The COOH-terminal End of R-Ras Alters the Motility and Morphology of Breast Epithelial Cells through Rho/Rho-Kinase

Ha-Won Jeong, Ju-Ock Nam, and In-San Kim

Cell and Matrix Biology National Research Laboratory, Department of Biochemistry, Kyungpook National University
School of Medicine, Daegu, Korea

Abstract

R-Ras has a high degree of sequence homology with Ras and other members of the Ras subfamily, including Rap, TC21, and M-Ras. Although R-Ras has been suggested to regulate cell adhesion, migration, and invasion, the biological mechanism has not been well assessed. In this report, we show that constitutively active R-Ras (38V) induces a more rounded cell shape and redistribution of focal adhesion, and enhances the phosphorylation of focal adhesion kinase and paxillin. Active R-Ras (38V) induces cell adhesion to type I collagen, but inhibits cell motility. In active R-Ras (38V) cells, the activity of RhoA is increased and accompanied with translocation to plasma membrane, but not that of Rac1 or Cdc42. In parallel, dominant-negative RhoA (N19RhoA) and Y27632, a specific inhibitor of Rho-associated kinase, dramatically reverse the rounded cell morphology to a spread cell shape and enhance motility. Furthermore, coincident with the formation of cortical actin filaments in active R-Ras (38V) cells, myosin light chain and Ser-19-phosphorylated myosin light chain are increased and accompanied with translocation to plasma membrane, but not that of Rac1 or Cdc42. In parallel, RhoA is increased and accompanied with translocation to plasma membrane, but not that of Rac1 or Cdc42. In parallel, RhoA activity is higher in R-Ras cells than control cells. In addition, introduction of dominant-negative R-Ras expressing cells are more rounded than parent cells, and lose their polarized morphology. RhoA activity is higher in R-Ras cells than control cells. Inhibition of RhoA and Rho-associated kinase (Rho-kinase) induces reversal of the rounded cell morphology to spread morphology and significant enhancement of motility. Additionally, using H-Ras/R-Ras and R-Ras/H-Ras hybrid constructs, we show that the COOH-terminal region of R-Ras contains the specific signal for inducing changes in motility and morphology. Our results suggest that R-Ras in breast epithelial cells disrupts cell polarity and motility through the Rho/Rho-associated kinase pathway triggered by a signal from the COOH-terminal end of R-Ras.

Introduction

R-Ras is a member of the superfamily of small GT-Pases, originally cloned through its homology to Ras (1). The R-Ras protein is highly homologous to Ras (55% identity), and has a 26-amino acid NH2-terminal extension. Its minimal effector region (amino acids 30–40) is identical to Ras, but the protein differs at nine amino acids within the extended effector region comprising amino acids 23–46 (2). R-Ras interacts in vitro with many of the same proteins as Ras, including the two GT-Pase-activating proteins, p120RasGAP, and neurofibromin; the exchange factor, RasGRF; and the three downstream effector proteins, Raf, PI3-kinase, and RaI-GDS; but does not interact with the Ras exchange factors, SOS1 or RasGRF (3–6). These similarities have generated speculation that, like Ras, R-Ras might activate similar signal transduction pathways and regulate important aspects of growth control.

Using the activated version of R-Ras (glycine to valine substitution at codon 38, equivalent to codon 12 in Ras), unlike Ras, it did not induce transformed foci in Rat1 fibroblasts and, furthermore, R-Ras–transformed NH3T3 do not display all of the morphologic effects associated with Ras-transformed cells (1, 7, 8). They are, however, able to proliferate in low amounts of serum, to form colonies in soft agar, and to give tumors in nude mice (7). Furthermore, these cells have elevated levels of ERK1/2 MAP kinases, suggesting that R-Ras can activate in parts similar to signal transduction pathways of Ras (7).

R-Ras has also been shown to affect integrin-mediated adhesion activity. Expression of active R-Ras in 32D.3 mouse myeloid cells, which normally grow in suspension, causes cells to become highly adherent. On the other hand, introduction of dominant-negative R-Ras to adherent Chinese hamster ovary cells reduces their adhesiveness (9). Zhang et al. showed that R-Ras induces an increase in cell adhesion by activating αβ3 and αβ1 integrins on the surfaces of Chinese hamster ovary and 32D cells, respectively. Others, however, suggest that changes in cell adhesion properties are not due to the direct activation of integrins by R-Ras, but rather a result of its ability to inhibit a Ras/Raf-mediated integrin suppression pathway (10). How R-Ras activates integrins is not known, but its effector loop and prenylation site, as well as the proline-rich sequence in the hypervariable region of R-Ras are essential for this activation process (11, 12). Although several studies on the roles of R-Ras in the regulation of cell functions have been reported, the effects of R-Ras on cell polarity and motility and its mechanisms are not yet clearly defined. Furthermore, which motif of R-Ras is responsible for these effects has not been studied yet.

In this study, we show that R-Ras has opposite effects on adhesion and motility activity in breast epithelial cells; enhancement of adhesion and disruption of motility. Active R-Ras–expressing cells are more rounded than parent cells, and lose their polarized morphology. RhoA activity is higher in R-Ras cells than control cells. Inhibition of RhoA and Rho-associated kinase (Rho-kinase) induces reversal of the rounded cell morphology to spread morphology and significant enhancement of motility. Additionally, using H-Ras/R-Ras and R-Ras/H-Ras hybrid constructs, we show that the COOH-terminal region of R-Ras contains the specific signal for changes in motility and morphology. Our results collectively suggest that R-Ras in breast epithelial cells disrupts cell polarity and motility through the Rho/Rho-kinase pathway triggered by a signal from the COOH-terminal end of R-Ras and thereby, R-Ras, in some respects, might oppose the oncogenic effects of H-Ras.

Materials and Methods

Materials. Y27632 was obtained from Calbiochem (La Jolla, CA). Epi- dermal growth factor, insulin, and hydrocortisone were purchased from Sigma (St. Louis, MO). Cell culture media and serum were acquired from...
Invitrogen (Carlsbad, CA). The following antibodies were used: anti-H-Ras, anti-RhoA, and anti-phospho myosin light chain (MLC) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-R-Ras, anti-Rac1, anti-Cdc42, anti-paxillin, and anti-ZO-1 from BD Transduction Laboratories (Lexington, KY); and anti-MLC from Sigma. Rhodamine-conjugated secondary antibody and FITC-conjugated phalloidin were obtained from Molecular Probes (Eugene, OR).

Cell Culture and Generation of Transfectant Cells. The human breast epithelial cell line, MCF10A, was cultured in DMEM/F12 supplemented with 5% horse serum, epidermal growth factor (2 ng/mL), insulin (10 μg/mL), hydrocortisone (0.5 μg/mL), cholera enterotoxin (0.1 μg/mL), fungizone (0.5 μg/mL), and 100 units/mL penicillin-streptomycin. MDA-MB-231, breast epithelial cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 units/mL penicillin-streptomycin. MCF10A and MDA-MB-231 cells were infected with retroviral vector encoding a wild form, an active form of R-Ras (38V), a dominant-negative form of R-Ras (43N), and empty vector (pBabe) as a control. Transfected cells were selected and maintained using 3 μg/mL puromycin (Sigma). To obtain coexpression of dominant-negative RhoA protein in R-Ras–expressing MCF10A cells, active R-Ras (38V) cells were transfected with control vector (pcDNA3) or pcDNA3-N19RhoA provided by Dr. J. Park (Hallym University, Korea; ref. 13). Double-transfected cells were selected in medium containing puromycin (3 μg/mL) and G418 (400 μg/mL) and expanded as pools of stably transfected cells.

Rho Family Activity Assay. Rho family activity was measured using a technique similar to that described by Ren et al. (14). Briefly, cells were lysed in 300 μL of 50 mmol Tris (pH 7.6), 500 mmol NaCl, 0.5% DOC, 1% Triton X-100, 0.5 mmol MgCl₂, and a protease inhibitor tablet (Roche, Indianapolis, IN). Lysates (500-700 μg) were cleared by centrifugation at 12,000 × g for 10 minutes, and the supernatant was rotated for 30 minutes (for Rac1, Cdc42) or 45 minutes (for RhoA) with 30 g of GST-PBD (for Rac1 and Cdc42 pull-down) or GST-RBD (for RhoA pull-down) bound to glutathione-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were washed thrice in 50 mmol Tris (pH 7.6), 150 mmol NaCl, 1% Triton X-100, 0.5 mmol MgCl₂, and protease inhibitor tablets, and immunoblotted with anti-Rac1, Cdc42, and RhoA monoclonal antibodies. Whole cell lysates were additionally immunoblotted for Rac1, Cdc42, and RhoA as loading controls.

Figure 1. Expression of constitutively active R-Ras (38V) induces rounded morphology of MCF10A cells. A, MCF10A cells were stably transfected with retroviruses encoding an active form of R-Ras (38V), dominant-negative R-Ras (43N), and empty vector (pBabe). Total cell extracts were analyzed by Western blotting. B, transfected cells in the culture dish were subjected to phase-contrast microscopy and photographed (magnification, 100 × ). Enlarged images are displayed in the inset. C, cells (5,000 cells) were seeded in a LabTek chamber slide (Nunc, Roskilde, Denmark) and incubated overnight. Cells in the coverslip were fixed and stained with anti-paxillin, and ZO-1 (BD Transduction Laboratories) for detection of the focal adhesion complexes and cell-cell contacts, respectively. Additionally, cells were stained with FITC-conjugated phalloidin (Molecular Probes) for the detection of filamentous actin. Bar, 20 μm.
Time-lapse Video Microscopy. A Nikon TE100 microscope equipped with Samsung CCD camera (Samsung, Korea) and Metamorph software (Universal Imaging, Downingtown, PA) were used to obtain images every 5 minutes for a total observation period of 12 to 16 hours. Cells were seeded the day before recording into a 35-mm dish. Temperature and carbon dioxide were adjusted to 37°C and 5%, respectively. A 100× magnification was applied for investigating a large area to obtain the required number of cases for a representative statistical analysis. To generate migratory tracks, the positions of nuclei of individual cells were marked on each image. The migratory speed was calculated, based on the sum of distances divided by the time of observation for each experimental condition. The motility of at least 60 cells were analyzed, and presented as mean ± SD.

Immunofluorescence. Cells grown on chamber slips were fixed at room temperature with 3.7% formaldehyde for 10 minutes, and permeabilized in 0.25% Triton X-100 for 5 minutes. FITC-phalloidin from Molecular Probes was employed to label filamentous actin. To analyze the morphologic changes in active R-Ras transfectants (data not shown) did not show any significant morphologic change. As shown in Fig. 1B, active R-Ras cells were round, symmetrical in shape, lost their polarity, and had poor contact in cell clustering, whereas wild MCF10A cells were polarized, asymmetric, and polarized, and formed islands clustering with the cells. To analyze the morphologic changes in active R-Ras cells in detail, we employed paxillin, a focal adhesion marker, and phalloidin to stain filamentous actin. Transfectant cells (5,000 cells) were seeded into LabTek chamber slide and then processed for immunofluorescence. More than 90% of the seeded transfectant cell population show identical results in Fig. 1B.

Adhesion Assay. The cell adhesion assay was done as described previously (15). Briefly, a 96-well plate (High Binding, Corning-Costar, Cambridge, MA) was coated with fibronectin and collagen I (10 μg/ml in PBS) at 4°C overnight. The plate was rinsed with PBS, and uncoated surfaces were blocked with 2% bovine serum albumin for 1 hour. Cells were suspended in medium at a density of 3 × 10^5 cells/ml, and 0.1 ml of the cell suspension was added to each well of the coated plates. Attached cells were used for hexosaminidase assay as described previously (15).

Results

R-Ras Disrupts Polar Morphology and Induces Redistribution of Focal Adhesion in Cells. To investigate the biological role of R-Ras, MCF10A cells were stably transfected with retroviruses encoding an active form of R-Ras (38V), a dominant-negative form of R-Ras (43N), or empty vector (pBabe). Western blot analysis of MCF10A cell extracts harboring these constructs revealed the presence of a 23-kDa protein. In parent or empty vector–transfected cells, the R-Ras protein was barely detectable (Fig. 1A). Interestingly, the expression of constitutively active R-Ras (38V) resulted in a dramatic change in cell shape (Fig. 1B), whereas dominant-negative form (Fig. 1B) and even wild-type R-Ras transfectants (data not shown) did not show any significant morphologic change. As shown in Fig. 1B, active R-Ras cells were round, symmetrical in shape, lost their polarity, and had poor contact in cell clustering, whereas wild MCF10A cells were polarized, asymmetric, and polarized, and formed islands clustering with the cells.

Disruption of Polarity and Motility in R-Ras Cells

Figure 2. Expression of active R-Ras modulates adhesion and motility of breast epithelial cells. A, active R-Ras (38V) enhances MCF10A cell adhesion to collagen I and fibronectin. Transfectant cells were seeded into 96-well plates coated with type I collagen (top) or fibronectin (bottom). Cells adhering to the well were quantified with a hexosaminidase assay, as described in MATERIALS AND METHODS. Results are presented as an average of experiments performed in triplicate (mean ± SD). B, active R-Ras (38V) disrupts the motility of MCF10A and MDA-MB-231 cells. Mock, active (38V), and dominant-negative (43N) R-Ras–expressing cells were plated at low confluency in a 35-mm culture dish, and incubated overnight. Cell motility was analyzed by time-lapse video microscopy and the migratory paths were tracked. The migratory pathways of MCF10A cells filmed for 5 hours are shown (B); the average migratory speed of MCF10A transfectants (C), and MDA-MB-231 transfectants (D), were calculated based on the sum of distances divided by the time of observation using the Metamorph program (Universal Imaging). Data are representative of three separate experiments. The motility of at least 60 cells were analyzed (mean ± SD).
MCF10A Cells.

Because morphologic and cytoskeletal changes were observed in active R-Ras cells, we investigated the effect of R-Ras on cell adhesion and motility using MCF10A cells. Active R-Ras (38V) cells exhibited enhanced adhesion to collagen I and fibronectin. Active R-Ras cells displayed a cortical actin ring and paxillin localization in the leading edge, whereas dominant-negative R-Ras cells did not. The addition of Y27632 altered the distribution of paxillin from a focal adhesion pattern to a spread fibroblast-like shape. Consistently, the motility of R-Ras cells was inhibited by Y27632.

Active R-Ras Modulates Cell Adhesion and Motility of MCF10A Cells. Because morphologic and cytoskeletal changes are associated with adhesion or migratory behavior of cells, we examined the effect of R-Ras on adhesion of cells to the extracellular matrix proteins, collagen I and fibronectin. Active R-Ras (38V) enhanced adhesion of MCF10A cells strongly to collagen I (top) and marginally to fibronectin (bottom; Fig. 2A).

Next, we tested the effect of R-Ras on cell motility. To determine single cell motility on a culture dish, we used time-lapse video microscopy and quantitatively analyzed the migratory paths of individual cells. As shown in Fig. 2B and C, mock cells in the culture dish moved randomly with an average velocity of 21 ± 0.3 μm/hour, whereas active R-Ras-expressing cells (38V) exhibited low motility, the velocity of random migration dropped to 1.8 ± 0.3 μm/hour. The motility of dominant-negative (43N) R-Ras cells was indistinguishable from that of mock cells. We also investigated these effects of R-Ras with another breast epithelial cell line, MDA-MB-231. A similar tendency was obtained for MDA-MB-231 showing that active R-Ras (38V) inhibited cell motility but dominant-negative R-Ras (43N) did not affect cell motility (Fig. 2D). Additionally, we did an in vitro wound healing assay. Similar results showing that R-Ras cells barely migrated into the scratched area were obtained (data not shown).

Migratory and Morphological Defects of Active R-Ras–Expressing Cells Are Related to Higher Rho/Rho-Kinase Activity. Rho GTPases, including RhoA, Rac1, and Cdc42, are critical for cell shape changes and adhesion dynamics. To elucidate whether Rho GTPases are related to the motility disruption of active R-Ras cells, we did activity assays for RhoA, Cdc42, and Rac1. We found that RhoA activity of active R-Ras cells was higher than that of control cells, whereas the activities of Rac1 and Cdc42 were not different in these two cells (Fig. 3A). Changes in the distribution of RhoA between membrane and cytosol have been used as an indication of Rho activation because increased membrane association of RhoA occurs when RhoA is activated (16–18). To address this issue, 5,000 control and R-Ras–expressing cells were seeded in a chamber slide, respectively, and immunostained with RhoA-specific antibody. Compared with control cells (mock), > 80% of R-Ras–expressing cells were flattened and had a spread fibroblast-like shape (Fig. 3B). Therefore, we investigated whether the morphologic and migratory changes of R-Ras cells is related to its higher RhoA activity. We transfected the dominant-negative form (N19RhoA) of RhoA in the active R-Ras (38V)–expressing MCF10A cells to suppress RhoA activity. Wound healing assay was used to study whether inhibition of RhoA activity could restore the suppressed cell motility in R-Ras–expressing cells. Compared with control cells (38V), wound repair was accelerated in the dominant-negative N19RhoA–transfected cells (38V-N19), showing significant differences at 6 hours (Fig. 4A). Consistently, the round morphology of the active R-Ras–expressing cells (38V) was abrogated by the transfection of dominant-negative N19RhoA (38V-N19; Fig. 4B). We next examined whether the inhibition of Rho-kinase, a target protein of RhoA, could also change morphology and motility in R-Ras–expressing cells using a well-known specific inhibitor of Rho-kinase, Y27632. In R-Ras cells (Fig. 4C), the rounded morphology of cells was rapidly altered upon the addition of Y27632 (15 μmol/L) to a spread fibroblast-like shape within 1 hour. Moreover, the spreading of R-Ras cells induced by Y27632 was concomitant with marked disruption of cortical actin bundles in the cell periphery. The addition of Y27632 altered the distribution of paxillin from a strong peripheral to weakly scattered pattern. Next, we did an in vitro wound healing assay. As shown in Fig. 4D, the motility of active R-Ras (38V) cells was enhanced by Y27632 (15 mol/L). Taken together, activation of RhoA/Rho-kinase signaling pathway is responsible for changes of morphology and motility in active R-Ras–expressing cells.

Because phosphorylation of the regulatory MLC was known to be critical for controlling actomyosin contractility in smooth muscle and nonmuscle cells (19), we examined the subcellular localization of MLC and its phosphorylated form in cells by

![Figure 3](cancerres.aacjrournals.org)
immunofluorescence staining using specific antibodies for MLC and Ser19-phosphorylated MLC. In mock cells, MLC and phosphorylated MLC were mainly detected in the perinuclear and cytosol area, with a similar distribution as actin (Fig. 5A, left). In active R-Ras cells, however, they were organized into long parallel filaments with a similar distribution pattern as actin (Fig. 5A, right). This distribution pattern was abolished in the presence of the Rho-kinase inhibitor, Y27632, suggesting that active actomyosin structures are assembled at the cell periphery through the Rho/Rho-kinase pathway (Fig. 5B).

The COOH-Terminal End of R-Ras Contains a Cell Motility Disruption Signal. Despite sequence similarities, activated versions of H-Ras and R-Ras confer distinct biological properties, including adhesion and migration (9, 20, 21). Therefore, to analyze whether the hypervariable region and NH2-terminal sequence of R-Ras (which are different from those of H-Ras) are essential for motility disruption, we created chimera constructs of H-Ras (12V)/R-Ras (38V). In the view of the extended NH2 terminus (amino acids 1-26) of R-Ras compared with other Ras molecules and sequence divergence in the COOH terminus of Ras proteins, COOH-terminal amino acids (amino acids 178-218) of R-Ras and equivalent residues (amino acids 151-189) of H-Ras were exchanged (RH, HR). Moreover, the 26 additional NH2-terminal amino acids of R-Ras were inserted into the NH2 terminus of H-Ras (R26H; Fig. 6A). All proteins contained activating mutations, and thus were anticipated to be in a constitutive GDP-bound form. Recombinant proteins were expressed at similar levels in MCF10A cells (Fig. 6B).

Morphologic changes were initially evaluated. Only HR cells exhibited a rounded shape similar to active R-Ras cells, whereas other cells displayed a fibroblast-like motile morphology (Fig. 6C).

Next, the motility of each cell was examined. Active H-Ras-expressing MCF10A cells (12V) showed enhanced motility (average...
It has been shown that the COOH-terminal end of R-Ras contains a focal adhesion targeting signal, and targeting and activation of R-Ras are linked processes in the formation of focal adhesion (22). Thus, it is likely that the targeting of R-Ras to plasma membrane is functionally linked to phenotypic changes in R-Ras–expressing MCF10A cells. Unfortunately, due to the failure of our anti–R-Ras antibody in immunofluorescence staining, we were unable to determine the specific localization of R-Ras in these cells. Instead, we investigated the localization of HR chimeric protein using anti–H-Ras antibody in HR chimeric cells. In Fig. 6E, we were able to show the membrane association of HR chimeric protein, which was found over the cell surface and along the cell margins similar to focal adhesion pattern. Therefore, this result indirectly supports that R-Ras is translocated to the membrane.

**Discussion**

R-Ras has been reported to induce cell proliferation, adhesion, migration, invasion, and phagocytosis, and has been further suggested to have opposite effects of oncogenic H-Ras despite their sequence similarity (9, 20, 21). However, the underlying mechanisms of R-Ras are largely unknown. In this study, we have investigated the biological consequence of R-Ras expression in a human breast epithelial cell line, and the possible mechanisms of action.

Expression of constitutively active R-Ras in MCF10A cells induced remarkable phenotype changes, including cell morphology, adhesion, and motility. Previous reports (4, 7, 23) suggest that similar to H-Ras, R-Ras plays an important role in cell growth by activating some elements of the H-Ras signaling pathways. However, in our experiments, R-Ras expression neither induced transformation in MCF10A cells nor affected cell proliferation (data not shown). Moreover, R-Ras did not stimulate ERK or PI3K activity (data not shown).

Focal adhesions are specialized signaling platforms on the cell surface that mediate cell-matrix interaction via integrins, which are associated with different cytoskeletal proteins (24). The turnover of focal adhesions are known to be regulated by Ras (25). Furuhjelm et al. (22) showed that GTP-bound R-Ras is preferentially targeted to focal adhesions. Here, we show that R-Ras cells display an increased number of focal adhesions and tight compact focal adhesions in the cell periphery. In addition, active R-Ras enhances the phosphorylation of focal adhesion complex proteins, FAK and paxillin, in collagen (data not shown). We obtained indirect evidence of R-Ras translocation to the membrane using HR chimeric protein (Fig. 6E). Although we cannot exclude the possibility of R-Ras localization in other regions, it is feasible that R-Ras targeted to focal adhesions induces an increase of integrin activity via phosphorylation of FAK and paxillin, leading to increased cell adhesion.

In contrast to the increase in adhesion activity, cell motility and polarity were disrupted in the R-Ras–expressing cells. Our results suggest that higher Rho/Rho-kinase activity in R-Ras cells could be responsible for the disruption of motility and morphology. Cell motility is a complex process involving the extension of membrane protrusions at the leading edge, formation of adhesions, detachment, and retraction at the rear of the cell (26). It has been well established that Cdc42, Rac1, and RhoA activities are crucial for the complex processes of cell motility (27). In our experiments, neither Rac1 nor Cdc42 were changed in R-Ras cells compared with control cells, whereas the RhoA activity of R-Ras cells were higher than that of control cells. In addition, blocking the Rho/Rho-kinase pathway reversed the disruption of cell polarity and motility. In a previous report (28), Chinese hamster ovary cells displayed increased RhoA activity at a high concentration of fibronectin. This higher RhoA activity was related to the motility disruption signal. In another investigation (29), dominant-negative p190RhoGAP elevated RhoA activity on fibronectin in Rat1 cells and inhibited migration. Although the detailed mechanism by which higher RhoA activity induces disruption of motility is not well characterized, the process...
may be explained by Rho-kinase, which is one of the RhoA targets. High Rho-kinase activity is known to phosphorylate two cytoskeletal proteins, adducin and moesin (30–32). This phosphorylation, in turn, enhances interactions with filamentous actin, which may subsequently facilitate the formation of cortical actin filaments. In addition, cortical actin bundles located in the periphery recruit myosin II and MLCK, leading to MLC phosphorylation and cell contraction (33, 34). In our experiments, MLC and phosphorylated MLC mainly accumulated at the cell periphery in R-Ras cells, similar to actin. This localization pattern was abolished by the Rho-kinase inhibitor, Y27632, suggesting that active actomyosin structures are assembled with cortical actin at the cell periphery through the Rho/Rho-kinase pathway. Thus, it is possible that an increase in the number and size of peripherally localized adhesions and enriched cortical actin bundles in active R-Ras–expressing cells is related to higher Rho-kinase activity. Because actomyosin filaments are distributed in the active R-Ras cell, contractile forces derived by these structures are possibly centripetal, which may result in cell rounding and a deficiency in cell motility.

Active R-Ras led to an increase in adhesion of MCF10A cells, which is consistent with previous results (35–37). In our experiments, on the other hand, active R-Ras disrupted cell motility, which seems to contradict the previous results (35–37) reporting that R-Ras induced increased migration of myoblast (C2C12) and epithelial cells (T47D, C33A). However, we found that in another mammary epithelial cell line, MDA-MB-231, active R-Ras significantly decreased cell motility compared with control cells (Fig. 2D), suggesting that reduced cell motility by active R-Ras expression is not restricted to MCF10A cells. The processes of cell motility involve both simultaneous and ordered changes in the activities and/or locations of hundreds of proteins. It indicates that R-Ras, like many other Ras superfamily members, may affect multiple effectors in regulating cell motility, and that some of these effectors interactions may be cell type–specific (38). In our transfectants (active R-Ras–expressing MCF10A and MDA-MB-231 cells), RhoA is activated but PI3K is not. On the contrary, most of the transfectants mentioned above were accompanied with the activation of PI3K, which is involved in cell migration. Therefore, this discrepancy could be due to using different cell types. Alternatively, it could be due to using different assays for cell motility. Meanwhile, previous reports measured cell migration using transwells (35–37); in our experiment, we measured single cell motility on culture dishes using time-lapse video microscopy. Haptotactic migration with the lower surface of the transwell membrane coated by substrate is a directional assay and measures the coordinate ability—not only motility but also

Figure 6. The COOH-terminal end of R-Ras contains a cell motility disruption signal. A, schematic outline of chimera, active forms of H-Ras and R-Ras are H-Ras (12V) and R-Ras (38V), respectively. R26H contains the first 26 amino acids of the NH₂ terminus from R-Ras and the complete sequence of H-Ras (12V). H-Ras (12V)/R-Ras hypervariable region (HR) comprises the NH₂-terminal 1-150 amino acids from H-Ras (12V) and amino acids 178-218 from the COOH terminus of R-Ras (38V). R-Ras (38V)/H-Ras hypervariable region (RH) includes 1-177 from R-Ras (38V) and amino acids 151-189 from the COOH terminus of H-Ras (12V). MCF10A cells were transfected with retrovirus encoding the above chimera constructs. Transfectant cells were immunoblotted with H-Ras and R-Ras antibodies for detecting expression (B). Chimera transfectant cells were visualized by phase-contrast microscopy (C). Chimera transfectant cells were seeded in culture dishes and incubated overnight. Next, cell motility was monitored by time-lapse video microscopy for 5 hours, and the migratory speed was calculated based on the sum of distances divided by the time of observation, using the Metamorph program (D). Data are representative of three separate experiments. The migration speeds of at least 60 cells were analyzed and represented as mean ± SD. E, chimeric transfectants, HR cells [H-Ras (12V)/R-Ras (38V) hypervariable region], were seeded on a chamber slide, fixed and immunostained with anti–H-Ras antibody (Santa Cruz). Bar, 20 µm.
adhesion—of a cell to sense and to respond toward substrate (39). Single cell motility on a culture dish using time-lapse video microscopy, is random and nondirectional (39).

Although active R-Ras (38V) inhibited cell motility in MCF10A cells, dominant-negative R-Ras (43N) did not affect cell motility. We have tried another cell line (MDA-MB-231) with the same mutant and also got the same results just like MCF10A cells. A previous report (37) supported our results and showed that the dominant-negative R-Ras (43N) did not affect the adhesion and migration of cervical epithelial cells.

The transforming Ras proteins promote neoplastic transformation, whereas R-Ras is far less efficient at doing so (7, 8). In spite of its structural similarity and sharing many signal transduction pathways, it is still unclear why R-Ras is less oncogenic than Ras proteins. The acquisition of motile behavior is one of the characteristics that occur in the process of malignant transformation of epithelial cells. Our study clearly shows that disruption of motility is induced by R-Ras in breast epithelial cells, which is distinct from the activity of transforming Ras proteins. The less motile property elicited by R-Ras may account for the less transforming activity of R-Ras than other Ras proteins.

R-Ras is closely related to H-Ras and K-Ras, which makes it possible to switch regions between these molecules without inducing gross changes in the protein architecture. These approaches indicate a role for the highly divergent COOH-terminal hypervariable regions of both GTPases in cell function. Replacement of hypervariable regions between R-Ras and H-Ras were recently reported to suppress R-Ras-mediated integrin activation (40) and targeting to focal adhesion (22). More evidence has been reported that the hypervariable regions of R-Ras confers the specificity of R-Ras to H-Ras (41). In our experiments, when the hypervariable region of H-Ras (amino acids 178-218) were replaced with the corresponding region of H-Ras (amino acids 151-189), the morphology and motility of cells were similar to those of active H-Ras cells. Conversely, replacement of the hypervariable region of H-Ras (amino acids 151-189) with the corresponding region of R-Ras (amino acids 178-218) induced a defect in cell motility and round cell shape, characteristic of active R-Ras cells. Our results collectively suggest that the hypervariable region of R-Ras has a key function in the regulation of cell morphology and motility. It has been known that the different effects of H-Ras and R-Ras on integrin activation and focal adhesion targeting come from the different lipid modification sites in the COOH-terminal region of R-Ras (22, 40). Recently, it was reported that R-Ras COOH-terminal sequences were sufficient to confer R-Ras specificity to H-Ras such as ROS production, transforming property, and integrin regulation (41). They suggested that regulatory events, such as binding of specific downstream effectors, must be involved in defining specificity of the COOH-terminal region of R-Ras (41). Therefore, it is possible that not only cellular localization, mainly driven by lipid modification, but also functional region in the COOH-terminal of R-Ras could explain the contrasting of effects H-Ras and R-Ras on cell migration in MCF10A cells. In conclusion, we have elucidated that R-Ras in breast epithelial cells disrupts cell polarity and motility through the Rho/Rho-kinase pathway triggered by a signal from the COOH-terminal end of R-Ras. These results suggest that disruption of motility by R-Ras may be at least partially responsible for the less transforming activity of R-Ras compared with Ras proteins.

Acknowledgments


Grant support: This work was supported by a program of the National Research Laboratory (M1010400036-01J000-01610), Korea.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

11. Oertli B, Han J, Marte BM, et al. The effector loop and the corresponding region of H-Ras (amino acids 151-189), hypervariable region of R-Ras (amino acids 178-218) were replaced with 18 U.S.C. Section 1734 solely to indicate this fact.


The COOH-terminal End of R-Ras Alters the Motility and Morphology of Breast Epithelial Cells through Rho/Rho-Kinase

Ha-Won Jeong, Ju-Ock Nam and In-San Kim


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/2/507

Cited articles
This article cites 41 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/2/507.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/2/507.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.