Functional Analysis of the Contribution of RhoA and RhoC GTPases to Invasive Breast Carcinoma

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ABSTRACT

Although the RhoA and RhoC proteins comprise an important subset of the Rho GTPase family that have been implicated in invasive breast carcinomas, attributing specific functions to these individual members has been difficult. We have used a stable retroviral RNA interference approach to generate invasive breast carcinoma cells (SUM-159 cells) that lack either RhoA or RhoC expression. Analysis of these cells enabled us to deduce that RhoA impedes and RhoC stimulates invasion. Unexpectedly, this analysis also revealed a compensatory relationship between RhoA and RhoC at the level of both their expression and activation, and a reciprocal relationship between RhoA and Rac1 activation.

INTRODUCTION

A critical step in cancer progression is the ability of tumor cells to invade surrounding tissues, a process requiring altered adhesion and polarization, cytoskeletal rearrangement, and basement membrane remodeling (1). Subsequent to invasion, tumors metastasize to distant organs, resulting in morbidity and mortality. Identifying the mechanisms by which cells acquire such invasive potential is crucial for developing strategies to impede carcinoma progression in a clinical setting (2). Analysis of tumor-derived cell lines and primary tumors from breast and other cancers has identified a number of candidate molecules that regulate cell migration and invasion as potential targets for intervention, especially members of the Rho GTPase family (reviewed in ref. 1). This family includes prototypic members RhoA, Rac1, and Cdc42 have been best characterized. These proteins regulate many cellular functions that underlie cancer progression including cell cycle, gene expression, focal adhesion assembly/disassembly, and matrix remodelling, and they are activated by signals transduced through tyrosine kinase receptors, G protein-coupled receptors and integrins (reviewed in ref. 3).

The Rho-related members, RhoA, RhoB, and RhoC share high sequence identity. These GTPases have been implicated in the progression of tumors from a broad range of cellular origins, and analyses at both the RNA and protein level have correlated their increased expression with tumor progression (4–6). Early studies in this field noted the transforming ability of active RhoA in 3T3 fibroblasts (7). More recently, RhoC has attracted substantial interest with its in vivo enhanced function. Given that the activity of Rho proteins dictates their functionality, the existing data from primary tumors, as well as from many cell lines, are limiting and cannot address this issue specifically.

Analysis of the contribution of the RhoA and RhoC isoforms to aggressive disease has been hampered by a lack of appropriate molecular tools. Seminal studies on Rho protein function have used dominant-negative and constitutive-active approaches, alongside biochemical ablation by Clostridium difficile toxin or Clostridium botulinum C3 exoenzyme treatment (reviewed in ref. 15). These methods however, are unable to distinguish among individual Rho isoforms because of their high sequence similarity, particularly at regions of functional importance. Recently, Wang et al. (16) showed that the Rho proteins are functionally distinct through the use of chimeric p190RhoGAP proteins specific for each isoform. However, this approach still relies on exogenously coexpressed proteins in model cell lines.

To advance our understanding of Rho protein biology, it has become increasingly clear that the functionality of the individual isoforms needs to be determined. To dissect the specific contributions of RhoA and RhoC to the behavior of invasive breast carcinoma cells, we used the SUM-159 cell line. This cell line model, which expresses both Rho proteins, was derived from a primary anaplastic breast tumor and has been shown to be highly invasive in both in vitro assays and in in vivo orthotopic models of mouse mammary tumorigenesis (17). We have used a stable retroviral small interfering RNA (siRNA) approach to selectively knock-down expression of the RhoA and RhoC isoforms. Using these cell lines, we were able to define distinct functions for these Rho isoforms and to uncover unexpected relationships between them.

MATERIALS AND METHODS

Antibodies and Reagents. The suppliers, catalogue number and working dilutions of all antibodies used are as follows: Anti-RhoA polyclonal (Sc-179, 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA); anti-Racl monoclonal (RS6220, 1:500, Transduction Laboratories, Newington, NH); antirabbit actin (A0266, 1:2,000, Sigma, St. Louis, MO); phallolidin-FITC (P-5282, 200 mmol/L, Sigma), antirabbit-horseradish peroxidase (711-035-152, 1:10,000, Jackson ImmunoResearch, West Grove, PA), antimonochrome peroxidase (715-035-151, 1:10,000, Jackson ImmunoResearch), Palmityl-lysophosphatidic acid was purchased from Avanti Polar Lipids (857-123, Avanti Polar Lipids, Alabaster AL).

Generation of siRNA Constructs. Nucleotide sequences of 19 bp specific to RhoA and RhoC were generated by Oligogene1 and BLAST2 to determine specificity. The RhoA-specific sequence was scrambled to generate a negative control, and confirmation of its nonspecificity was established by BLAST. The target sequences formed part of a larger 64 bp cassette when inserted in both the sense and antisense orientation within the context of a stem loop sequence structure as per Oligogene (Seattle, WA) design specifications. The 64 bp oligonucleotides were synthesized in the forward and reverse orientation, annealed, and ligated into the pSUPER vector backbone (18). The presence of the insert was determined by sequencing. After confirmation of target sequence specificity by exogenous overexpression approaches (data not shown), the cassette was excised by EcoRI and Xhol double digestion and subcloned into the prepared pSUPER.Retro backbone (19).

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Cell Lines. The SUM-159 and SUM-149 cell lines were obtained from Dr. Steve Ethier, University of Michigan (17), MCF-7 and MDA-MB-231 cells were obtained from the Lombardi Cancer Center (Georgetown University), and MCF-10A cells were obtained from Dr. Joan Brugge (Harvard Medical School). SUM-159 and SUM-149 cells were cultured in HamsF12 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% fetal bovine serum, insulin (5 µg/mL I5500, Sigma), hydrocortisone (1 µg/mL H4001, Sigma), penicillin (100 units/mL), and streptomycin (100 µg/mL, both from Life Technologies, Inc.), MCF-7, MDA-MB-231, and 293T cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum and antibiotics. MCF-10A cells were cultured as described previously (20). 293T cells were transfected at 50% confluence by lipid transfection (Life Technologies, Inc.) complex containing a ratio of envelope plasmid (1.75 µg), packaging plasmid (3.25 µg) and pSUPER, Retro express the RhoA, RhoC, and Sce sequence siRNAs (5 µg) in Optitrim (Life Technologies, Inc.). Two days after transfection, the virus was harvested, clarified, and the supernatant coated surfaces of the transwells were blocked with DMEM/BSA for 30 minutes by trypsinization and washed three times in low glucose DMEM containing Polybrene (8 µg/mL) of 1:1 and 1:8. Puromycin selection (4 µg/mL) in standard growth media was added 24 hours after infection and reduced to 2 µg/mL after 4 days.

Reverse Transcription-PCR. Oligonucleotides were designed to human RhoA, RhoC, and glyceraldehyde-3-phosphate dehydrogenase by the Primer c1 database. RhoA forward was 5'-ATGGCGTCCGATCAGGAAAG-3' and reverse 5'-TCACAAGACAGGACACGAGA-3', RhoC forward was 5'-ATGGCGTCCGATACTCAGGAAAG-3' and reverse 5'-TCAGAAGATGG-GACAGCCTG-3', and glyceraldehyde-3-phosphate dehydrogenase, forward was 5'CCTGCGCAAGGTCAATCCGAC-3' and reverse 5'TGCTTACAGCAGGAAATGAGCTTG-3'. Total RNA was extracted from adherent cells using RNeasy columns (Qiagen, Valencia, CA). Reverse transcription semi-quantitative PCR was performed using 1 µg of total RNA in the One-step RT-PCR reaction kit (Qiagen) as follows: 15 and 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 60°C for 60 seconds.

Invasion and Migration Assays. For invasion assays, the upper surface of transwell chambers (8-µm pore, Costar, Cambridge, MA) were precoated overnight with 0.5 µg Matrigel (Becton Dickinson, Franklin Lakes, NJ) diluted in PBS. For migration assays, both the upper and lower surfaces of the transwell chamber were coated overnight with Vitrogen collagen (15 µg/mL; Cohesion, Palo Alto, CA) diluted in PBS. Cells were harvested at 70 to 80% confluence by trypsinization and washed three times in low glucose DMEM containing heat inactivated fatty acid-free BSA (0.25%, DMEM/BSA). The coated surfaces of the transwells were blocked with DMEM/BSA for 30 minutes at 37°C. Cells (1 × 10³ or 3 × 10³ in a total volume of 100 µL) were loaded into the upper chamber and lysophosphatidic acid (LPA, 100 µmol/L in DMEM/BSA) or NIH-3T3 conditioned media was present in the lower chamber. Invasion assays proceeded for 2 hours, whereas migration assays went for 24 hours at 37°C. For both assays, the upper chamber was swabbed to remove residual cells and fixed with methanol. Invasion assays were stained with crystal violet (0.2% crystal violet in 2% ethanol), rinsed in water, and air-dried. Migration assays were mounted in 4',6-diamidino-2-phenylindole mounting media (Vector Laboratories). The number of cells penetrating the membrane were determined for five independent fields in triplicate with a 10× objective and bright-field optics or fluorescence and quantitated with IPLab spectrum software (Scanalytics, Fairfax, VA).

Morphological Analysis. Cells were harvested as described. Glass coverslips were coated with laminin-1 (20 µg/mL). The substrate was blocked with DMEM/BSA, and cells (1 × 10³) were plated and incubated at 37°C for 60 minutes then treated with or without LPA (100 µmol/L in DMEM/BSA) for 3 minutes. Cells were fixed [2% paraformaldehyde, 200 µmol/L KCl, 20 mmol/L Pipes (pH 6.8), 14% sucrose, 4 mmol/L MgCl2, EGTA; ref. 21], permeabilized in the presence of 0.2% Triton-X-100, blocked in 0.1% BSA in PBS, and stained with FITC-conjugated phalloidin. After washing three times with PBS, cells were mounted, and images were captured under oil immersion at 60× magnification by IPLab spectrum imaging software and a Nikon Diaphot 300 microscope. Quantitation of the lamellipodial area of LPA-treated cells was determined by outlining the area of the lamellae for at least 20 individual cells and quantitating the total surface area encompassed by the lamellipodia with IPLab spectrum software.

Adhesion Assays. Forty-eight well tissue culture dishes were coated with laminin-1 (20 µg/mL, generously supplied by Dr. Hynda Kleinman, National Institute of Dental Research, Bethesda Maryland) or BSA (20 µg/mL) diluted in PBS. Cells were harvested as described and 1 × 10³ cells plated per well for 60 minutes at 37°C. After incubation, cells were washed twice with PBS, fixed with methanol, stained with crystal violet (0.2% crystal violet in 2% ethanol), and washed twice with water. Plates were air-dried briefly, and the crystal violet was solubilized by the addition of 200 µL of 1% SDS. The absorbance of 100 µL was read at 595 nm in a microtiter plate reader. Nonspecific adhesion to BSA was used as a baseline to subtract from laminin-1-specific adhesion.

Immunoblotting. Whole cell lysates were prepared by lysis in ice-cold RIPA buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10 mmol/L EDTA, 1% NP40, 1% deoxycholate, 0.1% SDS, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 µg/mL aprotinin, leupeptin, and pepstatin]. Lysates (35 µg) were separated by electrophoresis through 12 or 15% SDS-PAGE and transferred to Hybond enhanced chemiluminescence membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked, hybridized overnight with anti-Rho or anti-Rac1 antibodies (Abs) followed by secondary peroxidase-conjugated antirabbit or antimouse Abs, and detection was by chemiluminescence.

Rho and Rac1 Activity Assays. Rho and Rac activity assays were done following published protocols (22, 23). The glutathione S-transferase (GST)-Rho binding domain (RBD) (for Rho activity) and GST-Pak binding domain (PKB) (for Rac activity) fusion proteins were used to coat glutathione Sepharose (GST) beads. Cells were harvested as described and between 2.5 and 3 × 10³ cells were attached to laminin-1 (20 µg/mL diluted in PBS) coated 60-mm dishes for 60 minutes followed by treatment with or without LPA (100 µmol/L) for 3 minutes. Cells were lysed by addition of ice-cold lysis buffer [Rho, 50 mmol/L Tris (pH 7.2), 500 mmol/L NaCl, 10 mmol/L MgCl₂, 1% Triton-X-100, 0.5% deoxycholate, and 0.08% SDS; Rac, 50 mmol/L tris (pH 7.4), 100 mmol/L NaCl, 1% NP40, 10% glycerol, 2 mmol/L MgCl₂, both containing 2 mmol/L phenylmethylsulfonyl fluoride, and 5 µg/mL each of aprotinin, leupeptin, and pepstatin]. Lysates were clarified and an aliquot (0.5 total volume for Rho and 0.1 total volume for Rac) removed to represent the total lysate control. GST-RBD- or GST-PKB–coupled beads were added to the remaining lysates for 35 minutes on a rotating platform at 4°C. Samples were washed three times, denatured, separated by electrophoresis, and transferred as described.

C3 Transferase Treatment. SUM-159 siRNA lines were cultured to 50% confluence in 6-well dishes and treated for 24 hours with human recombinant C3 Transferase (10 µg/mL; Cytoskeleton-CT03, Cytoskeleton, Denver, CO). Control untreated cells were cultured in parallel. Cells were harvested as described, washed three times in serum-free media, and invasion assays toward LPA (100 µmol/L, 1 × 10³ cells) done as outlined above.

Three-Dimensional Matrigel Assays. Wells of a 24-well dish were pre-coated with 200 µL of undiluted phenol-red free Matrigel (10.2 mg/mL; BD Biosciences, San Jose, CA). Cells were harvested as described, washed three times with PBS, and diluted to a concentration of 1 × 10⁶ per well in a volume of 200 µL. Cells were mixed with 100 µL of undiluted ice-cold Matrigel for a ratio 2:1, and laid over the bottom layer. After gelling, complete culture media was added and changed every 2 to 3 days. Morphology was assessed at day 11 by image capture at 10× magnification. For analysis of cell viability, the Matrigel was washed with PBS and digested by incubation with 500 µL of dispase (BD Biosciences) at 37°C for 2 hours. Cell aggregates were recovered by pipetting, diluted in 20 mL of PBS, and pelleted by centrifugation. A single cell suspension was obtained after trypsin-EDTA treatment for 5 minutes at 37°C. Cells were incubated with FITC-conjugated annexin-V (2.5 µg/mL; Biosource International, Camarillo, CA) for 20 minutes on ice, washed with 1× annexin-V buffer, and analyzed by flow cytometry.

RESULTS

Comparative Analysis of RhoA and RhoC Expression in Breast Cell Lines. Increased expression of RhoC at the mRNA level has been reported previously in a range of breast carcinoma cells (24);
however, the relative level of RhoA to RhoC expression has not been established. Because of the lack of a commercially available antibody specific for RhoC, we used a polyclonal Rho Ab that recognizes both RhoA and RhoC (see below) to examine expression of these isoforms. We screened breast carcinoma cell lines ranging from nonmotile to highly invasive and detected two bands that correspond to RhoA (~26 Mr) and RhoC (~27 Mr) in all lines (Fig. 1A). The relative expression of RhoA to RhoC varied for each cell line and neither the level of RhoA nor RhoC expression corresponded with increased invasiveness (Fig. 1A). We chose to pursue our studies using the SUM-159 cell line which was derived from a primary anaplastic breast tumor and is highly invasive both in vitro and in vivo (17). This line is distinct from inflammatory breast cancer and thereby represents an invasive cell line that may be less dependent on RhoC for its motility but more indicative of other highly invasive lines.

**Generation of Cell Lines Expressing RhoA and RhoC siRNA.** Despite the high level of sequence identity shared between RhoA and RhoC (25), we identified putative candidate sequences for the siRNA approach using Oligoengine software and confirmed their specificity by nucleotide BLAST searches. A negative control encoding the scrambled RhoA (Scr) siRNA sequence shared no homology to any human genes. Alignment of each siRNA sequence, beginning at nucleotide position 153 for RhoA, and position 193 for RhoC, revealed three nucleotide mismatches for each sequence (Fig. 1B). The sequences were synthesized as part of a 64 bp hairpin loop structure and cloned into the pSUPER.retro retroviral expression vector (Oligoengine). Viral stocks were generated and subsequently used to stably infect SUM-159 breast carcinoma cells.

Analysis of RhoA and RhoC expression in each siRNA line by semi-quantitative reverse transcription-PCR showed a specific reduction in mRNA levels for each gene relative to the Scr control (Fig. 1C). We observed no change in RhoB expression at the mRNA level (data not shown). Immunoblotting extracts from each siRNA line using a polyclonal Rho Ab revealed a complete reduction in expression of the appropriate isoform (Fig. 1D), confirming that this Ab recognizes both RhoA and RhoC (see Fig. 1A). Interestingly, we observed an increase in RhoC expression of 1.8-fold as a result of loss of RhoA expression and, conversely, a 2-fold increase in RhoA expression in the RhoC siRNA cells. Expression of Rac1 and Cdc42 remained unchanged in these lines. Importantly, loss of either RhoA or RhoC expression did not have a deleterious effect on the monolayer growth rate or survival of SUM-159 cells (data not shown).

**RhoA and RhoC Have Distinct and Inverse Roles in Invasion.** To investigate a role for RhoA and RhoC in invasion, we did Matrigel transwell assays using the siRNA cell lines. SUM-159 cells are highly invasive (17, 26). LPA, a component of serum and a known regulator of RhoA activation (15), stimulated robust invasion of the Scr control cells in a 2-hour assay (Fig. 2). This level of invasion was consistent with that observed for an empty vector pSUPER.retro control and the parental cell line (data not shown) indicating no functional change as a consequence of the Scr sequence. For all subsequent experiments, only the Scr control line was used. In comparison, a significant increase in invasion was observed for the RhoA siRNA cells. In contrast, the RhoC siRNA cells exhibited a significant decrease in invasion compared with the Scr control cells (Fig. 2). The reduction in invasion observed in the RhoC siRNA cells cannot be accounted for by cell death because no significant difference in the level of apoptosis, as measured by annexin-V staining, was observed (data not shown).

To extend these findings, MCF-7 cells were used to generate stable retroviral lines for RhoA siRNA and the Scr control. RhoA knockdown was robust (Fig. 3A) and resulted in a dramatic reduction in cell to cell contact and elongated morphology with large lamellae, indicative of motile cells (Fig. 3B). Consistent with this morphology, the

![Fig. 1. Cell line comparisons and generation of RhoA- and RhoC-specific siRNA.](image-url)
RhoB, and RhoC proteins, inhibiting downstream signaling interactions (15). C3 treatment of the Scr control cells slightly increased their invasive potential relative to untreated control cells (Fig. 5A), whereas the invasive potential of the RhoC siRNA cells was significantly enhanced after C3 treatment compared with the untreated cells (Fig. 5B). In contrast, invasion of the RhoA siRNA cells was significantly reduced by C3 treatment compared with untreated RhoA siRNA cells (Fig. 5C). However, the C3-treated RhoA siRNA cells were still more invasive than either the Scr control (by 2-fold) or RhoC siRNA C3-treated cells (data not shown). We infer from this approach that the increased expression of the opposing Rho isoform and subsequent activity in each Rho siRNA line contributes to the observed differences in invasion. Specifically, knock-down of RhoC by siRNA results in decreased invasion, and disruption of RhoC signaling by C3 treatment in the RhoA siRNA cells also results in a decrease in invasion. Similarly, disrupting RhoA signaling by C3 treatment in the

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**Figure 2.** RhoA expression is inhibitory to invasion, whereas RhoC expression is required for invasion. To determine the invasive capacity of the siRNA lines, cells (1 × 10^5) were loaded into the upper chamber of Matrigel-coated transwells and allowed to invade toward LPA (100 nmol/L) for 2 hours at 37°C. The average number of invaded cells in five independent fields was counted for each well and the mean of triplicate wells (±SD) is shown in this representative assay. RhoA siRNA cells invade significantly faster than the Scr control cells (54% in this assay; **P** < 0.01). In contrast, invasion was inhibited by 30% (**P** < 0.05) as a result of loss of RhoC expression. *P* values represent standard *t* test.

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**Figure 3.** Loss of RhoA expression in MCF-7 cells recapitulates SUM-159. MCF-7 cells were used to establish stable RhoA siRNA and Scr control cell lines. A, immunoblotting of total protein lysates (35 μg) by the polyclonal Rho Ab shows ablation of RhoA expression in the RhoA siRNA cells (individual isoforms are indicated by arrows). Actin hybridization is included as a loading control. B, phase-contrast images (10× magnification) of adherent cells growing on tissue culture plastic show the RhoA siRNA cells have a reduced requirement for cell to cell contact, are elongated, and have large lamellipodia. In comparison, the Scr control cells require cell to cell contact and exist in an epithelial colony network. C, analysis of the migratory potential of the RhoA siRNA cells compared with the Scr control by chemotaxis toward NIH-3T3 conditioned media for 24 hours. The RhoA siRNA cells showed a significant (**P** < 0.001) increase in migration of approximately 7-fold compared with the Scr control. The total number of migrating cells was counted for each well (in triplicate) and averaged for duplicate experiments (±SD). *P* values represent standard *t* test.

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**Distinct Roles for RhoA and RhoC in Invasion.** To determine the contribution of the RhoA and RhoC proteins to the invasion observed after the Rho-specific knock-downs, we treated the siRNA lines with C3 transferrase (10 μg/mL for 24 hours) and assessed their invasion toward LPA. The bacterial exotransferase from *Clostridium botulinum* (C3 transferrase) irreversibly ADP ribosylates RhoA, RhoB, and RhoC proteins, inhibiting downstream signaling interactions (15). C3 treatment of the Scr control cells slightly increased their invasive potential relative to untreated control cells (Fig. 5A), whereas the invasive potential of the RhoC siRNA cells was significantly enhanced after C3 treatment compared with the untreated cells (Fig. 5B). In contrast, invasion of the RhoA siRNA cells was significantly reduced by C3 treatment compared with untreated RhoA siRNA cells (Fig. 5C). However, the C3-treated RhoA siRNA cells were still more invasive than either the Scr control (by 2-fold) or RhoC siRNA C3-treated cells (data not shown). We infer from this approach that the increased expression of the opposing Rho isoform and subsequent activity in each Rho siRNA line contributes to the observed differences in invasion. Specifically, knock-down of RhoC by siRNA results in decreased invasion, and disruption of RhoC signaling by C3 treatment in the RhoA siRNA cells also results in a decrease in invasion. Similarly, disrupting RhoA signaling by C3 treatment in the

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**Figure 3.** Loss of RhoA expression in MCF-7 cells recapitulates SUM-159. MCF-7 cells were used to establish stable RhoA siRNA and Scr control cell lines. A, immunoblotting of total protein lysates (35 μg) by the polyclonal Rho Ab shows ablation of RhoA expression in the RhoA siRNA cells (individual isoforms are indicated by arrows). Actin hybridization is included as a loading control. B, phase-contrast images (10× magnification) of adherent cells growing on tissue culture plastic show the RhoA siRNA cells have a reduced requirement for cell to cell contact, are elongated, and have large lamellipodia. In comparison, the Scr control cells require cell to cell contact and exist in an epithelial colony network. C, analysis of the migratory potential of the RhoA siRNA cells compared with the Scr control by chemotaxis toward NIH-3T3 conditioned media for 24 hours. The RhoA siRNA cells showed a significant (**P** < 0.001) increase in migration of approximately 7-fold compared with the Scr control. The total number of migrating cells was counted for each well (in triplicate) and averaged for duplicate experiments (±SD). *P* values represent standard *t* test.
RhoC siRNA cells results in an increase in invasion, as seen in the RhoA siRNA cells. Thus, decreased invasion in the RhoC siRNA cells can be attributed to RhoA activity, whereas increased invasion in the RhoA siRNA cells can be attributed in part to RhoC activity. Given that the C3-treated RhoA siRNA cells continue to invade significantly faster than the Scr control C3 treated cells, other factors are likely influencing invasion in this line in addition to the Rho proteins.

**Distinct Morphology and Adhesion of the RhoA and RhoC siRNA Cells.** To gain insight into the distinct functions of RhoA and RhoC that could account for their opposing influences on invasion, we examined the morphology of the siRNA cells adherent to laminin-1 after stimulation with LPA. Scr control cells exhibited active membrane ruffling after attachment to laminin-1 for 60 minutes (data not shown), and increased ruffling and the formation of small lamellipodia were evident in response to LPA stimulation for 3 minutes (Fig. 6A). RhoA siRNA cells also showed active ruffling during attachment but formed extended, fine, veil-like lamellipodia in response to LPA treatment (Fig. 6A). Quantitation of the total lamellipodial area after LPA stimulation showed a significant increase in the RhoA siRNA cells (Fig. 6B). Intense phalloidin staining was seen at the edges of the RhoC siRNA cells, which is also indicative of extensive membrane ruffling (Fig. 6A). Interestingly, phalloidin staining showed that loss of either Rho protein did not impair the formation of actin stress fibers in response to LPA treatment (Fig. 6A). This observation suggests that all Rho proteins are equally capable of stress fiber formation or that compensation for this function exists between the Rho proteins. Taken together, the results suggest that loss of RhoA expression increases the size of lamellipodia that form in response to LPA. Increased membrane ruffling in the RhoC siRNA cells may be indicative of lamellipodia that are unable to extend, possibly explaining their limited motility. The observation that adhesion of the RhoA siRNA cells to laminin-1 after 60 minutes was significantly reduced compared with the Scr control and RhoC siRNA cells (Fig. 6C) is indicative of more motile cells and also suggests a functional role for RhoA in adhesion.

**Rac1 GTPase Activity Is Differentially Regulated in the RhoA and RhoC siRNA Cells.** Given that inhibition of Rho proteins by C3 treatment did not reduce the invasive capacity of the RhoA siRNA cells to the level of the Scr control, the interplay of other factors contributing to invasion is likely. The increased lamellipodial area observed in the RhoA siRNA cells in response to LPA stimulation is suggestive of increased Rac1 activity. Rac1 has been shown previously to be responsive to LPA (28). To establish whether the reported reciprocity in RhoA and Rac1 activity (29) might correlate with the increased invasion observed in the absence of RhoA expression, Rac1 activity assays were done under the same conditions as the Rho activity assays, and activity was measured as described previously (23). No change in total Rac1 expression was observed between the cell lines. Treatment with LPA for 3 minutes increased Rac1 activity in the Scr control cells by approximately 1.3-fold (Fig. 7). In contrast, Rac1 activation in the RhoA siRNA cells increased by a striking 2.5-fold after LPA treatment, whereas activity in the RhoC siRNA cells resulted in a 1.4-fold increase (Fig. 7). These data suggest there is cross-talk between RhoA and Rac1 and that RhoC does not impact Rac1 activity to the same extent.
outgrowth. RhoC siRNA cells, however, formed very small aggregates with limited invasive projections (Fig. 8). Cell death was excluded as the reason for reduced aggregate formation in the RhoC siRNA cells because no significant difference in the proportion of annexin-V–positive cells was seen (Fig. 8). These data substantiate the invasion assay data (Fig. 2), from which it would be predicted that the RhoC siRNA cells would be significantly impeded in their capacity to penetrate the Matrigel and conversely, the RhoA siRNA cells would be unrestricted.

**DISCUSSION**

We have used breast carcinoma cells and a RNA interference (RNAi) approach to dissect the functions of RhoA and RhoC, two Rho isoforms that share high sequence identity. Ablation of the expression of these Rho isoforms individually in SUM-159 cells, which are invasive and characteristic of late-stage tumors, enabled us to deduce that RhoA impedes and RhoC stimulates invasion. In addition, diminution of RhoA expression in the relatively nonmotile cells, MCF-7, induced their ability to migrate quite markedly. Together, these findings indicate that RhoA impedes the migration and invasion of breast carcinoma cells, a function distinct from RhoC. However, given that RhoA expression is maintained in invasive breast carcinomas (4), our findings argue that RhoA may contribute in other ways to breast cancer progression. For example, RhoA has been implicated in the epithelial-mesenchymal transition of carcinoma (30). In addition to demonstrating distinct functions for RhoA and RhoC, our studies unexpectedly revealed a compensatory relationship between RhoA and RhoC at the level of both their expression and activation.

Our findings should be considered within the context of the existing literature on Rho isoform expression in breast cancer. These studies have focused largely on the assessment of RhoA and RhoC expression in both cell lines and tumor specimens. A prevailing notion is that RhoC expression correlates with aggressive disease as evidenced by high levels of RhoC expression in inflammatory breast cancers, which are exceptionally aggressive tumors (31), and increased motility of normal mammary epithelial cells over-expressing RhoC (8). Clearly, our RNAi data implicating a proinvasive role for RhoC substantiate this notion. We note, however, that both RhoA and RhoC are expressed in all of the breast carcinoma cell lines we have analyzed and that neither of their expression patterns correlates strongly with invasion.

**CONTRIBUTION OF RHO PROTEINS TO THREE-DIMENSIONAL MORPHOLOGY.** To investigate Rho protein function in a more physiologically relevant setting, the siRNA cells were embedded in Matrigel. Matrigel mimics the mammary gland environment, being rich in basement membrane components such as laminin, collagen IV, heparan sulfate proteoglycans, and growth factors. Cells were embedded at low density and supplemented with standard growth medium for 11 days. The RhoA siRNA cells formed dense aggregates from which highly invasive cells emanated, forming a stellate morphology that almost completely filled the well by the termination of the experiment (Fig. 8). In comparison, the Scr control cells formed a combination of medium-sized cellular aggregates with disorganized edges and small stellate outgrowths. RhoC siRNA cells, however, formed very small aggregates with limited invasive projections (Fig. 8). Cell death was excluded as the reason for reduced aggregate formation in the RhoC siRNA cells because no significant difference in the proportion of annexin-V–positive cells was seen (Fig. 8). These data substantiate the invasion assay data (Fig. 2), from which it would be predicted that the RhoC siRNA cells would be significantly impeded in their capacity to penetrate the Matrigel and conversely, the RhoA siRNA cells would be unrestricted.
pression varies within these lines. This factor may be a critical determinant of functionality, particularly given the interplay observed between these proteins in the siRNA lines. This observation suggests that the regulation of RhoC function may be more complex than relative expression and that the RNAi approach we have developed will be useful to assess RhoC function in different types of breast carcinoma cells. Furthermore, studies that have concluded that RhoA expression increases in breast tumors are based either on the analysis of mRNA expression that may not necessarily reflect protein expression, or those studies have used Abs that may recognize multiple isoforms of Rho proteins. Despite these caveats, it is likely that RhoA contributes to aspects of breast tumorigenesis and progression other than invasion itself. Again, the RNAi approach will enable the elucidation of such contributions.

The compensatory relationship between RhoA and RhoC expression that we uncovered is unexpected and intriguing. The observation that this compensation occurs at the protein but not the mRNA level suggests a post-transcriptional mechanism that facilitates RhoC expression in the absence of RhoA and vice versa. This compensation is difficult to reconcile in light of our finding that these two isoforms have distinct functions. Indeed, the findings that loss of RhoC expression results in increased RhoA expression and activation and that this increase in RhoA activation impedes the invasion of the RhoC RNAi cells, as evidenced by the C3 data, indicate that the analysis of Rho isoform function is less straightforward than anticipated. This assessment has important implications for the generation and analysis of Rho knockout mice.

The regulation of RhoA and RhoC activation and the mechanisms by which these GTPases influence cell functions differently are two key issues that need to be addressed. Although RhoC has been implicated in the progression of many invasive carcinomas, its functional activation had not been reported. We show here that it is activated in response to LPA treatment, which also activates RhoA. Given their high sequence similarity, it is not surprising that the Rho proteins share similar modes of regulation. It will be interesting to determine whether RhoA and RhoC share the same exchange factors and, if so, whether the loss of expression of one isoform alters the balance of free guanine nucleotide exchange factors in favor of the remaining isoform. In this context, it is also notable that the exchange factor XPLN, detected in limited tissues, is specific for RhoA and RhoB but not for RhoC (32). What has not been addressed rigorously is the likely possibility that endogenous RhoA and RhoC differ in their subcellular localization, especially after activation. The RNAi approach, combined with fluorescent resonance emission technology (33), could be used to spatially localize the activated form of one isoform in the absence of the other.

Our RNAi data suggest distinct functions for RhoA and RhoC. In support of our data, recent studies using an overexpression model indicate that RhoA and RhoC differentially interact with the downstream effector Rho kinase (ROCK). RhoC exhibits a higher affinity for ROCK than does RhoA (34). ROCK, however, is one of the many downstream effector molecules that are known to interact with RhoA, and other examples of differential interactions are likely. Our data also suggest compensation functions exist within this family because no significant difference in stress fiber formation was observed after LPA treatment in either siRNA line, suggesting that both RhoA and RhoC have the capacity to regulate stress fibers. Indeed, in porcine endothelial cells active RhoA and RhoC equally induce stress fiber formation (35). Nonetheless, the critical issue from our perspective is the opposing effects of these two GTPases on breast carcinoma invasion. The fact that the RhoA and RhoC sequences differ significantly only in their COOH-termini (25) argues that the opposing effects of these two isoforms on invasion resides in these COOH sequences. The siRNA cells we have generated should be quite useful for assessing this hypothesis. For example, expression of COOH-terminal mutants of RhoC in the RhoC siRNA cells should facilitate the identification of specific RhoC amino acids that contribute to an invasive phenotype.

Although cross-talk between Rho and Rac activity has been described (23, 29, 36), no studies have addressed the involvement of individual Rho isoforms in this cross-talk specifically. In the absence of RhoA expression, we observed a significant increase in Rac1 activity after LPA treatment and increased lamellipodia formation consistent with a Rac1 induced phenotype (37). In preliminary studies, p21 activated kinase, a downstream Rac1 effector molecule was similarly activated by the loss of RhoA but not RhoC (data not shown). This finding is in agreement with reports on reciprocity between RhoA and Rac1 activities, with high RhoA activity being inhibitory to Rac1 activity and low RhoA activity resulting in high Rac1 activity and cell motility (29). C3 treatment of the RhoA siRNA cells partially reduced the invasive capacity of the RhoA siRNA cells, suggesting that the increased Rac1 activity may account for a proportion of the increased invasion. In contrast, disrupting RhoC expression did not impact Rac1 activity significantly, despite the high RhoA activity in these cells. Taken together, these results suggest that RhoA but not RhoC can influence Rac1 activation and intimates that these proteins can impact independent pathways. The mechanism involved awaits further investigation.

In summary, we have shown the successful application of RNAi to assess the functions of RhoA and RhoC in invasive breast carcinoma cells. The results obtained provide significant insight into their functions and suggest it is no longer sufficient to attribute Rho-associated functions to RhoA alone. This approach is directly applicable to a broad range of studies on Rho protein biology and should prove useful in dissecting the activation and downstream signaling pathways influenced by these GTPases. In addition, our results suggest that the
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