Polarized trafficking of E-cadherin is regulated by Rac1 and Cdc42 in Madin-Darby canine kidney cells

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Wang, Bo, Fiona G. Wylie, Rohan D. Teasdale, and Jennifer L. Stow. Polarized trafficking of E-cadherin is regulated by Rac1 and Cdc42 in Madin-Darby canine kidney cells. Am J Physiol Cell Physiol 288: C1411–C1419, 2005. First published February 2, 2005; doi:10.1152/ajpcell.00533.2004.—E-cadherin is a major cell-cell adhesion protein of epithelia that is trafficked to the basolateral cell surface in a polarized fashion. The exact post-Golgi route and regulation of E-cadherin transport have not been fully described. The Rho GTPases Cdc42 and Rac1 have been implicated in many cell functions, including the exocytic trafficking of other proteins in polarized epithelial cells. These Rho family proteins are also associated with the cadherin-catenin complexes at the cell surface. We have used functional mutants of Rac1 and Cdc42 and inactivating toxins to demonstrate specific roles for both Cdc42 and Rac1 in the post-Golgi transport of E-cadherin. Dominant-negative mutants of Cdc42 and Rac1 accumulate E-cadherin at a distinct post-Golgi step. This accumulation occurs before p120ctn interacts with E-cadherin, because p120ctn localization was not affected by the Cdc42 or Rac1 mutants. Moreover, the GTPase mutants had no effect on the trafficking of a targeting mutant of E-cadherin, consistent with the selective involvement of Cdc42 and Rac1 in basolateral trafficking. These results provide a new example of Rho GTPase regulation of basolateral trafficking and demonstrate novel roles for Cdc42 and Rac1 in the post-Golgi transport of E-cadherin.

Rho family GTPases; catenin; polarity; sorting; actin

E-CADHERIN IS THE PROTOTYPICAL member of the classic cadherin family and functions as a major cell-cell adhesion molecule in epithelia (2). E-cadherin has important roles in tissue patterning during development and is a powerful tumor suppressor in adult tissues (6, 21, 46). At the adherens junction, domains at the COOH terminus and in the juxtamembrane region of the cytoplasmic tail of E-cadherin support interactions with β-catenin/plakoglobin and with p120ctn, respectively (19). In turn, these catenins provide for indirect interactions with α-catenin to link the cadherin complex to the actin cytoskeleton and to a variety of signaling proteins (39).

The dynamic trafficking of E-cadherin to and from the lateral surface of epithelial cells is essential to initially deliver newly synthesized E-cadherin to the adherens junction and thereafter to balance and modulate cadherin-based adhesion. It is now widely recognized that cell surface cadherins can be internalized constitutively and recycled in confluent epithelia or endocytosed in response to growth factors via different endocytic carriers and pathways (3, 33). The exocytosis of newly synthesized E-cadherin requires sorting and polarized transport to the basolateral membrane in epithelial cells. We have shown that this trafficking of E-cadherin is mediated by a dileucine motif in the juxtamembrane tail domain and that a mutant (E-cadΔS1) lacking this motif is mistargeted to the apical membrane in Madin-Darby canine kidney (MDCK) cells (36, 37). Other events documented during the exocytic trafficking of E-cadherin include the sequential assembly of the E-cadherin-catenin complex. While β-catenin appears to bind to E-cadherin early in the biosynthetic pathway, p120ctn binds to the complex more distally, at or near the basolateral membrane in MDCK cells (8, 36). The trafficking of N-cadherin involves earlier binding of p120ctn and an interaction via p120ctn with kinesin and microtubules during transport to the cell surface (7, 47).

Other regulatory molecules involved in the sorting or basolateral transport of E-cadherin have not yet been defined. However, relevant insights have emerged from studies on the trafficking of other basolateral membrane proteins. Some proteins that are sorted via tyrosine-based motifs for basolateral trafficking interact with a specific adaptor protein (AP) complex, AP-1B, of which the μ1B-subunit is expressed only in polarized epithelial cells (13). The exocyst is a multisubunit complex of vesicle and target membrane-associated proteins with essential roles in polarized secretion in yeast (22) and in basolateral exocytic trafficking in epithelial cells, where it defines the apicolateral point of delivery for vesicle carriers moving to the lateral cell membrane (20, 34). Other proteins that interact with the exocyst complex, including RalA (44) and Rho GTPases, also have been shown to regulate basolateral trafficking (35).

The Rho family proteins are monomeric G proteins that are involved in signaling pathways throughout eukaryotic cells and often control actin polymerization in diverse contexts (12). Two members of this family, Cdc42 and Rac1, often have complementary functions and common effectors, and both have been implicated in regulating exocytic and endocytic trafficking pathways (43). Cdc42 and Rac1 also contribute to the establishment of cell polarity as reported in developing wing epithelia in Drosophila (10). Multiple lines of evidence, including activation of endogenous Cdc42, show that Cdc42 is associated with membranes and vesicles at the trans-Golgi network (TGN) (11, 14, 40). In addition, Cdc42 participates in regulating post-Golgi trafficking to the basolateral cell surface in polarized epithelial cells (9, 32). Dominant-negative mutants of Cdc42 disrupt polarity in epithelial cells, sending basolateral proteins to the apical membrane (32).

On the basis of the known functions of Cdc42 and Rac1 in polarized protein trafficking, we set out to investigate specific
roles for these GTPases in the basolateral trafficking of E-cadherin. An additional impetus for this study was the known function of Cdc42 and Rac1 in regulating the function and trafficking of E-cadherin once it reaches the cell surface. At the cell surface, RhoA, Rac1, and Cdc42 each act to directly regulate components of the cadherin-catenin complex to modulate cadherin-based adhesion and signaling (16). Rac1 has been shown to regulate the endocytosis of E-cadherin, specifically functioning to make nonadhesive E-cadherin available for internalization (27). While there is strong evidence linking Rho GTPases to E-cadherin at the cell surface, the participation of Cdc42 or Rac1 at an earlier stage during the biosynthesis or exocytic trafficking of E-cadherin has not been demonstrated. Our results show that both Rac1 and Cdc42 are required for the efficient post-Golgi sorting of E-cadherin and its delivery to the lateral cell surface. These findings show that Rac1 and Cdc42 are involved in determining the fate of E-cadherin at a much earlier stage than previously suspected.

MATERIALS AND METHODS

Cell culture. MDCK cells were grown and passaged in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (FCS) and 2% glutamine in 5% CO2 and 95% air (37). Cells were plated at subconfluent density on glass coverslips and used 24–48 h later. In some experiments, MDCK cells were incubated in medium containing cytochalasin D (10 μM; Sigma Chemical, St. Louis, MO) to depolymerize actin or Clostridium Chemical Research), Saitama, Japan) to inactivate Cdc42/Rac1 GTPases (48). MDCK cells expressing dominant-negative c-myc-tagged Rac1N17 under the control of a tetracycline-repressible transactivator (Tet-Off RacDN cells) were maintained in DMEM containing doxycycline (1 μg/ml), which was removed 24–48 h before use to induce expression of Rac1DN. Chinese hamster ovary (CHO) cells stably expressing full-length human E-cadherin (hECHO) (31) were grown and passaged in Ham’s F-12 medium (BioWhittaker, Australia) containing 10% FCS and 2% glutamine in 5% CO2 and 95% air. The hECHO cells were plated onto glass coverslips 24 h before use.

Plasmids. An EGFP vector (Clontech, Palo Alto, CA) encoding full-length human E-cadherin tagged with green fluorescent protein (GFP) at the COOH terminus was used as described previously (37). A mutant of this cDNA with the diencephal motif at amino acids 587 and 588 mutagenized to a dialanine motif (E-cadΔ51-GFP) also was used. This mutation results in the mistargeting of E-cadherin to the apical plasma membrane in polarized MDCK cells (36). Rac1 and Cdc42 cDNA were originally obtained from the laboratory of Dr. Alan Hall (Medical Research Council Laboratory of Cell Biology, London, UK). pcDNA3 expression vectors encoding wild-type Rac1 and Cdc42, dominant-active Rac1 (RacV12 mutation RacCA), constitutively active Cdc42 (Cdc42G12V mutation Cdc42CA) and dominant-negative forms of Rac1 (RacN17 mutation RacDN) and Cdc42 (Cdc42D57Y mutation Cdc42DN) tagged with c-myc were either transiently transfected or microinjected into MDCK cells. A pTet-Off plasmid (Clontech) was constructed to express tetracycline-inducible GFP and tetracycline-repressible c-myc-tagged Rac1DN bidirectionally and was kindly provided by Brooke Gardner and Richard Sturm (University of Queensland, Brisbane, Australia). This plasmid was used to generate stably expressing MDCK cells.

Transfection. MDCK cells were plated at 20–40% confluence onto glass coverslips 24 h before transfection with 2 μg of cDNA and Lipofectamin Plus, according to the manufacturer’s instructions (GIBCO-BRL, San Diego, CA). Briefly, cells were transfected in serum-free media and incubated for 3 h at 37°C in 5% CO2, followed by the addition of an equal volume of normal medium containing 20% FCS for 16–18 h (10% final serum concentration). The DNA-containing medium was then removed and replaced with standard growth medium. Cells were used for experiments 24–48 h after transfection.

Microinjection. Plasmids encoding cDNA of interest were diluted to a final concentration of 100 ng/μl in a microinjection buffer of 10 mM KH2PO4, pH 7.2, containing 75 mM KCl, and centrifuged for 15 min in a benchtop microfuge (Eppendorf, Hamburg, Germany) at 14,000 rpm. To remove aggregates, the cleared supernatants were loaded into microinjection syringes. Microinjection needles were pulled from thin-wall borosilicate glass capillaries. MDCK cells grown on coverslips were placed in CO2-independent medium (GIBCO-BRL) for injection. An Eppendorf Femtotjet microinjection apparatus mounted on a Zeiss Axiovert 100 microscope was used to inject ~200 cells per condition. In some experiments, two different cDNA were injected sequentially into the same cell nuclei by preinjecting MDCK cells with the mutant Rac1 or Cdc42 cDNA followed 5 h later by microinjection with E-cad-GFP cDNA. The sequentially injected cells were fixed and stained after 24 h when the cells had reached confluence.

Antibodies. The following primary antibodies were used: a mouse monoclonal antibody, HECD1, raised to the human E-cadherin ectodomain [provided by Alpha Yap, University of Queensland, with the permission of Dr. M. Takeichi, RIKEN (The Institute of Physical and Chemical Research), Saitama, Japan]; a rabbit polyclonal anti-E-cadherin (provided by Alpha Yap, University of Queensland); mouse monoclonal antibodies specific for p120ctn, the cis-medial Golgi-resident protein, GM130, and the Golgin family member p230 (Transduction Laboratories, Lexington, KY), which is a TGN protein; a mouse monoclonal antibody raised against lysobisphosphatidic acid (LBPA; provided by Sally Martin, University of Queensland, with permission of Jean Gruenberg, University of Geneva); and the 9E10 hybridoma cell line, which was harvested to produce monoclonal antibodies against the c-myc epitope tag. Alexa 488-conjugated phallolidin was used to stain F-actin (Molecular Probes, Eugene, OR). C3-4-conjugated sheep anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), and Alexa-488-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Molecular Probes.

Immunofluorescence. Cells grown on coverslips were fixed in 4% paraformaldehyde, permeabilized using 0.1% Triton X-100, and incubated in a blocking buffer of 0.5% bovine serum albumin in PBS, pH 7.4. Primary and secondary antibodies were diluted in blocking buffer, and the cells were incubated for 1–2 h at room temperature. Coverslips were mounted on glass microscope slides using either 90% glycerol-PBS containing 25 mg/ml 1,4-diazabicyclo[2.2.2]-octane or 50% glycerol-PBS containing 1% N-propyl gallate. Cells were viewed using epifluorescence on an Olympus Provis AX-70 microscope, and images were captured using a charge-coupled device 300ET-RCX camera (DAGE-MTI, Michigan City, IN), and analyzed using Adobe Photoshop software.

RESULTS

Overexpression of Rac1 or Cdc42 mutants disrupts post-Golgi trafficking of E-cad-GFP. E-cadherin and its tagged counterpart, E-cad-GFP, are sorted and trafficked in a polarized fashion to the basolateral membrane in epithelial cells (36). The localization of E-cad-GFP is similar to that of endogenous E-cadherin when expressed in both preconfluent cells and confluent MDCK monolayers (37). Newly synthesized E-cad-GFP appears first at the perinuclear Golgi complex, from which it is transported to the cell surface (33). Cdc42 has been implicated in post-Golgi trafficking in polarized cells (9, 32). Our study investigated whether Cdc42 and the closely related GTPase, Rac1, are also involved in the post-Golgi trafficking of E-cad-GFP by coexpressing Cdc42 or Rac1 mutants in
MDCK cells. Cells were cotransfected with E-cad-GFP and the epitope-tagged cDNA encoding either dominant-negative (RacDN, Cdc42DN) or constitutively active (RacCA, Cdc42CA) GTPases. After transfection, the cells were immunostained for c-myc to visualize the Rac1 expression 48 h after transfection. Coexpression of both GTPase mutant proteins caused reduced surface staining and an intracellular accumulation of E-cad-GFP. Mock-transfected cells were cotransfected with E-cad-GFP and empty vector (pcDNA3), and in these cells, newly synthesized E-cad-GFP was efficiently transported to the basolateral plasma membrane (Fig. 1A).

The overexpression of Rac1 mutants changed the distribution of E-cad-GFP by causing a marked accumulation of E-cad-GFP inside the cells and concomitantly reducing plasma membrane staining compared with mock-transfected cells or surrounding untransfected cells (Fig. 1A). Intracellular E-cad-GFP was concentrated in the perinuclear Golgi region and often appeared as large vesicular structures. In the case of

Fig. 1. A: Rac1 mutants disrupt the trafficking of E-cad-green fluorescent protein (GFP). E-cad-GFP was coexpressed by transfection in Madin-Darby canine kidney (MDCK) cells with DN and CA mutants of Rac1. Empty pcDNA3 vector (Mock) was cotransfected with E-cad-GFP as a control. The cells were immunostained for c-myc to visualize the Rac1 expression 48 h after transfection. Coexpression of both GTPase mutant proteins caused reduced surface staining and an intracellular accumulation of E-cad-GFP. B: Tet-inducible MDCK cells stably expressing RacDN were immunostained for endogenous E-cadherin and costained for c-myc and E-cadherin in the absence of doxycycline. In the presence of doxycycline, the transfected cells showed GFP expression with E-cadherin staining predominantly at the cell surface. Two days after the doxycycline was removed, the cells now expressed RacDN and the costaining of E-cadherin showed an intracellular accumulation with reduced staining at the cell surface. C: Cdc42 mutants disrupt the trafficking of E-cad-GFP. E-cad-GFP was cotransfected into MDCK cells with Cdc42DN and Cdc42CA mutants. After 48 h, the cells were immunostained for c-myc. The coexpressed Cdc42 mutants reduced membrane surface staining and caused an intracellular accumulation of E-cad-GFP. Scale bar, 10 μm.

Fig. 2. Sequential injection and expression of E-cad-GFP and Rac1 or Cdc42 mutants A: Rac1 or Cdc42 mutants were microinjected into MDCK cells, followed 5 h later by injection of E-cad-GFP. Cells were subsequently fixed and stained with a c-myc antibody to visualize the expressed Rac1 and Cdc42 mutants. In the RacDN- and Cdc42DN-preinjected cells, E-cad-GFP transport was blocked at the perinuclear Golgi region with little or no surface GFP visible. In the cells preinjected with RacCA and Cdc42CA, E-cad-GFP was present at the plasma membrane, but at a reduced level compared with controls, and a perinuclear accumulation of GFP signal was again observed. B: relative proportion of E-cad-GFP intracellular and surface staining in sequentially injected MDCK cells. The staining patterns of 50 cells from each condition were quantified using image analysis to measure fluorescence intensities. The results highlight the blocking effect of the Rac1 or Cdc42 activation mutants on the post-Golgi trafficking of E-cad-GFP. The most complete blocks resulted from expression of the DN mutants for both G proteins. Scale bar, 10 μm.
RacCA overexpression, the accumulation of intracellular E-cad-GFP was more diffuse (Fig. 1A). Thus overexpression of the RacDN and RacCA mutants by transient transfection caused an intracellular accumulation of E-cad-GFP, with an accompanying reduction of E-cad-GFP at the plasma membrane. MDCK cells from a cell line expressing RacDN under a repressible promoter (Tet-Off RacDN cells) were fixed and stained before and after induction of RacDN expression. Staining of endogenous E-cadherin in these cells appeared mostly at the cell surface before expression of RacDN, but intracellular accumulation of E-cadherin was observed in cells after induction of RacDN expression (Fig. 1B). Thus, under two transfection regimes, Rac1 mutants induced intracellular accumulation of endogenous and recombinant E-cadherin. Similar experiments were performed with plasmids encoding Cdc42DN and Cdc42CA, which were expressed in transiently transfected cells and were found to partially localize in the perinuclear Golgi region, which is consistent with previous studies showing association of Cdc42 with Golgi membranes (11). E-cad-GFP was also accumulated over the Golgi region in cells coexpressing Cdc42DN or Cdc42CA (Fig. 1C). Plasma membrane staining of E-cad-GFP was also reduced in these cells, but not as markedly as in cells overexpressing Rac1 mutants. Overexpression of wild-type Rac1 or Cdc42 produced an effect similar to, but less marked than, their constitutively active mutants (data not shown). The intracellular accumulation of E-cad-GFP with concomitant reduction in cell surface staining is suggestive of Rac1 and Cdc42 affecting post-Golgi transport.

In these experiments, overexpression of Rac1 and Cdc42 mutants by transfection accumulated some but not all of the E-cad-GFP intracellularly, possibly as a result of these GTPase mutants having only a partial or delayed effect on the trafficking of E-cad-GFP. Therefore, as an alternative approach, cells were sequentially microinjected to first express Rac1 or Cdc42 mutant cDNA, followed by injection of the E-cad-GFP cDNA at a later time. This ensured that E-cad-GFP was being made and trafficked in a preexisting environment of recombinant G protein expression (Fig. 2). In cells preexpressing RacDN, there was no surface staining of E-cad-GFP, while faint staining of the plasma membrane was still observed in cells injected with RacCA. In both cases, the majority of E-cad-GFP accumulated inside the cells was localized to large vesicular structures that were concentrated in the perinuclear region (Fig. 2A). Cdc42DN and Cdc42CA caused a near-complete absence of surface staining of E-cad-GFP in these sequential injection-expression experiments with an accumulation of E-cad-GFP inside cells (Fig. 2A). Semiquantitative image analysis of intracellular E-cad-GFP fluorescence vs. cell surface fluorescence indicated that the dominant-negative forms of Rac1 and Cdc42 had the strongest apparent effects on E-cadherin trafficking, while constitutively active Rac1 and Cdc42 were less effective in disrupting E-cad-GFP localization (Fig. 2B). Taken
E-cad-GFP trafficking.

Reduced cell surface staining, again consistent with an effect on E-cad-GFP trafficking.

E-cad-GFP accumulates in a juxta-Golgi compartment. In cells overexpressing Rac1 or Cdc42 mutants, E-cad-GFP appears to accumulate in structures at or near the location of the Golgi complex. To assess this hypothesis, cotransfected cells were also stained with antibodies specific for Golgi-associated proteins GM130 and p230 to label cis-medial Golgi and TGN, respectively (Fig. 3). Transfected cells were readily identified by their characteristic accumulation of E-cad-GFP. RacDN and RacCA overexpression accumulated E-cad-GFP in large vesicular structures that overlapped significantly with the staining of GM130 but also extended beyond the stained Golgi complex; this was particularly apparent with regard to the dispersed E-cad-GFP structures in RacDN-transfected cells. In cells overexpressing Cdc42CA or Cdc42DN, the accumulated E-cad-GFP was concentrated in structures closely overlapping with GM130 staining, suggesting that E-cad-GFP was blocked totally in the region of the Golgi complex (Fig. 3A). Similar results were obtained in MDCK cells coexpressing E-cad-GFP and Cdc42DN or RacDN, and stained for p230, a member of the Golgin family of proteins, which have been localized to the TGN (18). In these cells, the accumulated E-cad-GFP significantly overlapped with the region stained by p230. Similarly to the GM130-stained cells, E-cad-GFP did not show complete colocalization with p230 and the overlap was more apparent in the cells overexpressing Cdc42DN than in those expressing RacDN (Fig. 3B). Overexpression of these G proteins did not significantly affect the structure of the Golgi complex or the TGN as judged by the consistent appearance of GM130 and p230 staining in transfected cells compared with the surrounding untransfected cells. Together, these results confirm that E-cad-GFP is accumulated in structures that are associated with the Golgi complex as well as in more peripheral vesicular bodies.

E-cad-GFP does not accumulate in late endosomes. Given the perinuclear location of the Rac1- or Cdc42-accumulated E-cad-GFP, another potentially involved compartment is the late endosome. Therefore, we stained the coexpressing cells for LBPA, a marker of late endosomes (30) (Fig. 4). LBPA was found in large vesicular compartments in the perinuclear region; however, there was no significant colocalization of E-cad-GFP with LBPA staining in cells coexpressing either RacDN or Cdc42DN. We conclude that E-cad-GFP does not accumulate in late endosomes under the influence of these GTPases.

Toxin-mediated inactivation of G proteins blocks trafficking of endogenous E-cadherin. To verify that the results obtained above using E-cad-GFP are applicable to the trafficking of endogenous E-cadherin, we made use of C. difficile toxin B (TcdB), which inactivates Rho, Rac, and Cdc42 (29). Cells treated with TcdB typically show depolymerization of actin and loss of stress fiber staining as a measure of widespread inactivation of Rho family GTPases (48). Pre- and postconfluent MDCK cells were treated for 6 h with TcdB at a final concentration of 100 ng/ml and then stained with antibodies to detect endogenous E-cadherin and with Alexa-conjugated phalloidin to assess the F-actin morphology (Fig. 5). Preconfluent cells treated with TcdB showed a loss of stress fiber staining and diffuse, less intense phalloidin staining throughout the cell. E-cadherin was concomitantly depleted from the cell surface and accumulated inside the cells (Fig. 5, Aa–Af). In confluent MDCK cells (Fig. 5, Ag–Aa), toxin treatment induced the loss of stress fibers and a distinctive perinuclear accumulation of F-actin (Fig. 5Aa). E-cadherin also accumulated in the same perinuclear region in these cells and was depleted from the cell surface (Fig. 5Ak). Although the TcdB that we used does not discriminate between the Rho family GTPases, these results indicate that inactivation of these small G proteins in MDCK cells causes widespread actin depolymerization and simultaneously interferes with the post-Golgi trafficking of E-cadherin. Increased amounts of E-cadherin accumulated intracellularly in toxin-treated preconfluent cells compared with confluent cells (Fig. 5, Ae and Ak). This finding corresponds to the relative levels of trafficking of newly synthesized E-cadherin in each case and suggests that the toxin could affect exocytic transport. The effect of depolymerized actin on E-cad-GFP trafficking was directly tested by treating cells with...
cytochalasin D. This treatment also resulted in a perinuclear accumulation of E-cad-GFP (Fig. 5B). Thus the inactivation of Rho family GTPases with TcdB or by direct depolymerization of actin mimicked the effect obtained by overexpression of Rac1 and Cdc42 mutants, confirming that Rac1 and Cdc42 regulate the trafficking of both endogenous and recombinant E-cadherin. In addition, these results implicate actin polymerization as one possible mechanism of regulation by Rho GTPases.

Cdc42 and Rac1 relocate basolaterally directed E-cad-GFP, but not a missorted mutant. We previously identified and used a sorting mutant of E-cadherin, E-cadΔS1-GFP, to study the polarized trafficking of E-cadherin (36, 37). Absence of a critical dileucine motif in the cytoplasmic tail results in mis-sorting of E-cadΔS1-GFP to both the apical and basolateral membranes of MDCK cells (37). To examine the possible trafficking routes affected by Rac1 and Cdc42, we cotransfected MDCK cells with RacDN or Cdc42DN and E-cadΔS1-GFP. There was no accumulation of the E-cadΔS1-GFP in cells coexpressing either RacDN or Cdc42DN (Fig. 6) under the same conditions that block trafficking of E-cad-GFP (see Fig. 1). In both control (E-cadΔS1-GFP alone) and cotransfected cells, E-cadΔS1-GFP was efficiently delivered to the cell surface. The cells overexpressing both a G protein and E-cadΔS1-GFP also lose their polarized phenotype, making it difficult to distinguish the apical and basolateral domains of these MDCK cells. However, because neither RacDN nor Cdc42DN affected the trafficking of E-cadΔS1-GFP, we conclude that the wild-type and dileucine-mutated forms of E-cadherin travel to the cell surface via different or diverging routes, with only basolateral transport being affected by Cdc42 and Rac1.

Rac1 and Cdc42 accumulate E-cadherin trafficking before the binding of p120ctn. The E-cadherin-catenin complex is assembled by sequential binding of catenins to the complex during trafficking. Our studies using E-cadΔS1-GFP suggest that p120ctn does not bind to the cadherin complex until after

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Fig. 5. Toxin-mediated inactivation of G proteins. A: MDCK cells were treated with Clostridium difficile toxin B (TcdB) and then fixed and costained with Alexa phalloidin to label F-actin and with 3B8 monoclonal antibody to label endogenous E-cadherin. In both preconfluent (Aa–Af) and confluent (Ag–Al) cells, TcdB caused the cells to change shape and lose their characteristic actin staining pattern; loss of stress fibers and an intracellular accumulation of actin were observed (Aa, Ad, Ag, and Aj). The treated cells also showed reduced surface staining of E-cadherin (Ac and Ai) and an intracellular accumulation that partially overlapped with the intracellular actin staining. B: MDCK cells transiently transfected with E-cad-GFP were stained for F-actin after a 1-h treatment with the actin-depolymerizing drug cytochalasin D. Treated cells showed disruption of the F-actin compared with untreated control cells and a perinuclear accumulation of E-cadherin in cells also overexpressing E-cad-GFP. Scale bar, 10 μm.

Fig. 6. Apical transport of E-cadΔS1-GFP. MDCK cells were cotransfected with RacDN or Cdc42DN and E-cadΔS1-GFP, a mutant of E-cadherin that is missorted to the apical membrane in polarized MDCK cells. Neither of the G protein DN mutants altered the localization of the E-cadΔS1-GFP at the cell surface compared with control cells, which were transfected with E-cadΔS1-GFP alone. This suggests that the block in E-cad-GFP transport caused by the Rac1/Cdc42 mutants occurs before the polarized sorting of E-cadherin. Scale bar, 10 μm.
sorting has occurred and until it is at or near the basolateral membrane (36). Nonpolarized CHO cells endogenously express cytoplasmic p120\textsuperscript{ctn} but not E-cadherin. In a stably transfected CHO cell line, overexpression of hECHO recruits p120\textsuperscript{ctn} to the cell surface (36) (see Fig. 7). hECHO cells were transiently transfected with RacDN and Cdc42DN cDNA and then stained to localize recombinant human E-cadherin and endogenous p120\textsuperscript{ctn} (Fig. 7). Cells expressing RacDN or Cdc42DN were identified on the basis of their altered morphology and the characteristic and dramatic perinuclear accumulation of human E-cadherin. In these coexpressing cells, p120\textsuperscript{ctn} was not coincidentally accumulated with human E-cadherin. The dramatic accumulation of human E-cadherin without a concomitant accumulation of p120\textsuperscript{ctn}, along with other results presented herein, is consistent with Rac1 and Cdc42 mutants interfering with the trafficking of E-cadherin before it complexes with p120\textsuperscript{ctn}, either at or soon after leaving the TGN.

DISCUSSION

In this study, we have examined the potential roles of the Rho GTPases Rac1 and Cdc42 in the trafficking of E-cadherin to the basolateral plasma membrane. Disruption of Rac1 or Cdc42 by overexpression of mutant proteins or by treating cells with TcdB toxin consistently changed the localization of E-cad-GFP or endogenous E-cadherin by causing an intracellular accumulation of both forms of E-cadherin. This effect was confirmed using three different experimental approaches in MDCK cells, by cotransfection and steady-state expression of Rac1 and Cdc42 mutants with E-cad-GFP, by sequential microinjection to overexpress Rac or Cdc42 mutants and E-cad-GFP, and by the inactivation of endogenous Rho GTPases in TcdB-treated cells. Our results are consistent with an effect of both Cdc42 and Rac1 on the trafficking of E-cadherin at a post-Golgi step on its way to the plasma membrane. Additional results suggest that Rac1 and Cdc42 accumulate E-cadherin at or adjacent to the TGN and that this occurs functionally after dileucine-mediated sorting and before binding of p120\textsuperscript{ctn} near the basolateral membrane. Our results thus reveal a new and earlier point at which Rho GTPases could influence cadherin-based adhesion. Regulation of the delivery of E-cadherin to adherens junctions also presents an addition to the known subsequent roles for Rac1 and Cdc42 in mediating attachment of the cadherin complex to the actin cytoskeleton at the adherens junction and during cadherin endocytosis (16).

The effect of overexpressing Rac1 or Cdc42 in this study was to accumulate expressed E-cad-GFP in intracellular vesicular compartments. In previous studies, mutant forms of Cdc42 caused the intracellular accumulation of other basolateral membrane proteins (32, 38). In MDCK cells, dominant-negative forms of Cdc42, but not Rac1, selectively disrupted basolateral exocytic and endocytic trafficking, causing the mistargeting of VSVG protein (32). Furthermore, Cdc42 mutants were found to block the exit of basolateral membrane proteins such as neural cell adhesion molecule and low-density lipoprotein receptor from the TGN without affecting the polarized trafficking of soluble proteins (9, 32, 38). Our present results reveal E-cadherin as another physiologically important basolateral membrane protein whose post-Golgi transport is regulated by Cdc42. Jou and Nelson (28) observed E-cadherin in

![Fig. 7. The trafficking of E-cad-GFP is blocked before p120\textsuperscript{ctn} binding. Chinese hamster ovary (CHO) cells stably expressing full-length human E-cadherin (hECHO) were transfected with RacDN (middle) or Cdc42DN (bottom) and immunolabeled for endogenous p120\textsuperscript{ctn}, which is recruited to the cell surface in cells overexpressing E-cadherin. Untransfected control cells showed clear and overlapping p120\textsuperscript{ctn} and E-cadherin staining on the plasma membrane (top). In cells overexpressing either RacDN or Cdc42DN (arrows), E-cadherin showed the characteristic perinuclear accumulation and reduced cell surface staining, but the p120\textsuperscript{ctn} levels appeared to be downregulated compared with surrounding untransfected cells. This phenotype was more pronounced in the presence of RacDN than in the presence of Cdc42DN. Scale bar, 10 \mu m.](image-url)
intracellular vesicles in MDCK cells expressing Rac1 mutants. In addition, a recent study (41) found that knockdown of Rac1 using small interfering RNA reduced the surface staining of E-cadherin in polarized MDCK cells, although Western blot analysis indicated that total protein levels in the cells were unchanged. Thus our data are consistent with these observations and further show that Rac1 mutants also directly affect the localization and trafficking of E-cadherin. The expression of Cdc42 mutants induces mistargeting of basolateral proteins to the apical membrane in polarized cells (9, 38). In showing that both Cdc42 and Rac1 mutants selectively disrupted the basolateral transport of E-cadherin, but not that of the mis-sorted E-cadΔS1-GFP, our present findings are consistent with a specific role for Rho GTPases in basolateral trafficking (9, 38).

A possible site for Cdc42 and Rac1 to affect E-cadherin trafficking is at or near the TGN on the basis of the partial colocalization of accumulated E-cad-GFP and TGN markers. Interestingly, we consistently noted a difference in E-cad-GFP accumulated in cells expressing RacDN or Cdc42DN. Cdc42DN appears to block E-cad-GFP in a compartment overlapping more with the Golgi complex. This may imply that Cdc42 and Rac1 function in close but sequential steps to affect post-Golgi trafficking of E-cad-GFP. Setting a distal limit for the action of Rac1 and Cdc42 is the binding of p120ctn to the newly synthesized E-cadherin-β-catenin complex, which as we previously established happens only at or near the basolateral membrane (36). In the current experiments, p120ctn was not jointly accumulated intracellularly with E-cadherin, implying that the Rac1 block precedes the E-cadherin-p120ctn association. Our results predict that Cdc42 and Rac1 act at a subcompartment associated with or close to the TGN. The recent literature and our own data (35a) suggest that this may happen at the recycling endosome (1) or, equivalently, at the apical recycling endosome of MDCK cells (24). Ang et al. (1) reported that Cdc42 and Rab8 at the recycling endosome function in the trafficking of basolateral proteins and, moreover, that this occurs only for AP-1B-sorted basolateral proteins. The basolateral sorting of E-cadherin occurs via a dileucine-mediated, AP-1B-independent mechanism, although the specific adaptor complex involved has not been identified (36, 37). Thus a novel finding of the present study is that Cdc42 and Rac1 can regulate AP-1B-independent basolateral trafficking, implicating these Rho GTPases as regulators in more than one basolateral transport pathway. Our findings show that E-cadherin traffics through a Rab11-positive recycling endosome (35a) on its way to the cell surface and suggests this as an additional site for Cdc42 and Rac1 action.

Once at the surface, E-cadherin can be internalized and recycled to the surface or targeted to late endosomes (3). Rho GTPases also act to regulate the function and internalization of E-cadherin at the cell surface (17). In our experiments, the lack of colocalization of E-cad-GFP with LBPA in late endosomes suggests that under these conditions, Rac1 and Cdc42 accumulated E-cad-GFP in an exocytic rather than an endocytic pathway. Also, there is no evidence in these cells of the peripheral early endosome staining of E-cad-GFP typically observed during recycling of E-cadherin in MDCK cells (33). Thus the effects of Rac1 and Cdc42 shown in the present study represent functioning of these GTPases in an exocytic step in addition to their known roles at adherens junctions.

By what mechanisms do Rac and Cdc42 exert their effects on E-cadherin trafficking? Although Rho proteins can interact indirectly with the cadherin-catenin complex at the adherens junction (4), it is more likely that Rac and Cdc42 regulate E-cadherin sorting and trafficking through interactions with other components of the vesicle trafficking machinery. Cdc42, for instance, can interact with components of the exocyst to mediate basolateral trafficking (35) and participate in the Cdc42-Par6-Par3-αPKC complex for polarized trafficking (25, 26). Actin is a common effector for the Rho GTPases and is also involved in post-Golgi trafficking. Vesicle budding at the TGN involves actin and myosins (5, 45). There are biochemically distinct pools of actin associated with the Golgi complex (15) and short microfilaments demarked by isoforms of the Tm5 tropomyosin gene associated with transport vesicles budding off the TGN (23, 42). Interestingly, dynamic G-actin-rich microfilaments concentrated around the Golgi area were found to disappear in cells expressing activated Cdc42 (38). Future investigations will focus on whether and how Cdc42 and Rac1 work through the polymerization of actin to regulate steps in adaptor binding or vesicle formation at the recycling endosome, with the effect of controlling the basolateral transport and cell surface delivery of E-cadherin.

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