were counted per condition, and results were confirmed by a blinded observer.

**Data Analysis**—Bar graphs represent the mean ± S.E. of at least three independent experiments. Data were analyzed by one-way analysis of variance with Bonferroni post-test analysis. A p value of <0.05 was considered statistically significant.

**RESULTS**

**IQGAP1 Interacts with RhoA/C but Not RhoB**—We performed a proteomics screen in 293T cells transfected with equal amounts of epitope-tagged, constitutively active RhoA, B, and C (all with a glycine to valine mutation at position 14) to search for Rho isoform-specific interacting proteins. By mass spectrometric analysis of bands that were reproducibly and differentially associated with anti-epitope immunoprecipitates of the three Rho proteins, we identified IQGAP1 as a 190 kDa protein associated with RhoA and C, but not with RhoB (Fig. 1A). RhoA- and RhoC-specific association with IQGAP was confirmed in experiments in which the double epitope-tagged Rho proteins were re-immunoprecipitated using an antibody spe-
specific for the second epitope, but the protein yield was lower (supplemental Fig. S1A).

Co-immunoprecipitation of endogenous IQGAP1 with transfected, constitutively active RhoAV14 and RhoCV14, but not RhoBV14 was also confirmed by Western blotting using an IQGAP1-specific antibody, both in 293T cells (Fig. 1B) and MDA-MB-231 breast cancer cells (supplemental Fig. S1B). To determine whether the interaction between RhoA/C and IQGAP1 was direct, and to establish the IQGAP binding region, we incubated Flag-tagged RhoAV14 or RhoCV14 purified from 293T cells with GST-tagged IQGAP-N (amino acids 1–862) or IQGAP-C (amino acids 863–1657) purified from bacteria, with GST serving as a negative control. RhoA and C bound specifically to the C-terminal half, but not the N-terminal half of IQGAP1, whereas RhoB showed no specific binding to either construct (Fig. 1C). These data suggest direct binding of RhoA and C to the C-terminal half of IQGAP1, which contains the GAP-related domain required for CDC42 and Rac binding (20).

The IQGAP1/RhoA Interaction Requires GTP Binding and Prenylation of RhoA—To determine if the RhoA/IQGAP1 interaction was dependent on the activation state of RhoA, we transfected cells with expression vectors encoding Flag-tagged wild type (Wt), constitutively active (V14 or L63), dominant-negative (N19) RhoA proteins, or empty vector. Anti-Flag immunoprecipitates were analyzed by Western blotting with anti-Myc antibody for the presence of IQGAP1 (top panel), or with anti-Flag antibody for the amounts of RhoA (bottom panel). Input lysates (2.5%) are shown in the right lanes. B, cells were transfected with IQGAP1 and the Rho constructs described in panel A; absolute amounts of GTP bound to anti-Flag immunoprecipitates were measured using a luciferase-based assay, as described under “Experimental Procedures.” C and D, cells were transfected with IQGAP1 and Flag-tagged wild type RhoA as in panel A, but cells were serum-deprived for 8 h and treated with EGF (20 ng/ml) for 5 min. In panel C, anti-Flag immunoprecipitates were analyzed by Western blotting for the presence of IQGAP1, and in panel D, GTP bound to anti-Flag immunoprecipitates was measured enzymatically as in panel B. E and F, 293T cells were co-transfected with Myc-tagged IQGAP1 and vectors encoding Flag-tagged RhoA(V14) in its normal prenylated form (C190 is the cysteine modified by geranyl-geranylation), or with a mutation in the prenylation signal (S190); anti-Flag immunoprecipitates were probed for the association of IQGAP1 (E) or for the amount of GTP bound to RhoA(V14/C190) and RhoA(V14/S190) (F).

Interaction of Endogenous RhoA and RhoC with IQGAP1—To increase the amount of endogenous RhoA in the GTP-

FIGURE 2. The IQGAP1/RhoA interaction requires GTP binding and prenylation of RhoA. A, 293T cells were co-transfected with Myc epitope-tagged IQGAP1 and Flag-tagged RhoA constructs encoding wild type (Wt), constitutively active (V14 or L63), dominant-negative (N19) RhoA proteins, or empty vector. Anti-Flag immunoprecipitates were analyzed by Western blotting with anti-Myc antibody for the presence of IQGAP1 (top panel), or with anti-Flag antibody for the amounts of RhoA (bottom panel). Input lysates (2.5%) are shown in the right lanes. B, cells were transfected with IQGAP1 and the Rho constructs described in panel A; absolute amounts of GTP bound to anti-Flag immunoprecipitates were measured using a luciferase-based assay, as described under “Experimental Procedures.” C and D, cells were transfected with IQGAP1 and Flag-tagged wild type RhoA as in panel A, but cells were serum-deprived for 8 h and treated with EGF (20 ng/ml) for 5 min. In panel C, anti-Flag immunoprecipitates were analyzed by Western blotting for the presence of IQGAP1, and in panel D, GTP bound to anti-Flag immunoprecipitates was measured enzymatically as in panel B. E and F, 293T cells were co-transfected with Myc-tagged IQGAP1 and vectors encoding Flag-tagged RhoA(V14) in its normal prenylated form (C190 is the cysteine modified by geranyl-geranylation), or with a mutation in the prenylation signal (S190); anti-Flag immunoprecipitates were probed for the association of IQGAP1 (E) or for the amount of GTP bound to RhoA(V14/C190) and RhoA(V14/S190) (F).
bound form, we transfected 293T cells with the constitutively-active Rho guanine nucleotide exchange factor ΔNp115-Rho-GEF or the heterotrimeric G protein Go13(QL). In several cell types, Go13(QL) stimulates p115-RhoGEF activity, thereby activating RhoA (29, 35). In the absence of the activators, endogenous RhoA was barely detectable in IQGAP1 immunoprecipitates, but RhoA/IQGAP1 complexes were greatly enhanced in cells co-transfected with the constitutively active p115 or Go13 constructs, consistent with the increase in endogenous GTP-bound RhoA seen in the transfected cells (Fig. 3, A and B).

When we treated MDA-MB-231 breast cancer cells with EGF to stimulate GTP loading of endogenous RhoA, we detected small amounts of RhoA in IQGAP1 immunoprecipitates (supplemental Fig. S2, A and B). Treating MDA-MB-231 cells with EGF also increased GTP loading of RhoC, albeit with slower kinetics (Fig. 3D). Endogenous RhoC was associated with IQGAP1 in serum-starved cells, but EGF treatment increased the amount of RhoC in IQGAP1 immunoprecipitates (Fig. 3C).

In contrast, when MDA-MB-231 cells were transfected with an siRNA specific for IQGAP1, depletion of IQGAP1 greatly reduced RhoA activation in serum-stimulated cells (Fig. 4, B and C). There was a trend toward lower basal RhoA-GTP levels in IQGAP1-depleted cells compared with cells transfected with control siRNA, but this difference did not reach statistical significance. Similarly, IQGAP1 depletion blocked RhoC activation in EGF-treated cells (supplemental Fig. S2C). Thus, IQGAP1 over-expression enhanced Rho-GTP levels, whereas IQGAP1 knock-down largely prevented growth factor stimulation of RhoA/C activity, suggesting that IQGAP1 either stabilizes RhoA/C in the GTP-bound form or promotes RhoA/C activation by GEFs.

RhoA-induced Proliferation of Breast Cancer Cells Requires IQGAP1—In some breast cancers, RhoA, RhoC, or IQGAP1 are overexpressed compared with normal tissues, and all three proteins promote proliferation of breast cancer cells in vitro and stimulate tumorigenesis in vivo (1, 5, 7, 20, 22, 36, 37). To determine if IQGAP1 is necessary for RhoA to regulate cell proliferation (or vice versa), we used an siRNA approach.

IQGAP1 depletion inhibited DNA synthesis in MDA-MB-231 cells by ~50%, as assessed by nuclear BrdU uptake (Fig. 5, A and C, open bars, and Fig. 5B, compare top and bottom left panels); similar results have been reported in MCF-7 breast cancer cells (22). In MDA-MB-231 cells transfected with a control siRNA-targeting GFP, treatment with EGF or expression of RhoA1,63 doubled the number of cells in S-phase (Fig. 5A, filled bars, for EGF effects; Fig. 5B right panels and Fig. 5C, filled and dotted bars for the effects of RhoA1,63). However, in IQGAP1-depleted cells, neither EGF treatment, nor expression of RhoA1,63 was able to increase DNA synthesis (Western blots in Fig. 5, A and E show efficient IQGAP1 depletion, and Fig. 5E...
IQGAP1 is a Regulator and Effector of RhoA and RhoC

Figure 4. IQGAP1 regulates RhoA activation. A, 293T cells were co-transfected with expression vector encoding HA epitope-tagged wild type RhoA or RhoC and either empty vector or vector encoding Flag-tagged IQGAP1. Cell lysates were subjected to immunoprecipitation with anti-epitope antibody, and GTP bound to the immunoprecipitates was quantified as described in Fig. 2B. The amount of Rho-GTP found in cells transfected with empty vector was assigned a value of one; *, p < 0.05 for the comparison between the presence and absence of IQGAP1. B, MDA-MB-231 cells were transfected with siRNA specific for IQGAP1 or GFP (control) as described under “Experimental Procedures”; cells were serum-starved for 24 h, and stimulated with 20% FBS for 5 min to stimulate Rho activation. GTP-bound Rho was isolated from cell lysates as described in Fig. 3B. RhoA-GTP bound to Rho2kin-RBD was assessed by Western blotting with a RhoA-specific antibody (top panel). Cell lysates were also directly analyzed by Western blotting for total RhoA (middle panel), 1% of input, and IQGAP1 (bottom panel). C, Western blots from three independent experiments performed as in panel B were analyzed by densitometry, and the amount of endogenous RhoA-GTP (normalized to total RhoA) present in serum-starved, GFP siRNA-transfected cells was assigned a value of 1; *, p < 0.05 for the comparison to serum-starved, GFP siRNA-transfected cells; #, p < 0.05 for the comparison to serum-stimulated, GFP siRNA-transfected cells.

IQGAP1 also shows expression of virally transduced and endogenous RhoA.

We infected cells with two different concentrations of adenoviral vector encoding the constitutively active RhoA (2.63 to produce low and high amounts of GTP-bound RhoA, but even the lower dose of virus with less activated RhoA was sufficient to induce maximal stimulation of DNA synthesis in the presence of control siRNA (compare Fig. 5, C and D, filled and dotted bars on the left). In contrast, both doses of RhoA (2.63 were completely ineffective in IQGAP1-depleted cells (Fig. 5C, filled and dotted bars on the right). IQGAP1 depletion significantly decreased the amount of GTP bound to RhoA (2.63, suggesting that IQGAP1 is necessary for Rho-GEF-mediated GTP loading of the GTPase-deficient RhoA (2.63 protein (Fig. 5D). However, in IQGAP1-depleted cells infected with the higher (3×) virus concentration, the amount of GTP-bound RhoA (2.63 per mg cellular protein was similar to the amount of GTP-bound RhoA (2.63 found in control siRNA-transfected cells infected with the lower (1×) virus concentration (Fig. 5D). Since control siRNA-transfected cells showed a robust proliferative response to the 1× dose of RhoA (2.63 virus, we conclude that reduction in GTP-bound RhoA (2.63 does not explain the lack of response to RhoA (2.63 in IQGAP1-depleted cells. Thus, IQGAP1 appears to act as a RhoA effector inducing proliferation.

IQGAP1 Can Promote Proliferation Independently of RhoA—To test if increased levels of IQGAP1 can enhance cell proliferation independently of RhoA, we examined the effect of IQGAP1 overexpression in RhoA-depleted cells. RhoA depletion prevented EGF-induced DNA synthesis in MDA-MB-231 cells, but viral overexpression of IQGAP1 restored BrdU incorporation in EGF-treated cells (Fig. 6A; RhoA depletion and viral IQGAP1 expression is shown in Fig. 6B). Even in the absence of EGF, over-expression of IQGAP1 increased BrdU incorporation about 2-fold, and the effect was the same in MDA-MB-231 cells transfected with control or RhoA-specific siRNAs (Fig. 6, C and D). Thus, proliferation induced by IQGAP1 overexpression is independent of RhoA, but RhoA requires IQGAP1 to stimulate proliferation, placing IQGAP1 downstream of RhoA. Similar results were obtained with RhoC (2.14 (not shown).

RhoC-induced Migration of Breast Cancer Cells Requires IQGAP1—Overexpression of wild type RhoC in human mammary epithelial cells is sufficient to induce a motile and invasive phenotype, and expression of a constitutively active RhoC (2.14 has more dramatic effects (5, 8). IQGAP1 also promotes breast cancer motility and invasion, in vitro and in vivo (22–24). To determine if the two proteins operate in a linear pathway, we performed migration assays in siRNA-transfected MDA-MB-231 cells. EGF treatment or viral expression of constitutively active RhoC (2.14 stimulated transwell migration in control siRNA-transfected cells, but had minimal effects in cells transfected with IQGAP1-specific siRNA (Fig. 7A, filled bars, for EGF effects; Fig. 7C, filled and striped bars for the effects of RhoC (2.14; Fig. 7E shows IQGAP depletion and expression of virally transduced and endogenous RhoC).

IQGAP1 knockdown prevented the EGF-induced increase in endogenous Rho-GTP levels (Fig. 7B), and reduced the level of GTP bound to RhoC (2.14 by ~50% (Fig. 7D). However, the amount of GTP-bound RhoC (2.14 in IQGAP1-depleted cells infected with a high (3×) virus concentration was similar to the amount of GTP-bound RhoC (2.14 in control cells infected with 1× virus; thus RhoC-GTP remained high enough to expect an increase in migration (compare Fig. 7, C and D). We conclude that IQGAP1 is required for RhoC-induced migration, and that reduction in RhoC (2.14-GTP levels does not explain the lack of migratory response to RhoC (2.14 in IQGAP1-depleted cells.

IQGAP1 Can Promote Migration Independently of RhoC—RhoC knockdown blocked EGF-induced transwell migration in MDA-MB-231 cells (Fig. 8A, open and filled bars), consistent
with results reported in other breast cancer cell lines (5–7, 38). However, IQGAP1 overexpression restored EGF-induced migration in RhoC-deficient cells (Fig. 8A, cross-hatched bars; RhoC depletion and IQGAP1 expression is shown in Fig. 8B). We also assessed migration in a wound closure assay: RhoC depletion inhibited wound closure, and IQGAP1 overexpression enhanced wound closure in RhoC-deficient cells (Fig. 8C). Thus, overexpression of IQGAP1 is sufficient to induce migration of MDA-MB-231 cells independently of RhoC, but RhoC-induced migration requires IQGAP1, suggesting that IQGAP1 is an effector of RhoC (Fig. 8E).

**DISCUSSION**

We identified IQGAP1 as a Rho isoform-specific binding protein that may represent the first Rho isoform-specific effector to explain, at least in part, the differential effects of RhoA and C versus RhoB on tumor cell growth and motility (1, 2). IQGAP1 is a scaffolding protein that links multiple signaling pathways implicated in cancer: it regulates cell growth through interactions with growth factor receptors, B-Raf/mitogen-activated protein kinases, and Akt/mTOR; it modulates cell adhesions via β-catenin/E-cadherin interactions; and it stimulates cell motility via cytoskeletal rearrangements that involve bind-
Our findings now add RhoA and C to the small GTPases that directly bind IQGAP1. Sakurai-Yageta et al. (28) did not co-immunoprecipitate RhoAL63 with IQGAP1 from transfected 293 cells, but this was in the absence of magnesium, and we found magnesium to be essential for the Rho/IQGAP interaction. Other workers who examined the interaction of IQGAP1 with CDC42 and Rac1 used bacterially expressed, unprenylated Rho family proteins as affinity ligands, which may explain why they did not detect IQGAP1 binding to RhoA (18, 19, 26, 27). To our knowledge, the effect of prenylation on CDC42 and Rac1 binding to IQGAP1 has not been tested; CDC42 and Rac1 appear to bind IQGAP1 in the absence of lipid modifications, but prenylation could affect binding affinity. Prenylation of Rho proteins is also required for proper subcellular localization, and differences in RhoA/C versus RhoB subcellular localization may partly explain why RhoB does not interact with IQGAP1 in intact cells (2). However, more work is needed to explain why purified RhoB does not bind IQGAP1.

CDC42 and Rac1 bind to the GAP-related domain located in the C-terminal half of IQGAP1, and binding involves primarily the switch I and II regions of CDC42 and Rac1 (42). Since the switch regions of Rho family proteins are highly conserved (43), and we found that RhoA and C bind to the C-terminal half of IQGAP1, it is likely that they also bind to the GAP-related domain.

We found that IQGAP1 binds to RhoA only in the GTP-bound, active form, supporting the notion that it acts as an effector of the small GTPase. Similarly, IQGAP1 binds preferentially to GTP-bound CDC42 and Rac1, although variable degrees of IQGAP1 binding to GDP-bound CDC42 and Rac1 have been reported (18, 26, 27). IQGAP1 inhibits the intrinsic GTPase activity of CDC42 in vitro, stabilizing the protein in the GTP-bound form, and overexpression of IQGAP1 increases the pool of GTP-bound CDC42, but effects on Rac1-GTP are controversial (44, 45). The effect of IQGAP1 on CDC42 (and Rac1) GTP levels may explain why overexpression of IQGAP1 can increase cell proliferation and migration independently of RhoA/C (Fig. 8E).

We observed that overexpression of IQGAP1 increased GTP binding to RhoA and C, while IQGAP1 depletion prevented growth factor-induced activation of endogenous RhoA and RhoC. Similarly, a dominant-negative IQGAP1, missing the GAP-related domain required for Rac1 and CDC42 binding, decreases CDC42- and Rac-GTP levels and blocks CDC42 activation by bradykinin (44). We found a trend toward lower basal RhoA-GTP levels in IQGAP1 siRNA-transfected cells, which did not reach statistical significance; this may be due to the fact that basal RhoA-GTP levels in serum-starved cells were extremely low and difficult to measure. That IQGAP1 knockdown impaired growth factor-induced RhoA/C activation by Rho-GEFs suggests IQGAP1 may facilitate RhoA/C access to GEFs (Fig. 8E). Alternatively, IQGAP1 may stabilize RhoA-GTP by shielding the protein from GAPs, as suggested for Rac1 and CDC42 (44, 45). We favor the first explanation, because IQGAP1 depletion decreased GTP binding to the GTPase-deficient RhoAL63 and RhoCV14 mutants.

Expression of RhoA163 or RhoC14 increased DNA synthesis in MDA-MB-231 cells, similar to effects of RhoC14 seen in
MCF10A cells (8). RhoA and C stimulate cell cycle progression by increasing cyclin D expression; this occurs through multiple pathways that may involve IQGAP1 (1, 8, 46). In addition, RhoA down-regulates the cell cycle-dependent kinase inhibitor p21WAF1, which is crucial for promotion of G1-S transition (1, 46). Our data show that effects of RhoA and RhoC on breast cancer proliferation required IQGAP1, and suggest that IQGAP1 acts as a Rho effector in this pathway (Fig. 8E). While IQGAP1 depletion affected GTP-binding to RhoA1.63, residual amounts of GTP-bound RhoA remained high enough to allow us to conclude that lack of RhoA-induced proliferation in IQGAP-depleted cells could not be explained by a reduction in RhoA-GTP.

IQGAP1 overexpression stimulated DNA synthesis even in RhoA-depleted MDA-MB-231 cells, indicating that IQGAP1 affects cell proliferation independently of RhoA, most likely through its interactions with growth factor receptors, B-Raf/mitogen-activated protein kinases, and Akt/mTOR (20, 40, 41).
When overexpressed in MCF7 cells, IQGAP1 also increases soft agar colony formation and tumor growth in mice (22).

Expression of RhoC(V14) increased migration of MDA-MB-231 cells, consistent with previous reports in another breast cancer cell line (8). We found that the RhoC effect was blocked in IQGAP1-depleted cells, but again, this finding was not explained by the change in RhoC GTP-loading, suggesting that IQGAP acts as an effector for RhoC-induced migration (Fig. 8E). RhoC and IQGAP1 have both been linked to increased cancer cell motility and invasion; high RhoC or IQGAP1 expression strongly correlates with invasion and metastasis in several human cancers, including breast cancer (1, 17, 20, 36). RhoC overexpression is sufficient to enhance metastasis in poorly metastatic melanoma cells (47), and RhoC-null mice have a reduced number and size of lung metastasis in a murine breast cancer model (9). RhoC is overexpressed in 90% of inflammatory breast cancers, which exhibit a highly invasive and metastatic phenotype, and RhoC knock-down inhibits motility and invasiveness of inflammatory breast cancer-derived cell lines (5, 6, 38). The effects of RhoA knock-down on breast cancer migration are more varied (6, 38). We did not find a significant effect of RhoA(L63) on the motility of MDA-MB-231 cells, and a dominant-negative RhoA does not inhibit IQGAP1-induced migration in MCF7 cells (23). However, RhoA co-localizes with IQGAP1 to membrane protrusions termed invadopodia at the leading edge of invading breast cancer cells, and Rho A regulates the interaction of IQGAP1 with proteins involved in matrix metalloproteinase secretion (28). In glioma cells, IQGAP1 co-localizes with the small GTPase ADP-ribosylation factor-6 (ARF6) at the leading edge of invasive cells, and
IQGAP1 Is a Regulator and Effector of RhoA and C

Vega, F. M., Fruhwirth, G., Ng, T., and Ridley, A. J. (2011) RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. J. Cell Biol. 193, 655–665

Mircescu, H., Steuve, S., Savonet, V., Degraef, C., Mellor, H., Dumont, J. E., Maenhaut, C., and Pirson, I. (2002) Identification and characterization of a novel activated RhoB binding protein containing a PDZ domain whose expression is specifically modulated in thyroid cells by cAMP. Eur. J. Biochem. 269, 6241–6249

Zeng, P. Y., Rane, N., Du, W., Chintapalli, J., and Prendergast, G. C. (2003) Role for RhoB and PKR in the suppression of epithelial cell transformation by farnesyltransferase inhibitors. Oncogene 22, 1124–1134

White, C. D., Brown, M. D., and Sacks, D. B. (2009) IQGAPs in cancer: a family of scaffold proteins underlying tumorigenesis. FEBS Lett. 583, 1817–1824

McCallum, S. J., Wu, W. J., and Cerione, R. A. (1996) Identification of a putative effector for Cdc42Hs with high sequence similarity to the Ras-GAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. J. Biol. Chem. 271, 21732–21737

Hart, M. J., Callow, M. G., Souza, B., and Polakis, P. (1996) IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. EMBO J. 15, 2997–3005

Johnson, M., Sharma, M., and Henderson, B. R. (2009) IQGAP1 regulation and roles in cancer. Cell Signal. 21, 1471–1478

Brandt, D. T., and Grosse, R. (2007) Get to grips: steering local actin dynamics with IQGAPs. EMBO Rep. 8, 1019–1023

Jadeski, L., Mataraza, J. M., Jeong, H. W., Li, Z., and Sacks, D. B. (2008) IQGAP1 stimulates proliferation and enhances tumorigenesis of human breast epithelial cells. J. Biol. Chem. 283, 1008–1017

Mataraza, J. M., Briggs, M. W., Li, Z., Entwistle, A., Ridley, A. J., and Sacks, D. B. (2003) IQGAP1 promotes cell motility and invasion. J. Biol. Chem. 278, 41237–41245

Mataraza, J. M., Li, Z., Jeong, H. W., Brown, M. D., and Sacks, D. B. (2007) Multiple proteins mediate IQGAP1-stimulated cell migration. Cell Signal. 19, 1857–1865

Li, S., Wang, Q., Chakladar, A., Bronson, R. T., and Bernards, A. (2000) Gastric hyperplasia in mice lacking the putative Cdc42 effector IQGAP1. Mol. Cell. Biol. 20, 697–701

Kuroda, S., Fukata, M., Kobayashi, K., Nakafuku, M., Nomura, N., Iwamatsu, A., and Kaibuchi, K. (1996) Identification of IQGAP as a putative target for the small GTPases, Cdc42 and Rac1. J. Biol. Chem. 271, 23363–23367

McCallum, S. J., Erickson, J. W., and Cerione, R. A. (1998) Characterization of the association of the actin-binding protein, IQGAP, and activated Cdc42 with Golgi membranes. J. Biol. Chem. 273, 22537–22544

Sakurai-Yageta, M., Recchi, C., Le Dez, G., Sibarita, J. B., Daviet, L., Camonis, J., D’Souza-Schorey, C., and Chavrier, P. (2008) The interaction of IQGAP1 with the exocyst complex is required for tumor cell invasion downstream of Cdc42 and RhoA. J. Cell Biol. 181, 985–998

Chen, J. C., Zhuang, S., Nguyen, T. H., Boss, G. R., and Pilz, R. B. (2003) Oncogenic Ras leads to Rho activation by activating the mitogen-activated protein kinase pathway and decreasing Rho-GTPase-activating protein activity. J. Biol. Chem. 278, 2807–2818

Ren, X. D., Klosses, W. B., and Schwartz, M. A. (1999) Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. 18, 578–585

Turner, S. J., Zhuang, S., Zhang, T., Boss, G. R., and Pilz, R. B. (2008) Effects of lovastatin on Rho isoform expression, activity, and association with guanine nucleotide dissociation inhibitors. Biochem. Pharmacol. 76, 405–413

Rangaswami, H., Schwappacher, R., Marathe, N., Zhuang, S., Casteel, D. E., Haas, B., Chen, Y., Pfeifer, A., Kato, H., Shattil, S., Boss, G. R., and Pilz, R. B. (2010) Cyclic GMP and protein kinase G control a Src-containing mechanosens in osteoblasts. Sci. Signal. 3, ra91

Del Re, D. P., Miyamoto, S., and Brown, J. H. (2008) Focal adhesion kinase as a RhoA-activating signaling scaffold mediating Akt activation and cardiomyocyte protection. J. Biol. Chem. 283, 35622–35629

Zeng, Y., Zhuang, S., Gloddek, J., Tseng, C. C., Boss, G. R., and Pilz, R. B. (2006) Regulation of cGMP-dependent protein kinase expression by Rho
and Kruppel-like transcription factor-4. J. Biol. Chem. 281, 16951–16961
35. Gudi, T., Chen, J. C., Casteel, D. E., Seasholtz, T. M., Boss, G. R., and Pilz, R. B. (2002) cGMP-dependent protein kinase inhibits serum response element-dependent transcription by inhibiting Rho activation and functions. J. Biol. Chem. 277, 37382–37393
36. Karlsson, R., Pedersen, E. D., Wang, Z., and Brakebusch, C. (2009) Rho GTPase function in tumorigenesis. Biochim. Biophys. Acta 1796, 91–98
37. Bouzahzah, B., Albanese, C., Ahmed, F., Pixley, F., Lisanti, M. P., Segall, J. D., Condeelis, J., Joyce, D., Minden, A., Der, C. J., Chan, A., Symons, M., and Peestel, R. G. (2001) Rho family GTPases regulate mammary epithelium cell growth and metastasis through distinguishable pathways. Mol. Med. 7, 816–830
38. Wu, M., Wu, Z. F., Rosenthal, D. T., Rhee, E. M., and Merajver, S. D. (2010) Characterization of the roles of RHOC and RHOA GTPases in invasion, motility, and matrix adhesion in inflammatory and aggressive breast cancers. Cancer 116, 2768–2782
39. Brandt, D. T., Marion, S., Griffiths, G., Watanabe, T., Kaibuchi, K., and Grosse, R. (2007) Dia1 and IQGAP1 interact in cell migration and phagocytic cup formation. J. Cell Biol. 178, 193–200
40. Wang, J. B., Sonn, R., Tekletsadik, Y. K., Samorodnitsky, D., and Osman, M. A. (2009) IQGAP1 regulates cell proliferation through a novel CDC42-mTOR pathway. J. Cell Sci. 122, 2024–2033
41. White, C. D., Li, Z., Dillon, D. A., and Sacks, D. B. (2011) IQGAP1 protein binds human epidermal growth factor receptor 2 (HER2) and modulates trastuzumab resistance. J. Biol. Chem. 286, 29734–29747
42. Owen, D., Campbell, L. J., Littlefield, K., Evetts, K. A., Li, Z., Sacks, D. B., Lowe, P. N., and Mott, H. R. (2008) The IQGAP1-Rac1 and IQGAP1-Cdc42 interactions: interfaces differ between the complexes. J. Biol. Chem. 283, 1692–1704
43. Wennerberg, K., and Der, C. J. (2004) Rho-family GTPases: it’s not only Rac and Rho (and I like it). J. Cell Sci. 117, 1301–1312
44. Swart-Mataraza, J. M., Li, Z., and Sacks, D. B. (2002) IQGAP1 is a component of Cdc42 signaling to the cytoskeleton. J. Biol. Chem. 277, 24753–24763
45. Noritake, J., Fukata, M., Sato, K., Nakagawa, M., Watanabe, T., Izumi, N., Wang, S., Fukata, Y., and Kaibuchi, K. (2004) Positive role of IQGAP1, an effector of Rac1, in actin-meshwork formation at sites of cell-cell contact. Mol. Biol. Cell 15, 1065–1076
46. Liberto, M., Cobrinik, D., and Minden, A. (2002) Rho regulates p21cip1, cyclin D1, and checkpoint control in mammary epithelial cells. Oncogene 21, 1590–1599
47. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O. (2000) Genomic analysis of metastasis reveals an essential role for RhoC. Nature 406, 532–535
48. Hu, S., Shi, B., Jarzynka, M. J., Yin, J. J., D’Souza-Schorey, C., and Cheng, S. Y. (2009) ADP-ribosylation factor 6 regulates glioma cell invasion through the IQ-domain GTPase-activating protein 1-Rac1-mediated pathway. Cancer Res. 69, 794–801