R-cadherin Influences Cell Motility via Rho Family GTPases*

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Classical cadherins are the transmembrane proteins of the adherens junction and mediate cell-cell adhesion via homotypic interactions in the extracellular space. In addition, they mediate connections to the cytoskeleton by means of their association with catenins. Decreased cadherin-mediated adhesion has been implicated as an important component of tumorigenesis. Cadherin switching is central to the epithelial to mesenchymal transitions that drive normal developmental processes. Cadherin switching has also been implicated in tumorigenesis, particularly in metastasis. Recently, cadherins have been shown to be engaged in cellular activities other than adhesion, including motility, invasion, and signaling. In this study, we show that inappropriate expression of R-cadherin in tumor cells results in decreased expression of endogenous cadherins (cadherin switching) and sustained signaling through Rho GTPases. In addition, we show that R-cadherin induces cell motility when expressed in epithelial cells and that this increased motility is dependent upon Rho GTPase activity.

Classical cadherins are the transmembrane components of the adherens junction. E-Cadherin is found in epithelial cell junctions (E-cadherin),1 whereas other cadherins are found in similar structures in other cell types (1, 2). Disruption of E-cadherin function through mutations in E-cadherin itself, through modifications to the E-cadherin promoter or through disruption of the cadherin/catenin complex, contributes to the aggressive behavior of epithelial-derived tumor cells (1). Studies from our laboratory and others have shown that expression of an inappropriate cadherin in epithelial cells is another way that tumor cells can alter their adhesive function (3–6). In some cases, this is due to down-regulation of E-cadherin upon expression of the inappropriate cadherin (4). In other cases, neuronal cadherin (N-cadherin) can have a direct and dominant influence upon the phenotype of epithelial cells, despite their continued expression of E-cadherin. For example, expression of N-cadherin by oral squamous epithelial cells results in decreased expression of the endogenous cadherins, whereas expression of N-cadherin by some breast epithelial cells has no effect upon expression of endogenous cadherins, but none the less influences cell adhesion and behavior (4, 5). Thus, exogenous expression of N-cadherin can modulate the expression of endogenous cadherins in some cell types but not in others. Interestingly, exogenous expression of vascular endothelial cadherin (VE-cadherin) in a variety of cell types does not influence the expression levels of any endogenous cadherins (7).

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1 The abbreviations used are: E-cadherin, epithelial cadherin; N-cadherin, neuronal cadherin; VE-cadherin, vascular endothelial cadherin; R-cadherin, retinal cadherin; P-cadherin, placental cadherin; MEM, minimal essential medium; CRIB, Cdc42/Rac1 interactive binding; GST, glutathione S-transferase; TRITC, tetramethylrhodamine isothiocyanate; GFP, green fluorescent protein; DN, dominant-negative.
been reported to be a guanine exchange factor for RhoA, RhoG, Cdc42, and Rac1. Rac1 and Cdc42 seem to have similar functions in E-cadherin-mediated adhesion, whereas RhoA performs a separate function (2, 24–26).

Gauthier-Rouvire and co-workers (27) used myogenic cells to show that RhoA, but not Rac1 or Cdc42, was activated upon N-cadherin-mediated cell-cell contact. Mege and co-workers (28) used the same system to show that inhibiting Rac1 did not influence initial N-cadherin cell-cell contact, but that Rac1 was important in connecting the cadherin to the cytoskeleton. Dejana and co-workers (29) showed that Rac1, but not RhoA, was activated in VE-cadherin-expressing endothelial cells, and Kouklis et al. (30) showed that non-junctional VE-cadherin induced Cdc42 activation, which led to membrane protrusions. In the present study, we show that R-cadherin expression by a variety of cell types results in increased steady-state activity levels of Rac1 and Cdc42, that activation of these GTPases correlates with increased cell motility, and that dominant-negative forms of these GTPases inhibit R-cadherin-dependent cell motility. These studies are significant because they highlight the importance of cellular context in cadherin-mediated GTPase activation and demonstrate that R-cadherin GTPases and cadherins cooperate not only in cell adhesion but also in cell motility.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast cancer cell line BT-20 was obtained from American Type Culture Collection (ATCC) and maintained in minimal essential medium (MEM, Sigma) supplemented with 10% fetal calf serum (FCS, HyClone Laboratories). BT-20 cells are BT-20 cells retrovirally transduced to express R-cadherin. A431 cells (ATCC), Phoenix cells (a kind gift from Dr. Albert Reynolds, Vanderbilt University), and A431Ds were maintained in Dulbecco’s modified Eagle’s medium (Sigma) with 10% FCS. A431 is a squamous epithelial cell line derived from a cervical cancer. A431D1 cells were derived in our lab by long-term treatment of A431 cells with dexamethasone. These cells have lost expression of all classical cadherins and have been described previously in more detail (31). A431D1R and A431DE are A431D1 cells retrovirally transduced to express R-cadherin or E-cadherin, respectively. The human squamous carcinoma cell line UM-SCC-1 (SCC-1; a kind gift of Dr. Thomas Carey, University of Michigan) was maintained in MEM with 10% FCS, HT1080, HACAT, SkBr3, and MCF-7 (ATCC) were main-

Antibodies—Monoclonal antibodies against human E-cadherin and mouse R-cadherin were provided by Dr. Masatoshi Takeichi. Monoclonal antibodies against human placental cadherin (P-cadherin) (6A9), α-catenin (1G5), and β-catenin (15B8) have been described (40, 41). The mouse monoclonal antibody against the myc epitope (9E10) was a kind gift from Dr. Kathleen Green (Northwestern University, Chicago, IL). Antibodies against Rac1, Cdc42, and p120 catenin were purchased from BD Transduction Laboratories. Fluorescein isothiocyanate (FITC)- and TRITC-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratory. Secondary antibodies used for Western blot analysis were alkaline phosphatase-conjugated or horseradish peroxidase-conjugated anti-IgG (Jackson ImmunoResearch).

RESULTS

Exogenous Expression of R-Cadherin Down-regulates Endogenous Cadherins—To facilitate detection of R-cadherin in transfected cells, we myc-tagged mouse R-cadherin, cloned it into the viral expression vector LZRS-MS-Neo, and produced infective virus. Biological activity of the tagged construct was confirmed by stably expressing it in A431D cells, a cadherin-negative derivative of A431 cells (31). After infection, a population of cells was selected with G418 and designated A431DR. Control A431D cells infected with viral particles containing BanHI and 3′ EcoRI sites for insertion into pGEX-2T, resulting in a GST-CBD fusion. The plasmid encoding the rhotekin Rho-binding domain–GST fusion consisting of amino acids 7–89 of mouse Rhotekin fused to GST was a kind gift of Dr. Richard Cerione (Cornell University, Ithaca, NY) and was provided by Dr. Robert Lewis (Eppley Cancer Institute). The N-terminal GFP-tagged constructs Cdc42N17, Rac1N17, and RhoAN19 (kind gifts of Dr. Klaus Hohn, Scripps Institute, La Jolla, CA) were subcloned into LZRS-MS-Neo. Transfection, Virus Production, and Infection—Phoenix cells (37) were transfected using a calcium phosphate transfection kit (Stratagene) with LZRS-MS-Neo/GFP (35) and selected in 2 µg/ml puromycin (Sigma). Cells were cultured until confluent, split, and stored in liquid nitrogen. Transfected Phoenix cells were grown at 37 °C in selective media until 50–80% confluent, at which time the medium was replaced with fresh medium without puromycin and cells were grown overnight at 32 °C. Virus-containing medium was removed, filtered through a 0.45-µm filter, and supplemented with polybren (4 µg/ml, Sigma). Target cells were plated overnight at low density to ensure formation of single cells and then incubated in the virus-containing medium at 32 °C for 2–6 h. Infected cells were cultured until around 80% confluent and then either selected in 1 mg/ml G418 or sorted by fluorescence-activated cell sorting for GFP expression. Cells infected with the empty LZRS-MS-Neo virus were used to set the background fluorescence. Cell sorting was performed by the University of Nebraska Medical Center Cell Analysis Facility.

Pull-down Assays for Rac1/Cdc42—Fusion protein was prepared and coated onto beads as described (15, 36). The beads were resuspended in an equal volume of wash buffer and stored at −80 °C. Tissue culture cells were rinsed twice with cold Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and acerped in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The mixture was incubated on ice for several min, clarified by centrifugation, and used immediately. 10% of the cell lysate was used for the total protein control, whereas the remainder was incubated with beads coated with the GST-CRIB or GST-CBD fusion proteins for 45 min to 1 h at 4 °C. Beads were centrifuged, washed, and resuspended in sample buffer for SDS-PAGE (38). For RhoA pull-down assays, fusion protein was prepared and coated onto beads as described (39) with minor modifications. Tissue culture cells were treated the same as above except that the lysis buffer was 50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl2.

Fusion protein was prepared and coated onto beads as described (39) with minor modifications. Tissue culture cells were treated the same as above except that the lysis buffer was 50 mM Tris-HCl, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl2.

Aggregation and Motility Assays—Aggregation assays were performed as described (32). Briefly, trypsinized cells were resuspended in 50-µl drops of medium containing 5000 cells/drop and pipetted onto the inner surface of the lid of a 100-mm Petri dish and incubated over culture medium for 24 h at 37 °C. The drops were pipetted 10 times to disrupt weak aggregates prior to photography. Transwell filter motility assays were performed as described previously (5).

Constructs—PCR products utilized in any of the constructions described below were sequenced and shown to contain only the intended changes. The mouse R-cadherin cDNA (GenBank accession no. D14888) was a kind gift from Dr. Masatoshi Takeichi, pCR-PCR cloning (36). The R-cadherin stop codon was replaced with a ClaI site, allowing insertion of the modified cDNA was then inserted into LZRS-MS-Neo/GFP (35). The resulting sequence was identical to the one used by Benard et al. (36). The primers PAK1.1 5′-GAGGGATCCTAGGAGACGACGTCAGGACCTG-3′ and PAK2 5′-CTGAGAATTCAGGAGACGACGTCAGGACCTG-3′ added 5′ BamHI and 3′ EcoRI sites for insertion into pGEX-2T (Amersham Biosciences), resulting in a glutathione S-transferase (GST)-CRIB fusion. The Cdc42-binding domain (CBD) of the Wiskott-Aldrich syndrome protein (15) was amplified from IMAGE cDNA clone 344979 (ATCC). The primers 5′-ATATATG GAGGATCCTAGGAGACGACGTCAGGACCTG-3′ and 5′-GGGATCCTAGGAGACGACGTCAGGACCTG-3′ added 5′ and 3′ EcoRI sites for insertion into pGEX-2T, resulting in a GST-CBD fusion. The plasmid encoding the rhotekin Rho-binding domain–GST fusion of the LTR3-Rex vector was transfected into human T cells at high multiplicity of infection, and immunoprecipitated as described (42). The pellets were resuspended in 2× Laemmli buffer and subjected to SDS-PAGE. Membrane and cytosolic fractions were prepared as described (43).

Immunofluorescence, Cell Extraction, and Immunoprecipitation—Cells plated on glass coverslips for 24–48 h were fixed and stained as described (42), viewed on a Zeiss Axiovert 200M equipped with an ORCA-ER (Hamamatsu) digital camera, and processed using OpenLab software (Improvision Inc.). Membrane and cytosolic cell monolayers were extracted and immunoprecipitated as described (42). The pellets were resuspended in 2× Laemmli buffer and subjected to SDS-PAGE. Membrane and cytosolic fractions were prepared as described (43).

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Only the neomycin resistance gene was designated A431DNeo. Extracts of A431DNeo and A431DR cells were immunoblotted with monoclonal antibodies against myc, α-catenin, and β-catenin (Fig. 1A). A431DNeo cells did not exhibit a myc signal at the correct size for R-cadherin-2×-myc whereas the A431DR cell extract had such a signal. We have shown previously that the transfection of a cadherin into A431D cells results in the stabilization of α- and β-catenin (43). Thus, as expected, expression of R-cadherin in these cells resulted in increased signals for α- and β-catenin. To mediate full adhesion, classical cadherins must be attached to the cytoskeleton via catenins. Extracts from A431DNeo or A431DR cells were immunoprecipitated with anti-myc, resolved by SDS-PAGE, and immunoblotted for α-catenin (Fig. 1B) or β-catenin (data not shown). The catenins co-immunoprecipitated with R-cadherin-2×-myc, indicating that this cadherin was anchored to the actin cytoskeleton. To further show that R-cadherin-2×-myc was functional, we performed aggregation assays and immunofluorescence localization. Fig. 1C shows that A431DR cells form large aggregates, whereas A431Neo cells do not aggregate. In addition, A431DR cells show cell border staining with anti-myc antibodies (R-cadherin) and with anti-β-catenin antibodies (Fig. 1D). Together, the data presented in Fig. 1 show that R-cadherin-2×-myc is correctly processed, localized at cell borders, interacts with catenins, and mediates cell adhesion.

Previous work from our laboratory (4, 5) showed that exogenous expression of N-cadherin down-regulated expression of endogenous cadherins in some cells but not in others, suggesting that N-cadherin-mediated down-regulation of endogenous cadherins was cell-type specific. To determine whether R-cadherin influenced endogenous cadherin expression, we infected a number of cell lines with recombinant retrovirus encoding R-cadherin-2×-myc. In every case, endogenous cadherins were down-regulated. Fig. 2A shows immunofluorescence localization of cadherins in control BT-20Neo cells (panels a–c) and in BT-20R cells (panels d–f). The control cells did not exhibit plasma membrane staining with the myc antibody, but the R-cadherin-expressing cells showed membrane localization with this antibody. Timed exposures of BT-20R cells and the neomycin counterparts showed that the R-cadherin-expressing cells had diminished signal for both E- and P-cadherin at cell-cell borders (compare panels d and e with panels a and b, respectively). Immunoblot analysis further showed that BT-20R cells had a decreased expression of E-cadherin and P-cadherin when compared with control BT-20Neo cells (Fig. 2B). In addition to the BT-20 cells, exogenous expression of R-cadherin-2×-myc in A431 (E- and P-cadherin), HT-1080 (N-cadherin), HACAT (E-, P-, and N-cadherin), MCF-7 (E-cadherin), or SCC-1 (E- and P-cadherin) cells resulted in reduced expression of all endogenous cadherins (data not shown). These data suggest that R-cadherin differs from N-cadherin in its ability to regulate endogenous cadherin expression.

**Exogenous Expression of R-Cadherin Increases Cell Motility**—To examine the effect of R-cadherin expression on cell motility, 5 × 10⁵ BT-20Neo or BT-20R cells were plated in three separate transwell inserts and incubated overnight with NIH3T3-conditioned medium in the bottom chamber as a chemottractant. Nonmotile cells were removed from the top of the insert, and the cells on the bottom of the filter were counted. Fig. 3A shows the average number of cells per field of view from the three independent experiments. BT-20 cells expressing R-cadherin were significantly more motile than the control BT-20Neo cells. A complicating factor in this experiment was that R-cadherin expression resulted in down-regulation of E- and P-cadherin in BT-20 cells. To determine whether R-cadherin could influence cell motility independently of its ability to decrease endogenous cadherins, we investigated the influence R-cadherin had on motility in cadherin-null cells. The cadherin-null cell lines A431D and SkBr3 were infected with control neomycin resistance virus or with R-cadherin-2×-myc-expressing virus and used in motility assays. Expression of R-cadherin increased the motility of both A431D (Fig. 3B) and SkBr3 (data not shown) cell lines. Interestingly, in the case of A431D cells, the motility rate was only two times that of the parental cell line. Because the increase in motility was roughly 4-fold higher in BT-20R cells than in BT-20Neo cells, it is likely that the down-regulation of endogenous E- and P-cadherins in BT-20R contributed to the difference in motility. In any case, the experiments with A431D and SkBr3 cells showed that R-cadherin has an intrinsic ability to influence cell motility.

**R-Cadherin-based Motility Is Due in Part to the Activation of Rac1 and Cdc42**—A separate method to measure cell motility is a wound-healing assay, in which a confluent monolayer of cells is wounded and the cells are allowed to migrate into the denuded area. Such an assay is more difficult to quantify than...
R-cadherin and Rho GTPases

Fig. 2. R-cadherin-2×-myc expression in epithelial cells down-regulates endogenous E- and P-cadherin. Recombinant retrovirus was used to stably express R-cadherin-2×-myc together with the neomycin resistance gene or the neomycin resistance gene alone in BT-20 cells (BT-20R or BT-20Neo, respectively). A, immunofluorescence. BT-20Neo cells (panels a–c) or BT-20R cells (panels d–f) were stained for E-cadherin, P-cadherin, or R-cadherin(myec). FITC-conjugated anti-mouse secondary was used to detect the primary antibodies. a and d, E-cadherin localization; b and e, P-cadherin localization; c and f, R-cadherin localization. B, immunoblots. 40 μg of total protein were resolved by SDS-7% PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against E-cadherin, P-cadherin, or R-cadherin(myec).

the assays used above, but it can provide information about how the cells move. When A431Neo cells were compared with A431R cells in a wound-healing assay, the R-cadherin-expressing cells not only migrated significantly further than the A431Neo cells but also displayed robust lamellipodia at the leading edge of the wound (Fig. 4, compare B with A). Similarly, the expression of R-cadherin in A431D cells caused these cells to display membrane protrusions (Fig. 1D), and immunofluorescence of BT-20 cells showed that E-cadherin contacts were more linear than R-cadherin contacts, which were jagged and suggestive of membrane protrusions (Fig. 2A). Immunofluorescence localization of R-cadherin (Fig. 4D) and E-cadherin (Fig. 4C) in A431D cells using higher magnification suggested that R-cadherin-expressing cells interact through overlapping membranous structures, whereas E-cadherin interactions produce a smoother interface. Indeed, even when we expressed R-cadherin in A431 cells, which endogenously express E- and P-cadherin, the cell borders displayed long membrane protrusions (Fig. 4F), resembling filopodia or microspikes.

Experiments from other laboratories have shown a connection between cadherins and the activity of Rho GTPases (15, 16, 29, 30, 44). The increased motility of R-cadherin-expressing cells, together with the increased membrane ruffling observed in these cells, led us to hypothesize that expression of R-cadherin may alter the steady-state activity levels of one or more of the Rho GTPases Cdc42, Rac1, or RhoA. To assay for activated GTPases, we employed GST fused to the GTPase-binding domain of PAK1, WASP, and Rhotekin, which bind respectively to the active, GTP-bound forms of Rac1, Cdc42, and RhoA, and we performed pull-down assays with extracts of A431D cells, A431D cells expressing E-cadherin or R-cadherin, BT-20 cells, and BT-20 cells expressing R-cadherin.

The A431D cell line is a good model system because it lacks endogenous cadherin expression, allowing us to investigate the absolute influence of cadherin expression on GTPase activation. 3 × 10^6 cells were plated overnight, then lysed and incubated with fusion proteins conjugated to glutathione-Sepharose beads. A431D cells expressing neo-resistance alone or neo-resistance plus E-cadherin showed approximately equal levels of Rac1 activation, whereas expression of R-cadherin by these cells significantly increased the steady-state activity level of Rac1. There was a slight elevation in the level of activated Cdc42 in A431DR cells, but it was not statistically significant (Fig. 5A). Activated RhoA could not be detected in A431Dneo, A431DE, or A431DR cells (data not shown).

Of equal interest was determining if R-cadherin was capable of altering the steady-state activity levels of the Rho GTPases in the motility model cell line BT-20. The parental BT-20 cells express E-cadherin and P-cadherin, each of which may also influence the activity of small GTPases. If R-cadherin-mediated motility is driven by the activation state of these molecules, we would expect there to be elevated levels of the active forms of one or more of the small GTPases in BT-20R cells when compared with parental BT-20Neo cells. 2.5 × 10^6 BT-20Neo or BT-20R cells were plated and processed for pull-down assays (Fig. 5B). There was a 1.5-fold increase in Rac1 activation and a 2.5-fold increase in the activation of Cdc42 in the R-cadherin-expressing cells. Interestingly, there was a decrease in steady-state RhoA GTP levels in the BT-20R cells compared with the parental cell line. This finding is consistent with the idea that Rac1 and Cdc42 activities tend to promote similar cellular traits, whereas RhoA opposes their action (24–26).

To further investigate the role of Rac1 and Cdc42 in R-cadherin-dependent motility, a dominant-negative form of each GTPase was expressed in BT-20R cells. Recombinant viruses containing dominant-negative Rho GTPases fused to green fluorescent protein (GFP) or GFP alone were used to infect BT-20R cells. Cells expressing GFP or the GFP fusions were sorted from non-expressing cells by fluorescence-activated cell sorting. Fig. 6A shows phase microscopy of living cells and the
corresponding GFP signal after sorting. The dominant-negative GTPases were both diffuse in the cytosol and at the plasma membrane, suggesting that at least a portion is in junctions, which is surprising because it is typically the GTP-bound form of these molecules that is junctional (14, 25). Whether R-cadherin-containing junctions differ in this regard from E-cadherin-containing junctions has yet to be determined.

Total protein from each cell line was resolved on a 7% polyacrylamide gel and immunoblotted for R-cadherin, E-cadherin, and P-cadherin (Fig. 6B). Importantly, dominant-negative Rho GTPases did not significantly influence the expression levels of the cadherins. Cell extracts were resolved on a 14% polyacrylamide gel and blotted with antibodies against Rac1 or Cdc42 (Fig. 6C). Expression of the dominant-negative Rho GTPases resulted in a slight down-regulation of the corresponding endogenous protein.

To determine whether BT-20R cell motility was altered by the expression of dominant-negative Rho GTPases, BT-20R cells expressing dominant-negative (DN) Rho GTPase fusion proteins were subjected to motility assays. 5 x 10^5 cells from each cell line (BT-20Neo, BT-20R, DN Rhoa1, and DNCdc42) were plated overnight in a motility assay (Fig. 6D). As expected, the BT-20RGFP cells displayed a motility rate higher than that of the parental BT-20Neo cells. BT-20R cells expressing DN Rhoa1 or DNCdc42 were less motile than the BT-20RGFP and were not significantly different from the parental cells.

A role for Rho GTPases in cell motility has been established (45). Thus, to rule out the possibility that dominant-negative forms of Rac1 and Cdc42 were inhibiting general BT-20 cell motility and not motility specifically due to R-cadherin expression, we compared the motility of BT20 cells with that of BT-20 cells expressing either DN Rhoa1 or DNCdc42 (Fig. 6E). There was no significant difference in the motility of any of these cell lines, further supporting a role for Rac1 and Cdc42 in R-cadherin-mediated cell motility.

One link between cadherins and Rho GTPases is p120 catenin. A mechanism whereby cadherins could differentially activate Rho GTPases would be through differing affinities for p120 catenin. That is, each cadherin family member may differentially bind p120 catenin and as a result have different influences on Rho GTPase activity. To test the hypothesis that R-cadherin and E-cadherin have different affinities for p120 catenin, we transduced 2HOE11003-myc-tagged E-cadherin or 2HOE11003-myc-tagged R-cadherin into the cadherin-null A431D cells and selected populations of cells that expressed approximately equal levels of cadherin as judged by immunoblots using the myc antibody directed against the tag on each cadherin. The populations also expressed approximately equal levels of p120 catenin and E-cadherin (Fig. 7A). When the cells were partitioned into membrane and cytosolic fractions, virtually all of the cadherin and p120 catenin were in the membrane fraction, whether the cells expressed E-cadherin or R-cadherin (Fig. 7B). However, when cell extracts were immunoprecipitated with antibodies against p120 catenin and immunoblotted for p120 catenin and myc, there was less R-cadherin than E-cadherin for an equal amount of p120 (Fig. 7C). Likewise, when approximately equal amounts of cadherin were immunoprecipitated...
with anti-myc antibodies, less p120 catenin co-immunoprecipitated with R-cadherin than with E-cadherin (Fig. 7D).

**DISCUSSION**

Animal development frequently employs “cadherin switching” to facilitate the epithelial to mesenchymal transitions that are necessary for normal tissue structure (46). Cells undergo a similar epithelial to mesenchymal transition during tumorigenesis (46, 47). This sometimes occurs when the epithelial tumor cell turns on the expression of an inappropriate non-epithelial cadherin, which results in down-regulation of endogenous cadherins, decreased cell-cell adhesion, and increased motility and invasion (3–6, 48, 49). N-cadherin has been shown to be inappropriately expressed by cells derived from squamous epithelial tumors (4) and by breast cancer cells (5). Interestingly, exogenous expression of N-cadherin in squamous epithelial cells results in decreased expression of the endogenous cadherins, whereas breast cancer cells often continue to express their endogenous cadherins when they are transfected with N-cadherin (4, 5). R-cadherin plays an important role in epithelial to mesenchymal transitions in developing kidney and bladder and has been shown to be expressed in a number of cancer cell lines (12). In addition, R-cadherin is highly homologous to N-cadherin, which led us to hypothesize that transfection of R-cadherin into epithelial tumor cells may also influence expression of endogenous cadherins. Surprisingly, exogenous expression of R-cadherin consistently resulted in a drastic reduction of all endogenous cadherins in every cell line we checked. This was the case even for cells like HT1080 and HACAT that endogenously express N-cadherin. Thus, it seems that R-cadherin has an even higher capacity than N-cadherin to down-regulate expression of other cadherins.

It is clear that, in addition to their role in cell adhesion, cadherins play important roles in other cellular characteristics including cell motility, cellular polarity, cytoskeletal organization, and cellular signaling (1, 2). Investigations into the role
cadherins play in cell motility have produced results that may at first glance seem contradictory. In Frixen et al. (50), treatment of Madin-Darby canine kidney cells with anti-E-cadherin antibodies resulted in cells with a more invasive phenotype, suggesting that inhibiting the activity of E-cadherin induced cell motility. In a separate publication, wound healing assays

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**Fig. 6. Expression of dominant-negative Rho GTPases in BT-20R cells.** BT-20R cells were infected with recombinant retroviruses encoding dominant-negative Rac1GFP, Cdc42GFP, or GFP alone (A). a, c, e, and g, phase microscopy of living infected cells. b, d, f, and h, GFP signal in living cells. a and b, BT-20Neo cells; c and d, BT-20RGFP cells; e and f, DNRac1 cells; g and h, DNCdc42 cells. 40 μg of total protein were resolved on SDS-7% PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against R-cadherin (myc), E-cadherin, or P-cadherin (B). Rac1 or Cdc42 (C). D, 5 × 10^5 BT-20Neo cells or BT-20R derivatives were plated on motility inserts in 3 ml of medium. After overnight incubation, non-motile cells were removed from the top of the chamber, and the cells adhering to the bottom surface were fixed and stained. Ten random fields from three experiments were counted. The average number of cells and the standard deviation are shown.
cells. Thus, Rho GTPase family members may play an important role in the signaling events that regulate cadherin-dependent motility in addition to the role they play in cadherin-mediated adhesion. Other laboratories have suggested links between Rho GTPases and cell motility (54). This is the first suggestion that increased cell motility via differential cadherin expression was due to altered activity of Rho GTPases. Importantly, R-cadherin-induced cell motility was reduced to that of parental BT-20Neo cells by expression of dominant-negative Rho GTPases. In addition, the levels of endogenous cadherins in BT-20R cells did not increase when dominant-negative Rho GTPases were expressed, suggesting that the dominant-negative effect was truly due to diminished GTPase activity and not to restoration of endogenous cadherins. These data support the idea that R-cadherin-induced cell motility is at least partially due to increased Rho GTPase activity.

BT-20 cells express E-cadherin and P-cadherin, and yet expressing R-cadherin in these cells resulted in increased activation of Rac1 and Cdc42, suggesting that cadherins may differentially activate Rho GTPases. A number of recent studies have shown that cadherins activate Rho GTPases; however, this is the first indication of differential activation of Rho GTPases by different cadherin family members. The mechanism whereby cadherins might differentially activate Rho GTPases has yet to be determined. One possibility might be through their interactions with p120 catenin, a protein which has been shown by a number of laboratories to be involved in cadherin activation of Rho GTPases (20–23, 25, 44). In addition, the Dejana (55) lab has shown that VE-cadherin and N-cadherin have different affinities for p120 catenin, suggesting that cadherin-p120 catenin interactions do differ between different cadherins and lending credence to this hypothesis. The experiments shown in Fig. 7 indicate that almost all of the p120 catenin in A431DE and A431DR cells is membrane-associated and that there seems to be no differences in the behavior of the two p120 catenin isoforms expressed by these cells. Because less p120 catenin co-immunoprecipitates with R-cadherin than with E-cadherin (Fig. 7C), the simplest explanation is that p120 catenin is lost during the immunoprecipitation washes. However, an alternative possibility is that, although p120 catenin is membrane-associated in A431DR cells, it interacts with membrane-associated proteins in addition to R-cadherin. Perhaps the overall consequence is that R-cadherin and p120 catenin form more dynamic complexes than do E-cadherin and p120, and it is this dynamic aspect that contributes to the increased steady-state activity level of activated Rho family GTPases in cells expressing R-cadherin. An additional possibility is that when cells make contact with E-cadherin, there is eventually a reduction in the levels of activated Rho family members that contributes to contact inhibition. In contrast, this does not happen when cells make contact using R-cadherin, resulting in sustained membrane ruffling or other activities at sites of cell-cell contact. Our lab is currently investigating these possibilities.

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REFERENCES

1. Wheelock, M. J., and Johnson, K. R. (2003) Annu. Rev. Cell Dev. Biol. 19, 207–235
2. Wheelock, M. J., and Johnson, K. R. (2003) Curr. Opin. Cell Biol. 15, 509–514
3. Hazan, R. B., Kang, L., Whosley, B. P., and Borgen, P. I. (1997) Cell Adhes. Commun. 4, 399–411
4. Islam, S., Carey, T. E., Wolf, G. T., Wheelock, M. J., and Johnson, K. R. (1996) J. Cell Biol. 135, 1643–1654
5. Nisen, M. T., Prudoff, R. S., Johnson, K. R., and Wheelock, M. J. (1999) J. Cell Biol. 147, 631–644
6. Fushvaian, M. J., Feltes, C. M., Thompson, P., Bussemakers, M. J., Schalken,
R-cadherin and Rho GTPases

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J. A., and Byers, S. W. (1999) Cancer Res. 59, 947–952
7. Jaggi, M., Wheelock, M. J., and Johnson, K. R. (2002) Cell Commun. Adhes. 9, 103–115
8. Shan, W. S., Tanaka, H., Phillips, G. R., Arndt, K., Yoshida, M., Colman, D. R., and Shapiro, L. (2000) J. Cell Biol. 148, 579–590
9. Inuzuka, H., Redies, C., and Takeichi, M. (1991) Development 113, 959–967
10. Redies, C., Engelhart, K., and Takeichi, M. (1993) J. Comp. Neurol. 333, 398–416
11. Rosenberg, P., Ezmi, F., Sjodin, A., Larue, L., Carlsson, L., Gullberg, D., Takeichi, M., Kemler, R., and Semb, H. (1997) Dev. Biol. 187, 55–70
12. Girolami, I. A., Shimazui, T., Schalken, J. A., Yamasaki, H., and Bringuier, P. P. (2000) Morphologie 84, 31–38
13. Bussemakers, M. J., Van Bokhoven, A., Tomita, K., Jansen, C. F., and Schalken, J. A. (2000) Int. J. Cancer 85, 446–450
14. Yap, A. S., and Kovacs, E. M. (2000) J. Cell Biol. 150, 11–16
15. Kim, S. H., Li, Z., and Sacks, D. B. (2000) J. Biol. Chem. 275, 36999–37005
16. Kovacs, E. M., Ali, R. G., McCormack, A. J., and Yap, A. S. (2002) J. Biol. Chem. 277, 6708–6718
17. Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001) J. Biol. Chem. 276, 33305–33308
18. Betson, M., Lezcano, E., Zhang, J., and Braga, V. M. (2002) J. Biol. Chem. 277, 36962–36969
19. Ehrlich, J. S., Hansen, M. D., and Nelson, W. J. (2002) Dev. Cell 3, 259–270
20. Goodwin, M., Kovacs, E. M., Thoreson, M. A., Reynolds, A. B., and Yap, A. S. (2003) J. Biol. Chem. 278, 20533–20539
21. Anastasiadis, P. Z., Moon, S. Y., Thoreson, M. A., Mariner, D. J., Crawford, H. C., Zheng, Y., and Reynolds, A. B. (2003) Nat. Cell Biol. 2, 637–644
22. Noren, N. K., Liu, B. P., Burridge, K., and Kreft, B. (2000) J. Cell Biol. 150, 567–580
23. Grushev, I., Shutman, M., Elbaum, M., and Bershadsky, A. D. (2001) J. Cell Sci. 114, 695–707
24. Fukata, M., and Kaibuchi, K. (2001) Nat. Rev. Mol. Cell Biol. 2, 887–897
25. Anastasiadis, P. Z., and Reynolds, A. B. (2001) Curr. Opin. Cell Biol. 13, 604–610
26. Braga, V. M. (2002) Curr. Opin. Cell Biol. 14, 546–556
27. Charrasse, S., Merian, M., Comunale, F., Blangy, A., and Gauthier-Rouviere, C. (2002) J. Cell Biol. 158, 955–965
28. Lambert, M., Choquet, D., and Mege, R. M. (2002) J. Cell Biol. 157, 469–479
29. Lampugnani, M. G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V., and Dejana, E. (2002) Mol. Biol. Cell 13, 1175–1189
30. Koukis, P., Konstantoulaki, M., and Malik, A. B. (2003) J. Biol. Chem. 278, 16230–16236
31. Lewis, J. E., Wahl, J. K., 3rd, Sass, K. M., Jensen, P. J., Johnson, K. R., and Wheelock, M. J. (1997) J. Cell Biol. 136, 919–934
32. Redfield, A., Nieman, M. T., and Knudsen, K. A. (1997) J. Cell Biol. 138, 1323–1331
33. Matsunami, H., Miyatani, S., Inoue, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Takeichi, M. (1993) J. Cell Sci. 106, Pt 1, 401–409
34. Falcone, D., and Andrews, D. W. (1991) Mol. Cell. Biol. 11, 2656–2666
35. Ireten, B. C., Davis, M. A., van Hengel, J., Mariner, D. J., Barnes, K., Thoreson, M. A., Anastasiadis, P. Z., Matrisian, L., Bundy, L. M., Sealy, L., Gilbert, B., van Roy, F., and Reynolds, A. B. (2002) J. Cell Biol. 159, 465–476
36. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) J. Biol. Chem. 274, 13196–13204
37. Grigioni, F., Kinnella, T., Mencarelli, A., Valtieri, M., Riganelli, D., Lanfrancone, L., Peschle, C., Nolan, G. P., and Pelicci, P. G. (1998) Cancer Res. 58, 14–19
38. Laemml, U. K. (1970) Nature 227, 680–685
39. O’Conner, K. L., Nguyen, B. K., and Mercurio, A. M. (2000) J. Cell Biol. 148, 253–258
40. Johnson, K. R., Lewis, J. E., Li, D., Wahl, J., Soler, A. P., Knudsen, K. A., and Wheelock, M. J. (1993) Exp. Cell Res. 207, 252–260
41. Sacco, P. A., McGranahan, T. M., Wheelock, M. J., and Johnson, K. R. (1995) J. Biol. Chem. 270, 21921–21926
42. Wahl, J. K., 3rd, Kim, J. B., Cullen, J. M., Johnson, K. R., and Wheelock, M. J. (2003) J. Biol. Chem. 278, 17269–17276
43. Nieman, M. T., Kim, J. B., Johnson, K. R., and Wheelock, M. J. (1999) J. Cell Sci. 112, Pt 10, 1621–1632
44. Noren, N. K., Arthur, W. T., and Burridge, K. (2003) J. Biol. Chem. 278, 13615–13618
45. Kiemene-Manneville, S., and Hall, A. (2002) Nature 420, 629–635
46. Thiery, J. P. (2002) Nat. Rev. Cancer 2, 444–454
47. Bissell, M. (2001) Crit. Care Nurs. Q. 24, 39–43
48. Wang, A. S., Maine-Bandiera, S. L., Rosen, B., Wheelock, M. J., Johnson, K. R., Leung, P. C., Roskelley, C. D., and Auerberg, N. (1999) Int. J. Cancer 81, 180–188
49. Tomita, K., van Bokhoven, A., van Leenders, G. J., Ruijter, E. T., Jansen, C. F., Bussemakers, M. J., and Schalken, J. A. (2000) Cancer Res. 60, 3650–3654
50. Frizen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, R., Warda, A., Lodner, D., and Birchmeier, W. (1991) J. Cell Biol. 113, 173–185
51. Chen, W. C., and Obrink, B. (1991) J. Cell Biol. 114, 319–327
52. Hazan, R. B., Phillips, G. R., Qiao, B. P., Norton, L., and Aaronson, S. A. (2000) J. Cell Biol. 148, 779–790
53. Suyama, K., Shapiro, I., Guttman, M., and Hazan, R. B. (2002) Cancer Cell 2, 301–314
54. Hall, A. (1998) Science 279, 509–514
55. Navarro, P., Ruco, L., and Dejana, E. (1998) J. Cell Biol. 140, 1475–1484
Additions and Corrections

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MalK, the ATP-binding cassette component of the *Escherichia coli* maltodextrin transporter, inhibits the transcriptional activator MalT by antagonizing inducer binding.

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Page 33124, lines 7 and 8 under “Experimental Procedures”: The name and sequence of the upstream primer used to amplify the *malK* gene, rather than KU001 (5′-GCCGCGCCATGGGGACCCACGATCAGGTCGA-3′, should be: KU006 (5′-CGC-CCGCGCCATGGGGATGGCGAGCGTACAGCTGC-3′).

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Binding specificity of sea anemone toxins to Na$_v$ 1.1–1.6 sodium channels. Unexpected contributions from differences in the IV/S3–S4 outer loop.

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Page 33323, line 10 of the summary: L36A should be changed to K36A.

Page 33331, next to the last line in the left-hand column: The first Asp-Ile should be changed to Asp-Leu, to read “The Asp-Leu instead of the Asp-Ile motif . . .”

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
R-cadherin influences cell motility via Rho family GTPases.

Emhonta Johnson, Christopher S. Theisen, Keith R. Johnson, and Margaret J. Wheelock

Page 31046: The wrong Fig. 5 was printed. The correct figure is shown below:

**FIG. 5**