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Cdc42-dependent Modulation of Tight Junctions and Membrane Protein Traffic in Polarized Madin-Darby Canine Kidney Cells

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Polarized epithelial cells maintain the asymmetric composition of their apical and basolateral membrane domains by at least two different processes. These include the regulated trafficking of macromolecules from the biosynthetic and endocytic pathway to the appropriate membrane domain and the ability of the tight junction to prevent free mixing of membrane domain-specific proteins and lipids. Cdc42, a Rho family GTPase, is known to govern cellular polarity and membrane traffic in several cell types. We examined whether this protein regulated tight junction function in Madin-Darby canine kidney cells and pathways that direct proteins to the apical and basolateral surface of these cells. We used Madin-Darby canine kidney cells that expressed dominant-active or dominant-negative mutants of Cdc42 under the control of a tetracycline-repressible system. Here we report that expression of dominant-active Cdc42V12 or dominant-negative Cdc42N17 altered tight junction function. Expression of Cdc42V12 slowed endocytic and biosynthetic traffic, and expression of Cdc42N17 slowed apical endocytosis and basolateral to apical transcytosis but stimulated biosynthetic traffic. These results indicate that Cdc42 may modulate multiple cellular pathways required for the maintenance of epithelial cell polarity.

INTRODUCTION

The normal function of epithelial cells depends on the asymmetrical organization of cellular components such as the plasma membrane, organelles, and the cytoskeleton. The achievement of this cellular polarity is initiated by temporal and spatial cues that induce the formation of specialized cell adhesion complexes (reviewed by Yeaman *et al.*, 1999). Cell adhesion complexes are stabilized, in turn, by assembly of cytoskeletal and signaling proteins at sites of contact, and this subsequently leads to the repositioning of the secretory apparatus. Associated with these global morphological changes is the division of the plasma membrane into two distinct domains termed the apical and basolateral plasma membranes. These are separated by tight junctions that impede the lateral diffusion of lipids and proteins between opposite domains. This membrane asymmetry is reinforced and maintained by sorting and transport of proteins from the *trans*-Golgi network (TGN) to the appropriate membrane domain. Epithelial cells can maintain polarity at the membrane by additional mechanisms (reviewed by Yeaman *et al.*, 1999). One of these entails the internalization of plasma membrane proteins and lipids via endocytosis, followed by the subsequent recycling, degradation in lysosomes, or delivery to the opposite surface domain by transcytosis. An alternative mechanism involves tethering proteins at the plasma membrane. In each of these mechanisms, the cytoskeleton has been shown to play an important role (reviewed by Yeaman *et al.*, 1999).

The Rho family of GTPases modulates multiple membrane traffic events as well as the cytoskeleton and may play important roles in the establishment/maintenance of epithelial cell polarity (Van Aelst and D'Souza-Schorey, 1997; reviewed by Hall, 1998). Members of this family include Cdc42 (Cdc42Hs and G25K splice variants), TC-10, Rac subfamily (1, 2, and 3 isoforms), Rho subfamily (A, B, and C isoforms), Rnd subfamily (Rnd 1, 2, and 3 isoforms), RhoD, RhoE, and RhoG. In yeast, Cdc42 plays an important role in the regulation of actin organization and the polarized formation of growing buds (Adams *et al.*, 1990). In mammalian cells Cdc42 has been implicated in the regulation of a variety of

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Abbreviations used: BSA, bovine serum albumin; DC, doxycycline; EGF, epidermal growth factor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; LCM, low calcium medium; MDCK, Madin-Darby canine kidney cells; MEM, minimal essential medium; PI-3-K, phosphatidylinositol 3-kinase; pIgR, polymeric immunoglobulin receptor; TER, transepithelial resistance; TGN, *trans*-Golgi network; Tf, transferrin.

functions. These include actin rearrangement, receptor-mediated signal transduction pathways, cell cycle progression and apoptosis (reviewed by Johnson, 1999), and multiple membrane trafficking events including phagocytosis, exocytosis, and endocytosis (Cox *et al.*, 1997; Brown *et al.*, 1998; Li *et al.*, 1998; Massol *et al.*, 1998; Gasman *et al.*, 1999; Kroschewski *et al.*, 1999; Garrett *et al.*, 2000; Hong-Geller and Cerione, 2000; Lee *et al.*, 2000; Wu *et al.*, 2000). Furthermore, there is growing evidence that Cdc42 may govern pathways required for establishment and maintenance of cellular polarity (Adams *et al.*, 1990; Stowers *et al.*, 1995; Kroschewski *et al.*, 1999).

A connection between Cdc42 signaling and the establishment/maintenance of epithelial polarity and regulation of membrane trafficking pathways in epithelial cells is only now being explored. Cell-cell contact is the first step in the establishment of epithelial polarity and may be regulated by Cdc42 (Kuroda *et al.*, 1997). In Madin-Darby canine kidney (MDCK) cells expression of dominant-active mutants of Cdc42 alters tight junction morphology (Kroschewski *et al.*, 1999; Joberty *et al.*, 2000), but the effects of these mutants on tight junction function is unknown. In MDCK cells mutated forms of Cdc42 cause missorting of a basolateral resident membrane protein gp58 (Kroschewski *et al.*, 1999) to the apical cell surface. In contrast, the distribution of gp114, an apical membrane protein, is not affected by the expression of a dominant-negative mutant of Cdc42 but becomes nonpolarized in cells expressing a dominant-active mutant of this GTPase. It has been suggested that Cdc42 may control secretory transport to the basolateral membrane of MDCK cells, perhaps by regulating traffic from the Golgi apparatus (Kroschewski *et al.*, 1999). It remains to be determined whether Cdc42 plays a role in regulating other membrane trafficking events in epithelial cells.

We have investigated whether Cdc42 modulates the gate and fence function of tight junctions in MDCK cells, as well as endocytic and biosynthetic pathways that direct proteins to the apical and basolateral plasma membrane domains of this cell line. We used MDCK cells that express constitutively active or dominant-negative mutants of Cdc42 under the control of the tetracycline-repressible system. There are two distinct advantages of this system. First, the expression of the exogenous protein can be regulated by varying the level of tetracycline. Second, the generation of stable cell lines with uniform expression makes quantitative biochemical experiments possible. Here we report that expression of dominant-active Cdc42V12 or dominant-negative Cdc42N17 perturbed tight junction function. In addition, Cdc42V12 expression slowed endocytic and biosynthetic traffic. The dominant-negative mutant of Cdc42 (Cdc42N17) slowed apical endocytosis and basolateral to apical transcytosis but stimulated biosynthetic traffic. Our results indicate that Cdc42 may govern multiple pathways that are important in the establishment and maintenance of cell polarity.

MATERIALS AND METHODS

Generation of Cell Lines Expressing Wild-type and Mutant Cdc42

The myc epitope-tagged Cdc42V12 and Cdc42N17 sequences were amplified by polymerase chain reaction from the template plasmids pCMVneo-myc-Cdc42V12 and pCMVneo-myc-Cdc42N17 (courtesy of Dr. Matt Hart and Dr. Arie Abo at Onyx Pharmaceuticals, Richmond, CA) with the use of the forward primer CCGAATTCACCATGGAGCAGAAGCTGATC, which matches the codon sequence of the first six amino acids sequence of myc epitope with an upstream *Eco*RI site (which is underlined), and the reverse primer CGTCTAGAGGGTACCATGCATGATATCGC, which matches the 3′ multiple cloning sites sequence of pCMVneo-myc with a downstream *Xba*I site (which is underlined). The amplified fragments were then digested with *Eco*RI and *Xba*I and gel purified before being inserted into the *Eco*RI and *Xba*I sites of pUHD10–3 (courtesy of Dr. Manfred Gossen and Dr. Hermann Bujard at University of Heidelberg, Heidelberg, Germany) to generate pUMCdc42V12 and pUMCdc42N17. Stable MDCK cells expressing Cdc42 mutant GTPases were generated with the use of a tetracycline-repressible system as described previously (Jou and Nelson, 1998). In brief, parental T23 MDCK cells, which express a tetracycline-regulated transactivator, were cotransfected with pUMCdc42V12 or pUMCdc42N17 and pHMR272, which carries a hygromycin B selection marker. The transfected cells were selected in the presence of 200 μg/ml hygromycin B and 20 ng/ml doxycycline (DC) for 10 d before surviving clones were picked. Screening was performed on cells that were grown in the absence of DC, and expression of Cdc42 mutant proteins was assessed by immunoblotting or immunofluorescence with the use of a monoclonal antibody specific for the myc epitope tag (9E10). The data presented are from an individual, representative clone that uniformly expressed high levels of either myc-Cdc42V12 or myc-Cdc42N19 when cells were cultured in the absence of DC.

Cell Culture

The cell lines were routinely cultured in DMEM medium (Life Technologies-BRL, Gaithersburg, MD) containing 10% (vol/vol) fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin/streptomycin, and 20 ng/ml DC at 37°C in a humidified atmosphere containing 5% CO_2. Expression of Cdc42V12 or Cdc42N17 was performed as described previously (Leung *et al.*, 1999). Briefly, mutant Cdc42 protein expression was induced by plating cells in 15-cm dishes at low density into DMEM/FBS medium lacking DC and incubating them for 36–48 h at 37°C. At the end of this incubation period the cells had reached ~30% confluency. Cells treated in an identical manner, but incubated in the presence of 20 ng/ml DC, served as controls. The cells were trypsinized and washed with culture medium containing 5 μM calcium chloride (low calcium medium [LCM]), and 1×10^6 cells (resuspended in 0.5 ml of LCM) were added to the apical chamber of rat-tail collagen-coated 12-mm diameter Transwells (Corning-Costar, Cambridge, MA) in LCM containing either 20 ng/ml DC (control) or no DC. Cells were incubated in LCM for 3–4 h (±DC), rinsed twice with phosphate-buffered saline, and then incubated in DMEM/FBS containing either 20 ng/ml DC (control) or no DC and normal amounts of calcium chloride (1.8 mM) for 18–48 h.

Antibodies, Proteins, and Other Markers

The following reagents were used: mouse anti-myc hybridoma 9E10 ascites (Dr. S.W. Whiteheart, University of Kentucky, Lexington, KY), rat anti-ZO-1 hybridoma R40.76 culture supernatant (Dr. D.A. Goodenough, Harvard University, Cambridge, MA), purified rabbit anti-Cdc42 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), affinity-purified rabbit polyclonal anti-human immunoglobulin (Ig) A antibody (Jackson Immunoresearch Laboratories, West Grove, PA), affinity-purified rabbit polyclonal anti-giantin antibody (Dr. A. Lindstedt, Carnegie Mellon University, Pittsburgh, PA; Lindstedt and Hauri, 1993), affinity-purified rabbit anti-canine Tf antibodies (Apodaca *et al.*, 1994), mouse anti-LAMP2 hybridoma AC17 (Dr. E. Rodriquez-Boulain, Cornell University, New York, NY; Nabi *et al.*, 1991), rabbit polyclonal anti-occludin antibody (Zymed Laboratories, South San Francisco, CA), rabbit polyclonal anti-mouse furin antibody (Alexis Biochemicals, San Diego, CA),

affinity-purified and minimal cross-reacting fluorescein and Cy5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories), and human polymeric IgA (purchased from Dr. J.-P. Vaerman, Catholic University of Louvain, Louvain, Belgium).

Western Blot Analysis

Western blot analysis was performed as described previously (Maples *et al.*, 1997). Samples were quantified by densitometry.

Immunofluorescence Labeling and Scanning-Laser Confocal Microscopy

Cells were fixed and processed with the use of a pH shift protocol (Apodaca *et al.*, 1994). Imaging was performed on a TCS confocal microscope equipped with krypton, argon, and helium-neon lasers (Leica, Dearfield, IL). Images were acquired with the use of a 100× plan-apochromat objective (NA 1.4) and the appropriate filter combination. Settings were as follows: photomultipliers set to 600–800 mV, 1.0-μm pinhole, zoom = 2.0–3.0, Kalman filter (n = 4). The images (1024 × 1024 pixels) were saved in a tag-information-file-format (TIFF), and the contrast levels of the images were adjusted in the Photoshop program (Adobe, Mountain View, CA) on a Power PC G-4 Macintosh (Apple, Cupertino, CA). The contrast-corrected images were imported into Freehand (Macromedia, San Francisco, CA) and printed from a Kodak (Rochester, NY) 8650PS dye sublimation printer.

Measurement of Trans-Epithelial Resistance (TER), Paracellular Diffusion of [^{14}C]Inulin, ^{125}I-IgA, and ^{125}I-Transferrin (Tf)

TER was quantified and [^{14}C]inulin flux was measured as described previously (Apodaca *et al.*, 1995; Jou and Nelson, 1998). The flux of ^{125}I-IgA or ^{125}I-Tf was quantified as follows. Filter-grown cells were washed three times with minimal essential medium (MEM)/bovine serum albumin (BSA; MEM containing 0.6% [wt/vol] BSA, 0.35 g/l $NaHCO_3$, 20 mM HEPES, pH 7.4, penicillin/streptomycin), and ^{125}I-IgA or ^{125}I-Tf, diluted in 0.5 ml of MEM/BSA containing a 100-fold excess of unlabeled IgA or Tf, was added to the apical chamber of the Transwell. Cold-competing ligand was added to prevent receptor-mediated internalization and apical to basolateral transcytosis. MEM/BSA (0.5 ml) was placed in the basolateral chamber. At the designated times, the basolateral MEM/BSA medium (0.5 ml) was collected and replaced with fresh MEM/BSA. After the final time point, filters were washed twice with ice-cold phosphate-buffered saline and cut out of the insert, and the amount of ^{125}I-IgA or ^{125}I-Tf that remained in the apical medium, that had diffused into the basolateral chamber, or that remained cell associated was quantified in a gamma counter. The results are expressed as the percentages of ^{125}I-IgA or ^{125}I-Tf initially added to the apical chamber that was released basolaterally.

Electron Microscopic Analysis of Cells Expressing Cdc42 Mutants

The cells were incubated for 60 min at room temperature in fixative containing 2% (vol/vol) gluteraldehyde, 2% (wt/vol) paraformaldehyde, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, in 100 mM sodium cacodylate buffer, pH 7.4. Cells were rinsed three times with 100 mM sodium cacodylate buffer, pH 7.4, and then treated with 1% (wt/vol) OsO_4, 1% (wt/vol) $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in 100 mM sodium cacodylate, pH 7.4, for 90 min at 4°C. After several rinses with H_2O the samples were incubated in 1% (wt/vol) aqueous tannic acid for 5 min, rinsed with H_2O, and then en bloc stained overnight with 0.5% (wt/vol) uranyl acetate in H_2O. Filters were dehydrated in a graded series of ethanol, embedded in the epoxy resin LX-112 (Ladd, Burlington, VT), and sectioned with a diamond knife (Diatome, Fort Washington, PA). Sections, silver in color, were stained with 2% (wt/vol) aqueous uranyl actetate and 0.8% (wt/vol) lead citrate and then mounted on butvar-coated nickel grids and viewed at 80 kV in a JEOL (Tokyo, Japan) 100 CX electron microscope.

Labeling of the Plasma Membrane with Fluorescent Lipid or IgA

BODIPY-FL-C_5 sphingomyelin (Molecular Probes, Eugene, OR) and defatted BSA (fatty acid ultra-free; Boehringer Mannheim, Indianapolis, IN) were used to prepare 5 μM sphingomyelin/0.5 μM BSA complexes in P buffer (10 mM HEPES, pH 7.4, 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, and 145 mM NaCl) as previously described (Pagano and Martin, 1994). Lipid labeling was performed as described before (Jou *et al.*, 1998). In brief, duplicate confluent monolayers of MDCK cells grown on Transwell filters were washed twice in prechilled P buffer, and 0.5 ml of the sphingomyelin/BSA solution was added to the apical compartment and 1 ml of P buffer was added to the basal compartment. The incubation was performed for 10 min on ice, and then the sphingomyelin/BSA complexes were removed, and the filters were washed five times in cold P buffer. One filter was immediately processed for confocal microscopy, and the other one was left on ice in P buffer for 1 h before processing for confocal microscopy. Similar experiments were performed with IgA. The IgA ligand (100 μg/ml), diluted in P buffer, was bound to the apical or basolateral surfaces of the cell for 60 min at 4°C. Nonadherent ligand was removed by several washes with chilled P buffer. One filter was immediately fixed, immunolabeled, and then processed for confocal microscopy. The other filter was incubated in P buffer for 1 h at 4°C before fixation, immunolabeling, and processing.

Endocytosis of ^{125}I-IgA

Endocytosis of ^{125}I-IgA was measured as previously described (Apodaca *et al.*, 1994)

Analysis of ^{125}I-IgA Postendocytotic Fate

The postendocytotic fate of a preinternalized cohort of ^{125}I-IgA (at 5–10 μg/ml) was analyzed as described by Maples *et al.* (1997). In preliminary experiments we performed assays in the presence of a 100-fold excess of cold-competing IgA. Because the amount of fluid-phase internalization was <5% of the signal observed in polymeric Ig receptor (pIgR)-expressing cells, this step was omitted in subsequent experiments.

Analysis of ^{125}I-Tf Recycling

Iron-saturated Tf was iodinated to a specific activity of 5.0–9.0 × 10^6 cpm/μg with the use of ICl as described by Apodaca *et al.* (1994). The cells were washed with warm (37°C) MEM/BSA three times, and unlabeled Tf was allowed to dissociate from the cell surface and filter for 60 min in MEM/BSA. ^{125}I-Tf (5 μg/ml) was internalized from the basolateral surface of the cells for 45 min at 37°C in a humid chamber. The cells were washed three times for 5 min with ice-cold MEM/BSA and then warmed to 37°C for 2.5 min to allow for receptor internalization as described previously (Apodaca *et al.*, 1994). The medium was aspirated, fresh medium was added, and the postendocytic fate of ^{125}I-Tf was assessed as described above. ^{125}I-Tf uptake was inhibited >95% when the radioactive ligand was internalized in the presence of a 100-fold excess of cold ligand.

Analysis of ^{125}I-EGF Degradation

^{125}I-Epidermal growth factor (EGF) was purchased from NEN Life Science Products (Boston, MA; 150–200 μCi/ug) and used at a final concentration of 40 ng/ml. The cells were washed with warm (37°C) MEM/BSA three times and ^{125}I-EGF was internalized from the baso-

lateral surface of the cells for 10 min at 37°C. The cells were washed rapidly three times with MEM/BSA, the apical and basolateral medium was aspirated and replaced with fresh MEM/BSA, and the cells were then incubated for 3 min at 37°C. The medium was aspirated, fresh medium was added, and the postendocytic fate of ^{125}I-EGF was determined as described previously (Leung *et al.*, 1999).

Measurement of gp80 Secretion, Basolateral Delivery of the pIgR, and New Protein Synthesis

Secretion of gp80 was measured as described before (Apodaca *et al.*, 1993). Basolateral delivery of newly synthesized pIgR was quantified with the use of our previously published methods (Apodaca *et al.*, 1993). Data were quantified with the use of a Molecular Imager FX phosphorimager (Bio-Rad, Hercules, CA) and associated Quantity One software. Radioactivity was quantified with the use of the volume rectangle tool. Values (volume counts × cm^2) were corrected by subtracting background counts. Total protein synthesis was measured as follows. Cells were starved for 15 min in DMEM medium that lacked cysteine and methionine and contained 10% (wt/vol) dialyzed FBS, 0.35 g/l of $NaHCO_3$, and 20 mM HEPES, pH 7.4. The cells were then pulse-labeled with 0.5 mCi/ml [^{35}S]-Express protein label mix (1175 Ci/mmol; New England Nuclear, Boston, MA) from both the apical and basolateral cell surfaces for 5 min at 37°C. The cells were washed with MEM/BSA three times, the filter with attached cells was cut out of the Transwell holder, and the cells were lysed in 0.5 ml of SDS lysis buffer (0.5% [wt/vol] SDS, 100 mM triethanolamine, pH 8.6,and 5 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride and a peptide inhibitor cocktail (5 μg/ml leupeptin, 5 μg/ml antipain and 5 μg/ml pepstatin). The samples were boiled for 5 min and then vortex shaken for 15 min at 4°C. An aliquot of the lysate was spotted on 3 MM paper (Whatman, Tewksbury, MA) and allowed to dry, and ^{35}S-labeled proteins precipitated with 10% (vol/vol) trichloroacetic acid. The samples were placed in vials containing scintillation fluid, and the radioactivity was quantified in a liquid scintillation counter (Wallach, Gaithersburg, MD).

RESULTS

Expression of Cdc42V12 and Cdc42N17 in Polarized MDCK Cells

Our goal was to test the hypothesis that Cdc42 regulates trafficking pathways known to be important in the maintenance of cell surface polarity. To fulfill this objective we generated stable cell lines that expressed either a dominant-active mutant of Cdc42 (Cdc42V12) or a dominant-negative mutant of Cdc42 (Cdc42N17) in an inducible manner. In these studies we used the parent T23 clone of MDCK cells that stably expresses the tetracycline transactivator (Barth *et al.*, 1997). These cells were transfected with cDNA encoding either amino-terminal myc-tagged dominant-active Cdc42V12 or dominant inactive Cdc42N17. These cells also express the rabbit pIgR. The level of expression of mutant Cdc42 was regulated by the addition of the tetracycline analogue DC. In the presence of 20 ng/ml DC, expression of Cdc42V12 or Cdc42N17 was not detected by Western blotting of cellular lysates (Figure 1A) even after an extended exposure of the blot, indicating efficient inhibition of expression in this tetracycline-repressible system.

When the Cdc42N17 cells were cultured on Transwells in the absence of DC for 48 h, the amount of Cdc42N17 expression was ~600% of the endogenous Cdc42 levels (Figure 1A). The myc-tagged version of this mutant has a lower mobility on SDS-PAGE than the endogenous protein (marked with an arrow in Figure 1A). The distribution of myc-tagged Cdc42N17 was assessed by immunofluorescence with the use of the myc tag-specific monoclonal antibody 9E10. Myc-Cdc42N17 was found at the apical pole of the cell (Figure 1, B and C) and along its lateral margins (Figure 1D). Much of the staining appeared cytosolic, but some of the myc-tagged Cdc42N17 was associated with vesicular structures. These vesicles were observed above the level of the nucleus (Figure 1C) and to a lesser extent at the base of the cell (Figure 1E).

The amount of myc-tagged Cdc42V12 expression 48-h postplating was determined to be ~120% of the level of endogenous Cdc42 expression (Figure 1A). In contrast to myc-tagged Cdc42N17, little of the myc-tagged Cdc42V12 was found at the apical pole of the cell (Figure 1F). Instead, it was associated with small punctate vesicles at the base of the cell (Figure 1I), along the lateral cell surfaces (Figure 1, G and H), as well as in an aggregate (marked with arrows in Figure 1H) that was found in focal planes above the nucleus. This aggregate was either located at the center of the cell or near the lateral surfaces. This central aggregate was observed in many, but not all, cells. A similar aggregate is observed in cells expressing a dominant-active mutant of Rac1 (Jou *et al.*, 2000). Like that aggregate, the one observed in cells expressing Cdc42V12 was labeled by the endosomal markers IgA, Tf, and Rab11 but not by the late endosomal marker LAMP-1, the Golgi marker giantin, or the TGN marker furin (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations).

Expression of Mutant Cdc42 Causes Actin Cytoskeleton Reorganization in MDCK Cells

It is hypothesized that Rho-GTPases may modulate protein traffic in epithelial cells by regulating the organization of actin filaments and the activity of actin-associated motor proteins. In fact, RhoA and Rac1 regulate the organization of the actin cytoskeleton associated with basal, lateral, and apical membrane domains of MDCK cells (Jou and Nelson, 1998). We examined whether the distribution of F-actin in MDCK cells was altered by expression of Cdc42N17 or Cdc42V12. Control cells (grown in the presence of DC) or those expressing either Cdc42V12 or Cdc42N17 were cultured for 48 h on Transwell filters. The distribution of F-actin was determined by incubating fixed and permeabilized cells with fluorescein isothiocyanate (FITC)-labeled phalloidin and examining the cells by scanning-laser confocal microscopy (Figure 2). The cells were also incubated with an antibody that recognizes occludin, an integral membrane protein in tight junctions that marks the border between the apical and basolateral plasma membrane domains (Faruse *et al.*, 1993). In control cells F-actin was distributed across the tops of the cells in a punctate pattern that likely reflects FITC-phalloidin staining of the actin-rich core of the apical microvilli (Figure 2A). F-actin was also associated with the lateral surfaces of the cell, both at the level of the tight junctions (Figure 2B) and in sections taken at the level of the nucleus (Figure 2C). F-actin was also detected at the base of the cells where it was associated with bundles of stress fibers (Figure 2D). These results are in agreement with prior reports of F-actin distribution in MDCK cells (Stevenson and Begg, 1994; Maples *et al.*, 1997). Finally, it was noted that the tight junctions surrounded each cell in a continuous band

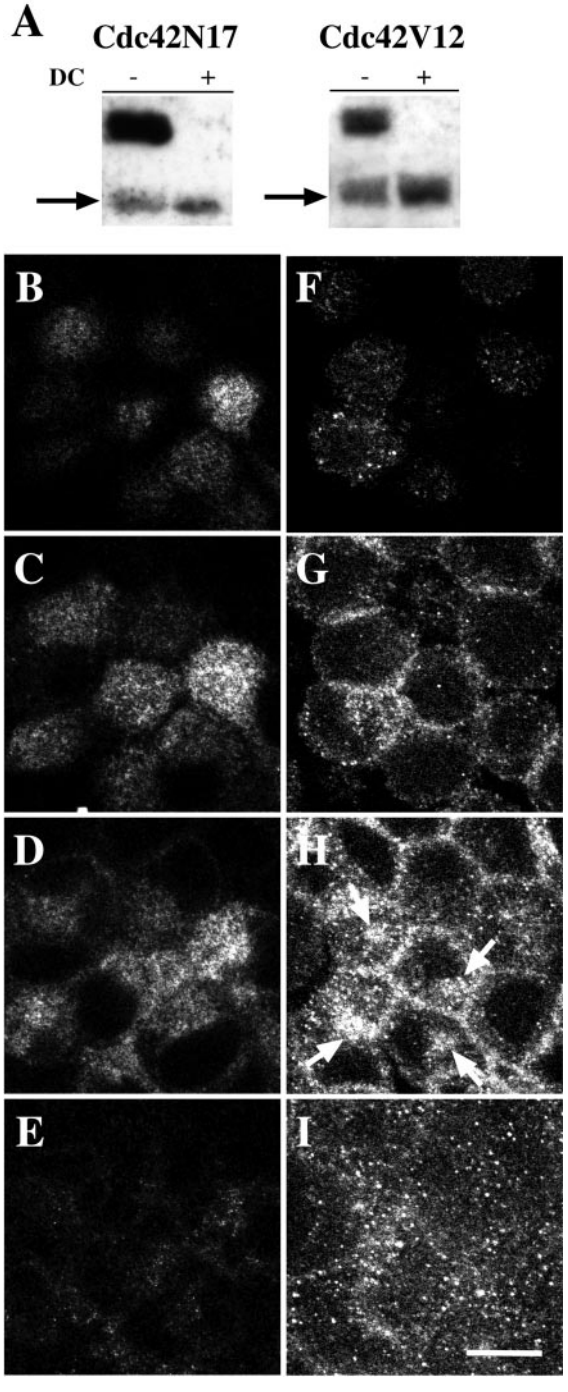

Figure 1. Inducible expression of myc-tagged Cdc42N17 or Cdc42V12 and their distribution in polarized MDCK cells. Cdc42N17 or Cdc42V12 cells were plated at low density in medium lacking DC (−) or containing DC (+)and then were plated on Transwell filter supports (±DC) for 48 h. (A) The filter-grown cells were solubilized in SDS lysis buffer, the lysates were resolved by SDS-PAGE, and Western blots of the cellular lysates were probed with an anti-Cdc42 polyclonal antibody to detect induction of the myc-tagged Cdc42 mutant proteins as well as endogenous Cdc42. Endogenous Cdc42 is marked with an arrow. The addition of the myc tag to Cdc42N17 and Cdc42V12 causes these proteins to migrate more slowly than endogenous Cdc42 on SDS-PAGE. Similar levels of protein expression were observed 18 h postplating on Transwells. Cdc42N17 (B–E) and Cdc42V12 (F–I) cells were fixed with the use of a pH-shift protocol, incubated with a myc tag-specific antibody, and then a goat anti-mouse secondary antibody conjugated to FITC. Images were captured with the use of a scanning-laser confocal microscope. Shown are optical sections taken at the apical pole of the cells (B and F), 2 μm below the previous section (C and G), around the level of the nucleus (D and H), or at the base of the cells (E and I). Central aggregates are marked with arrows. Bar, 10 μm.

and were usually distributed in a relatively thin plane of focus (Figure 2F).

Expression of Cdc42N17 did not appear to alter the distribution of F-actin structures associated with the apex or base of the cells (Figure 2, I and L). However, in cells expressing this mutant protein, F-actin was observed in cytoplasmic aggregates that formed in the apical cytoplasm (arrowheads in Figure 2J), and thickening and disorganization of the cortical F-actin cytoskeleton was observed along the lateral surfaces of the cell (Figure 2, J and K). The tight junctions in cells expressing Cdc42N17 were no longer distributed in a thin plane of focus; instead, they often extended deeper into the cell (Figures 2, N and O).

Expression of Cdc42V12 also resulted in alterations in the distribution of F-actin in MDCK cells. In some cells there was a loss of the punctate F-actin staining at the apex of the cell (Figure 2Q). The cortical F-actin appeared thickened at some points along the lateral surfaces of the cells (small arrow in Figure 2R). This thickening was also noted at regions of multiple cell-cell contact (small arrow in Figure 2S). In contrast, the actin-rich bundles of stress fibers at the base of the cells appeared unaltered by expression of Cdc42V12 (Figure 2T). In many regions of the monolayer the distribution of occludin was significantly disrupted, and this protein was now observed in all planes of focus (Figure 2, U–X). The organization of the tight junctions was especially impaired at regions where multiple cells formed sites of contact (large arrow in Figure 2W).

Tight Junction Morphology Is Altered in Cells Expressing Cdc42V12

The above results indicated that expression of Cdc42N17 or Cdc42V12 may alter tight junctions. Because large-scale diffusion of ligands or proteins between the two cell surface domains would significantly alter the interpretation of our assays of polarized biosynthetic and endocytic traffic, we further examined whether tight junction morphology and function were affected by expression of Cdc42V12 or Cdc42N17.

First, we assessed the effects of mutant Cdc42 expression on tight junction morphology. When examining individual optical sections (Figure 2) it was not apparent whether the tight junctions formed a continuous band around the cells expressing Cdc42N17 or Cdc42V12. To assess the continuity of the tight junction rings in these cells, the cells were grown for 48 h on Transwell filters, were fixed, and then stained with anti-occludin and anti-ZO1 antibodies. A Z-series was captured with the confocal microscope and the total image volume was summed and then projected as a single image

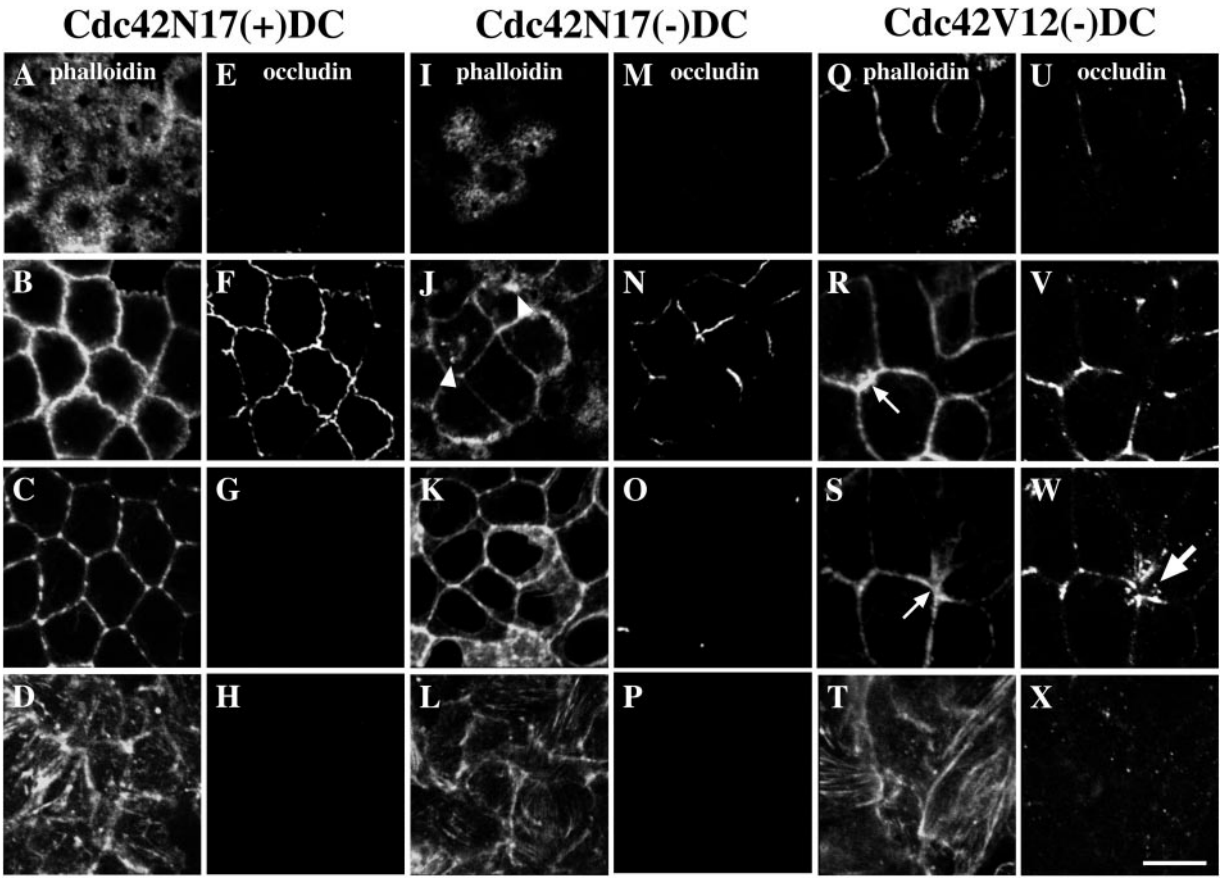

Figure 2. Distribution of occludin and F-actin in Cdc42N17 cells grown in the presence of DC (A–H), Cdc42N17 cells grown in the absence of DC (I–P), or Cdc42V12 cells grown in the absence of DC (Q-X). The distribution of FITC-phalloidin and occludin in Cdc42V12 cells grown in the presence of DC was similar to A–H and is not shown. Cells were cultured on Transwells for 48 h, fixed with the use of a pH-shift protocol, incubated with rabbit anti-occludin antibody, and then reacted with goat anti-rabbit Cy5 and FITC-phalloidin. The FITC and Cy5 emissions were captured simultaneously with the use of a scanning-laser confocal microscope. Staining for FITC-phalloidin is shown in A–D, I–L, and Q–T. Staining for occludin is shown in E–H, M–P, and U–X. Single optical sections are shown from the base of the cells below the nucleus (D, H, L, P, T, and X), from the lateral surface of the cell at the level of the nucleus (C, G, K, O, S, and W), from the apical region of the cell above the nucleus (B, F, J, N, R, and V), and from the apical pole of the cell at or above the level of the tight junctional complex (A, E, I, M, Q, and U). Arrowheads in J point to accumulations of actin in the apical cytoplasm. Small arrows in R and S point to thickening of actin at the sites of cell-cell contact. The large arrow in W shows a region of cell-cell contact where the distribution of occludin is altered. Bar, 10 μm

(Figure 3). In control cells, occludin formed a continuous ring that encircled each cell (Figure 3A) and was localized near the apical pole of the cell (Figure 3G). A similar distribution of occludin was observed in cells expressing Cdc42N17 (Figure 3, B and H). In contrast, in cells expressing Cdc42V12 the junctional rings appeared irregular in shape and the thickness of the junctions appeared to vary (Figure 3C). Increased amounts of occludin were observed at the regions where multiple cells formed contacts. In X-Z sections occludin was found at the apical borders of some cells expressing Cdc42V12, but in other cells it was found at multiple sites along the lateral borders (Figure 3I).

In addition to the transmembrane protein occludin, we also examined the distribution of ZO-1, a cytoplasmic protein associated with tight junctions (Figure 3, D–F and J–L). In both control cells and those expressing Cdc42N17 or Cdc42V12, the distribution of ZO-1 was identical to that observed for occludin (in Figure 3, compare A–C with D–F and G–I with J–L). Finally, we examined the ultrastructure of the tight junctions in control cells or those expressing Cdc42N17 or Cdc42V12. Expression of Cdc42N17 had no visible affect on the morphology of tight junctions (compare control cells in Figure 3M with cells expressing Cdc42N17 in Figure 3N). Junctions with normal morphology were also observed in cells expressing Cdc42V12 (Figure 3O). However, in cells expressing Cdc42V12 we also observed junctions in which the membranes appeared to fuse well below the position where tight junctions normally form (Figure 3P; sites of membrane fusion are marked with arrows).

Mutants of Cdc42 Alter the "Gate" and "Fence" Function of Tight Junctions

Next, we assessed whether expression of Cdc42N17 or Cdc42V12 altered the development of TER, a measure of the monolayer's ability to impede ion flow. The TER of

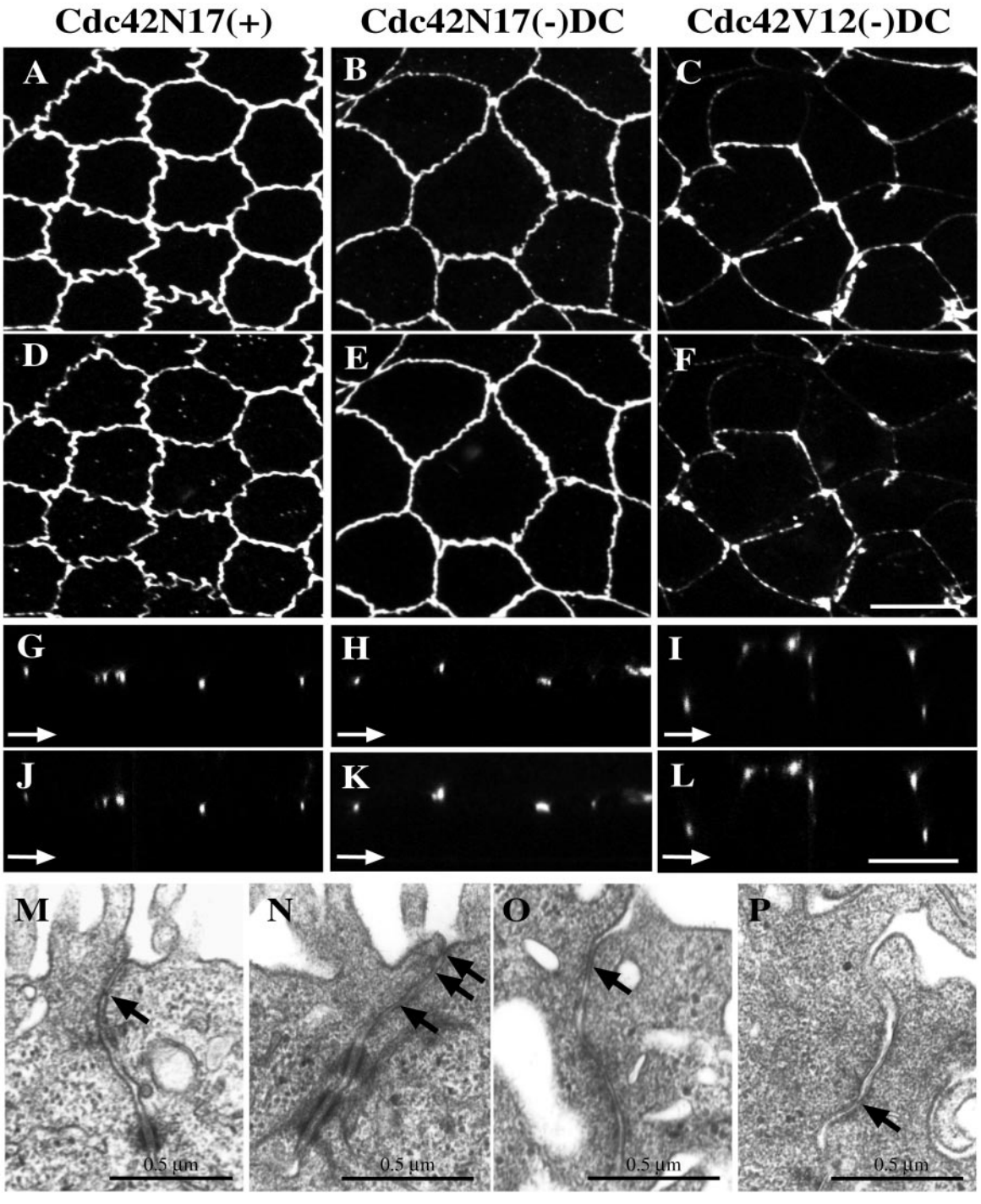

Figure 3. Ultrastructural analysis of tight junctions and distribution of the tight junction-associated proteins occludin and ZO-1 in cells expressing Cdc42N17 or Cdc42V12. (A–L) Shown are Cdc42N17 cells cultured in the presence of DC (A, D, G, and J), Cdc42N17 cells grown in the absence of DC (B, E, H, and K), and Cdc42V12 cells cultured in the absence of DC (C, F, I, and L). The distribution of occludin and ZO-1 in Cdc42V12 cells grown in the presence of DC was identical to A, D, G, and J and is not shown. Cells were fixed with the use of a pH-shift protocol, incubated with antibodies against occludin (A–C and G–I) and ZO-1 (D-F and J–L), and then reacted with goat anti-rabbit secondary antibody conjugated to FITC and goat anti-rat secondary antibody conjugated to Cy5. Images were captured simultaneously with the use of a scanning-laser confocal microscope. Projections of the total image volume are shown in A–F. X–Z sections are shown in G–L. The position of the filter is marked with an arrow in G–L. Bar, 10 μm. (M–P) Transmission electron micrographs of Cdc42N17 cells grown in the presence (M) or absence of DC (N) or Cdc42V12 cells grown in the absence of DC (O–P). The tight junctions of Cdc42V12 cells grown in the presence of DC looked similar to those shown in M and are not shown. Arrows point to regions of the junctional complex where the membranes appear to be fused.

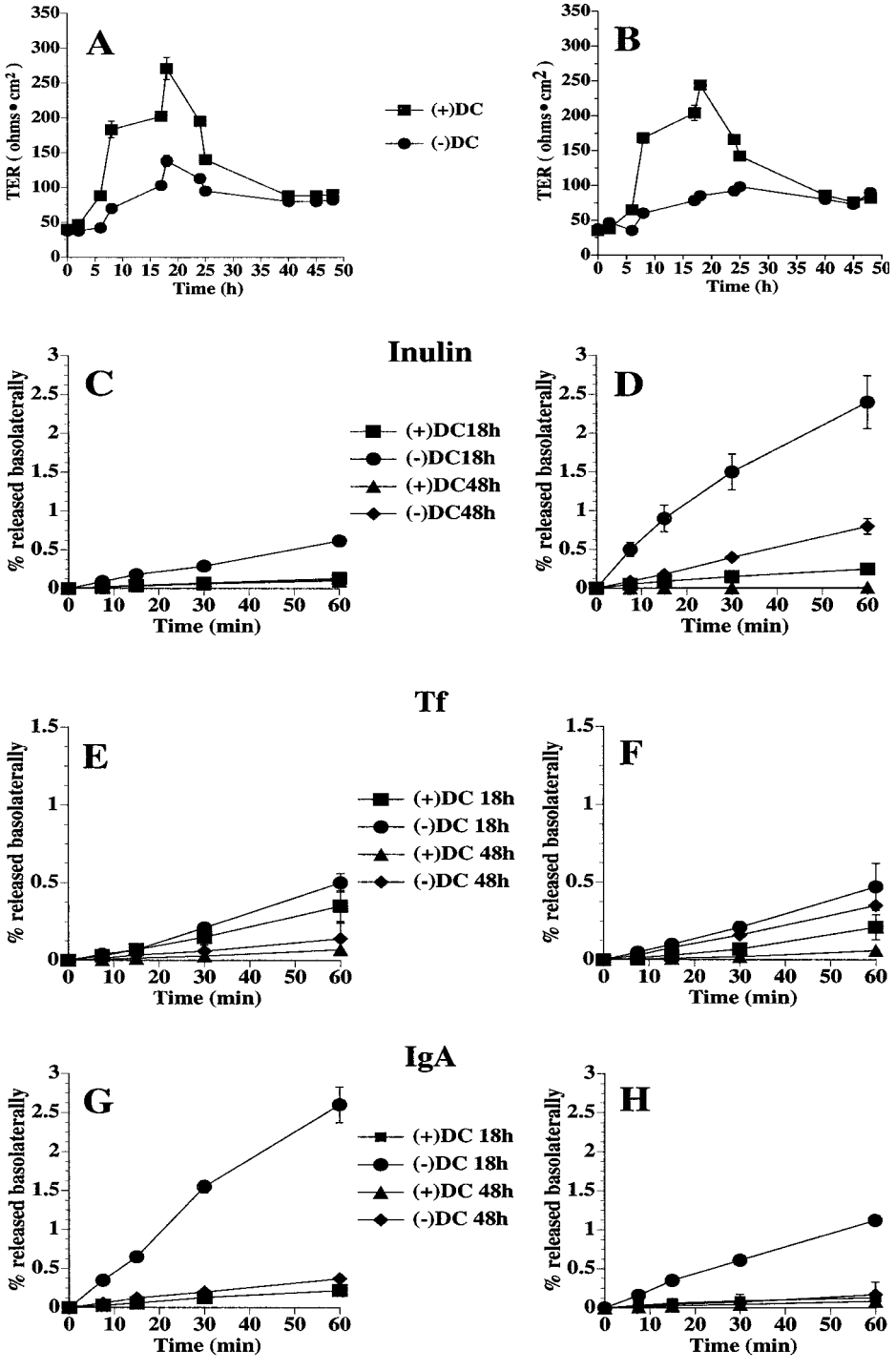

Figure 4. Development of TER and measurement of [^{14}C]inulin, ^{125}I-Tf, and ^{125}I-IgA flux in Cdc42N17 or Cdc42V12 cell lines. (A and B) Cdc42N17 (A) or Cdc42V12 (B) cells were plated on Transwells in low calcium medium (±DC), and cell-cell contact was synchronously induced by switching to normal calcium medium (±DC). TER (Ωcm^2) was measured at the designated time points after induction of cell-cell contact. The data are reported as the means ± SEM; n = 18. In some cases the error is smaller than the symbol. (C–H) Cdc42N17 (C, E, and G) or Cdc42V12 (D, F, and H) cells were cultured on Transwells in the presence (+) or absence (−) of DC for 18 or 48 h. [^{14}C]inulin (C and D), ^{125}I-Tf (E and F), or ^{125}I-IgA(G and H) was added to the apical chamber of the Transwell unit and the percentage of initially added marker that diffused into the basal chamber was quantified over time. Shown are data (means ± SEM; n = 3) from a representative experiment. In some cases the error is smaller than the symbol. In G the data from Cdc42N17 cells grown in the absence of DC for 48 h overlaps completely the data from cells grown in the presence of DC for 18 h.

Cdc42N17 or Cdc42V12 cells was monitored during a 48-h period after induction of cell-cell contacts. When Cdc42N17 or Cdc42V12 cells were cultured in the presence of DC (+DC) the TER reached 250–275 Ωcm^2 after 18 h and then decreased to 100 Ωcm^2 after 40 h (Figure 4, A and B). After 18 h, expression of Cdc42V12 or Cdc42N17 decreased TER by ~40–60% (Figure 4, A and B). However, after 40 h or longer TER values were comparable to those of the cells grown in the presence of DC (Figure 4, A and B). These results indicate that expression of Cdc42N17 and Cdc42V12 may alter early steps in the formation of tight junctions.

Because TER is dependent on paracellular transport of ions across tight junctions as well as transcellular transport via ion channels and pumps, we confirmed that paracellular

flux of solutes was altered across these monolayers. To measure any changes in tight junction permeability to small molecules we quantified the diffusion of [^{14}C]inulin between the apical and basolateral chamber of cells cultured on Transwells for 18 or 48 h. In Cdc42N17 cells grown in the presence of DC, ~0.15% of apically added [^{14}C]inulin was found in the basolateral compartment of the Transwell after a 60-min incubation at 37°C (Figure 4C). A fivefold increase in [^{14}C]inulin flux was observed in Cdc42N17 cells cultured on Transwells for 18 h in the absence of DC (Figure 4C). However, 48-h postplating on Transwells, Cdc42N17 expression had no affect on diffusion of [^{14}C]inulin between the apical and basolateral compartments of the Transwell (Figure 4C). In contrast, in cells expressing Cdc42V12 paracellular flux of inulin was increased above control levels by ~9- to 10-fold at both the 18- and 48-h time points (Figure 4D). Although the absolute magnitude of inulin flux observed in cells expressing Cdc42V12 was significantly decreased at the 48-h time point, inulin flux still remained elevated (Figure 4D), whereas TER was at control levels (Figure 4B). One possible explanation for this discrepancy is that at the 48-h time point transcellular ion flow was decreased in cells expressing Cdc42V12.

We also measured the paracellular movement of large solutes between the two poles of the cells. ^{125}I-IgA (M_r > 325,000 for multimeric IgA) or ^{125}I-Tf (M_r ~76,000) was added to the apical compartment of the Transwell (in the presence of a 100-fold excess of unlabeled competing ligand) and the appearance of ^{125}I-IgA or ^{125}I-TF in the basolateral compartment was assessed over a 60-min incubation at 37°C (Figure 4, E–H). Unlabeled ligand was added to prevent receptor-mediated endocytosis and apical to basolateral transcytosis of ligand. Under control conditions <0.35% of apically added ^{125}I-IgA or ^{125}I-Tf appeared in the basolateral compartment of filter-grown Cdc42V12 or Cdc42N17 cells grown in the presence of DC (Figure 4, E–H). In cells expressing Cdc42N17 the flux of these large solutes, especially IgA, was significantly increased 18-h postplating on Transwells (Figure 4, E and G). However, by 48-h postplating the flux of these solutes was near that of control cells (Figure 4, E and G). This observation is consistent with the return of inulin flux and TER to near control levels by this time point. In cells expressing Cdc42V12 the paracellular flux of these large solutes increased significantly above control levels at 18 h (Figure 4, F and H), but the flux of these markers was decreased when the cells were incubated for 48 h before the assay.

In addition to serving as a gate that regulates the paracellular flow of ions and solutes, the tight junction can also form a fence that prevents the lateral diffusion of proteins and lipids between the outer leaflet of the apical and basolateral plasma membrane domains. Expression of mutants of other members of the Rho family, such as RhoA and Rac1, perturb tight junction fence function (Jou *et al.*, 1998). Therefore, we examined whether fence function was disrupted in cells expressing Cdc42N17 or Cdc42V12, by analyzing lipid diffusion in polarized MDCK cells grown on Transwell filters for 18 or 48 h (Figure 5). To test fence function, BODIPY-sphingomyelin was added to apical membranes for 10 min on ice, and after extensive washing, the cells were either processed immediately for confocal microscopy or incubated for an additional 60 min on ice in the absence of labeled lipid.

Similar results were observed at the 18- and 48-h time points. In Cdc42N17 cells grown in the presence of DC, BODIPY-sphingomyelin was restricted to the apical surface after 10 min of addition (Figure 5, A and C) or after the 60-min chase (Figure 5, B and D). Similarly, in cells expressing Cdc42N17 (−DC), BODIPY-sphingomyelin was restricted to the apical membrane after the 10-min pulse (Figure 5, E and G) or after the 60-min chase (Figure 5, F and H). In Cdc42V12 cells grown in the presence of DC, BODIPY-sphingomyelin was similarly restricted to the apical surface (Figure 5, I–L). However, in cells expressing Cdc42V12, BODIPY-sphingomyelin labeled only the apical membrane after the initial 10-min pulse (Figure 5, M and O), but after 60 min both the apical and basolateral plasma membrane were labeled by the fluorescent lipid (Figure 5,N and P). These observations indicate that expression of Cdc42V12 disrupted the barrier to lipid diffusion. Next, we examined whether membrane proteins could similarly diffuse across the tight junctions of cells expressing this mutant protein 18 or 48 h postplating. pIgR molecules at the apical or basolateral surface were labeled with IgA, and the appearance of IgA-labeled pIgR at the opposite cell surface was assessed after a 60-min chase (see MATERIALS AND METHODS for details). No appearance of IgA-labeled pIgR at the opposite cell surface was observed (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished results). These results indicate that, although expression of Cdc42V12 is permissive for lipid diffusion, the barrier for membrane proteins may remain intact.

In summary, our observations indicate that expression of Cdc42N17 altered TER and paracellular flux during the early stages of MDCK polarization, but by 48 h these effects were significantly lessened. Expression of Cdc42V12 significantly decreased TER and increased solute flux at 18 h. By the 48-h time point the absolute magnitude of flux remained relatively small (<1.5%/h). We also observed that expression of Cdc42V12 disrupted tight junction fence function, and increased lipid diffusion was observed at 18 or 48 h postplating. However, diffusion of apical membrane proteins was not observed under these conditions. As such, by 48-h the alterations in tight junction function observed in Cdc42V12 and Cdc42N17 cells were unlikely to affect the assays described below.

Endocytosis Is Altered by Mutant Cdc42 Expression

In addition to tight junctions, the cell uses other mechanisms to maintain cellular polarity. One such pathway is endocytosis (reviewed by Yeaman *et al.*, 1999). We measured endocytosis of ^{125}I-IgA, which is internalized by a clathrin-dependent mechanism (Breitfeld *et al.*, 1990), from either the apical or basolateral pole of cells expressing either Cdc42V12 or Cdc42N17. Dominant-negative Cdc42N17 expression had little affect on basolateral endocytosis (Figure 6A). However, the amount of apical endocytosis was selectively decreased by ~50% relative to control cells (Figure 6C). In cells expressing Cdc42V12, the rate and extent of both apical and basolateral endocytosis was significantly inhibited compared with that observed in control cells (Figure 6, B and D). The effect was most pronounced at the 1- and 2.5-min time points, when 35–60% inhibition was observed. These results

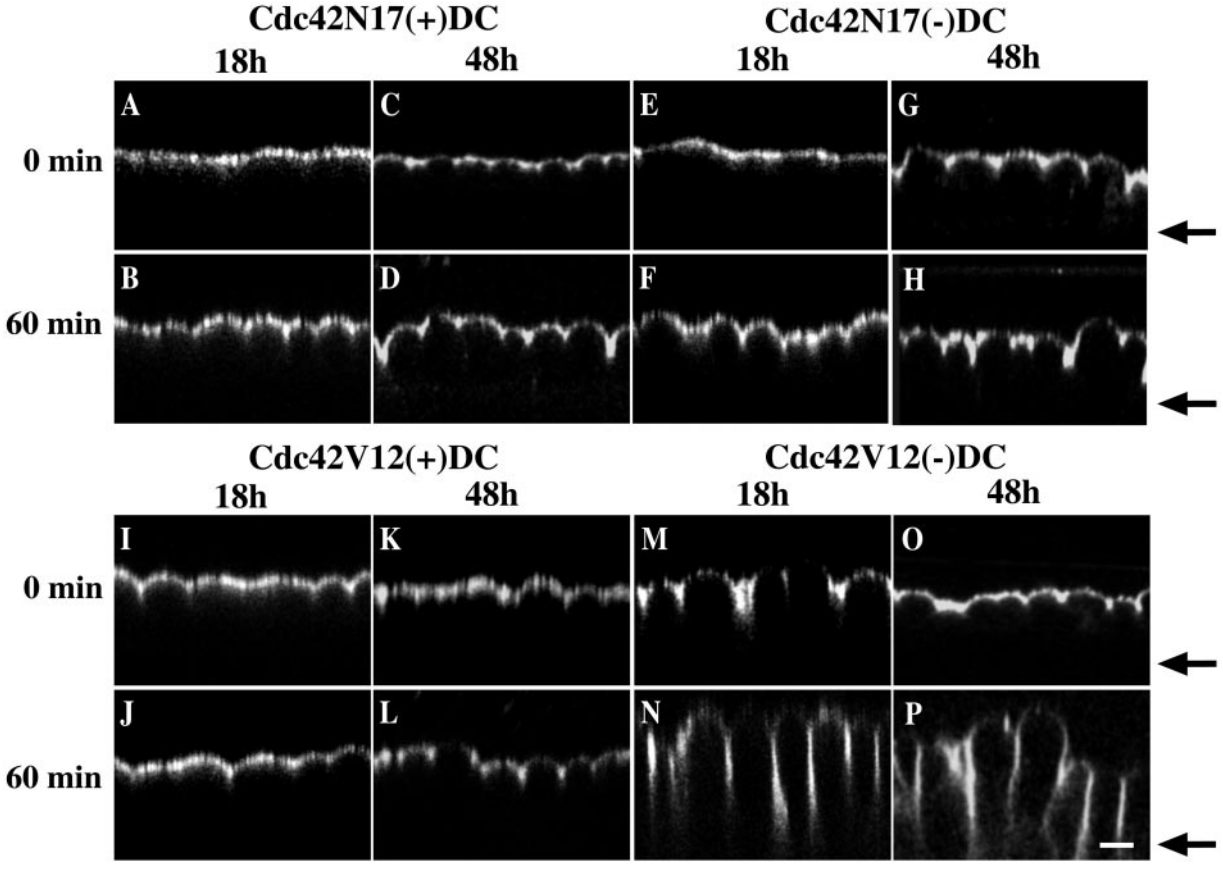

Figure 5. Lateral diffusion of BODIPY-sphingomyelin from the apical to basolateral membrane of cells expressing Cdc42N17 or Cdc42V12. Cdc42N17 (A–H) or Cdc42V12 (I–P) cells were cultured on Transwells in the presence (A–D and I–L) or absence (E–H and M-P) of DC for 18 h (A and B, E and F, I and J, and M and N) or 48 h (C and D, G and H, K and L, and O and P). The apical cell surface was labeled with BODIPY-sphingomyelin on ice for 10 min. The cells were washed and then were either immediately examined by confocal microscopy (A, C, E, G, I, K, M, and O) or incubated for an additional 60 min (B, D, F, H, J, L, N, and P) on ice before examination. X–Z projections from a representative experiment are shown. The position of the filter is marked with arrows. Bar, 10 μm

indicate that Cdc42 may regulate clathrin-dependent endocytosis in polarized MDCK cells.

Postendocytic Traffic Is Impaired in Cells Expressing Mutant Cdc42

We next explored the effect of Cdc42V12 and Cdc42N17 expression on the postendocytic trafficking pathways that occur after internalization. These pathways are also required to maintain cellular polarity. Tf was used as a marker of the basolateral recycling pathway. In control cells, Tf is internalized almost exclusively from the basolateral pole of the cell and rapidly recycles back to this cell surface via basolateral early endosomes and the common endosome (reviewed by Apodaca, 2001). There was little affect of Cdc42N17 expression on polarized Tf traffic (Figure 7A), although a small but reproducible slowing of the rate of recycling was observed at the earliest time point. The rate and extent of Tf recycling was significantly decreased in cells expressing Cdc42V12 (Figure 7B). This decrease was coupled with a significant increase in the amount of ligand released from the apical pole of the cell (~3% in control vs. ~30% in cells expressing Cdc42V12). This latter observation indicated that a significant fraction of the Tf receptor may reside at the apical surface of cells expressing Cdc42V12. In fact, relative to control cells there was a sixfold increase in apical uptake of ^{125}I-Tf in cells expressing Cdc42V12, confirming a significant increase in apical expression of the Tf receptor in these cells (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations). There was little difference in the amount of ^{125}I-Tf degraded (~2–3% in both cases), but there was a small increase in the amount of ligand remaining cell associated (~3% in control vs. ~8% in Cdc42V12 expressors). These observations indicate that Cdc42V12 expression alters the efficient sorting of Tf into the basolateral recycling pathway of polarized MDCK cells.

As a marker of the degradative pathway we followed the postendocytic fate of basolaterally internalized EGF. On endocytosis, EGF is primarily delivered to late endosomes/lysosomes where it is degraded. In MDCK cells, a fraction of the ligand (~20–25%) also recycles at the basolateral pole of the cell or is released at the apical pole of the cell (~5–10%; Brandli *et al.*, 1991). The majority of ^{125}I-EGF internalized

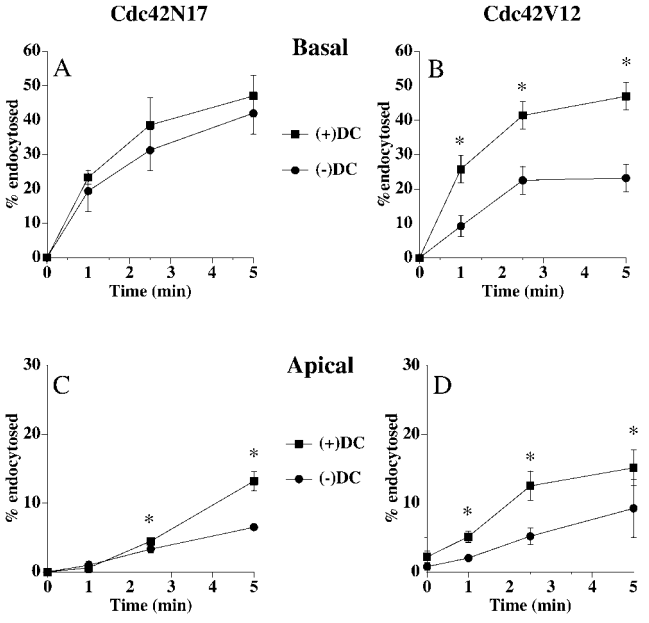

Figure 6. Apical and basolateral endocytosis of IgA in Cdc42N17 or Cdc42V12 cells. Cdc42N17 (A and C) or Cdc42V12 (B and D) cells were cultured on Transwells in the presence (+) or absence (−) of DC for 48 h. ^{125}I-IgA was bound to the basolateral (A and B) or apical (C and D) surface of the cells for 60 min at 4°C. The cells were washed and then incubated at 37°C for the times indicated. The media were collected and the cells were then rapidly cooled on ice. ^{125}I-IgA was stripped from the cell surface by a sequential treatment with trypsin and acid at 4°C and the filters were cut out of their holders. Total ^{125}I-IgA initially bound to the cells included ligand released into the medium, ligand stripped from the cell surface with trypsin and acid, and cell-associated ligand protected from stripping (endocytosed) and was quantified in a gamma counter. Data are from three separate experiments (means ± SEM; $n \geq 8$). *Values were significantly different from control ($p < 0.05$).

from the basolateral surface of Cdc42N17 cells grown in the presence of DC was degraded (~60%; Figure 7C), ~20% was recycled to the basolateral surface, ~10% was transcytosed, and the balance remained cell associated. Cdc42N17 expression did not affect EGF degradation (Figure 7C). Cdc42V12 expression slowed the degradation of ^{125}I-EGF (Figure 7D) but had no effect on the extent of ligand degradation. A small increase in the amount of ligand released at the apical pole of the cell (~10% in control and ~15% in cells expressing Cdc42V12), and a decrease in the amount of EGF that recycled (~18% in control vs. ~13% in Cdc42V12 expressors) was noted in these Cdc42V12-expressing cells.

IgA internalized from the apical pole of pIgR-expressing cells was used as a marker of the apical recycling pathway. The pIgR moves by transcytosis from basolateral early endosomes to common endosomes, to the apical recycling endosomes, and finally to the apical pole of the cell where it is cleaved to secretory component (reviewed by Apodaca, 2001). However, a significant fraction of the receptor escapes cleavage and can be internalized from the apical cell surface (Breitfeld *et al.*, 1989). This apically internalized pool of IgA primarily recycles to the apical membrane; little of the IgA is transcytosed and released at the basolateral pole of the cell (Apodaca *et al.*, 1994). Cdc42N17 expression had little effect on apical IgA recycling (Figure 7E). In cells expressing Cdc42V12 there was a decrease in the kinetics of apical recycling versus control cells and an overall 20% decrease in the total amount of IgA recycled (Figure 7F). Compared with control cells there was no difference in the amount of ligand that was degraded (~7%), but the amount of IgA remaining cell associated increased from 7% in control cells to ~23% in cells expressing Cdc42V12.

Finally, we measured the effect of Cdc42V12 and Cdc42N17 expression on the postendocytic fate of basolaterally internalized ^{125}I-IgA. Expression of Cdc42N17 significantly slowed the kinetics of IgA transcytosis; however, the extent of transcytosis was unaltered (Figure 7G). Expression of this mutant protein had no effect on ligand recycling but increased the amount of degraded IgA from ~5% to ~8% and cell associated IgA from ~3% to ~9%. In cells expressing Cdc42V12 there was also a significant slowing in the kinetics of basolateral to apical transcytosis (Figure 7H), and the total amount of ligand transcytosis was inhibited by ~15%. Recycling of the ligand was unaffected by Cdc42V12 expression, but the total cell-associated or degraded ligand was increased from ~3–4% in control cells to ~8.5% in cells expressing Cdc42V12.

Golgi Morphology Is Modified in Cells Expressing Cdc42V12 or Cdc42N17

It was previously reported that Cdc42 is associated with the Golgi of liver and kidney-derived epithelial cells (Erickson *et al.*, 1996; Kroschewski *et al.*, 1999). We assessed whether Cdc42V12 and Cdc42N17 were associated with the Golgi of our transfected cell lines and also determined whether Golgi morphology was perturbed by expression of these proteins. In control cells, giantin, a resident Golgi protein (Lindstedt and Hauri, 1993), is distributed in a ribbon-like structure that is found between the nucleus and apical membrane (Figure 8, A–C). Cdc42N17 expression resulted in the dispersion of the Golgi into vesicular elements that were distributed throughout the cytosol at the apical pole of the cell (Figure 8, D–F). Much of the Cdc42N17 staining was diffuse and did not colocalize with giantin-labeled Golgi. However, some colocalization of myc-tagged Cdc42N17 and giantin was observed on vesicular structures present in focal planes above the nucleus (see arrows in Figure 8, D and E). In Cdc42V12-expressing cells the Golgi also appeared somewhat fragmented and was distributed throughout the cell (Figure 8, G–I). Although there was an occasional region of colocalization between these two proteins, the majority of Cdc42V12 protein was associated with other intracellular compartments, including the central aggregate, and did not colocalize with giantin-labeled Golgi. We have also examined the distribution of the TGN marker protein furin and either myc-tagged Cdc42V12 or Cdc42N17. Although the distribution of furin was fragmented relative to control, there were only small amounts of furin-labeled TGN that colocalized with Cdc42V12 or Cdc42N17 (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations).

Expression of Cdc42V12 or Cdc42N17 Alters Biosynthetic Targeting of Proteins

The disruption of Golgi morphology by Cdc42V12 and Cdc42N17 indicated that biosynthetic traffic might be altered

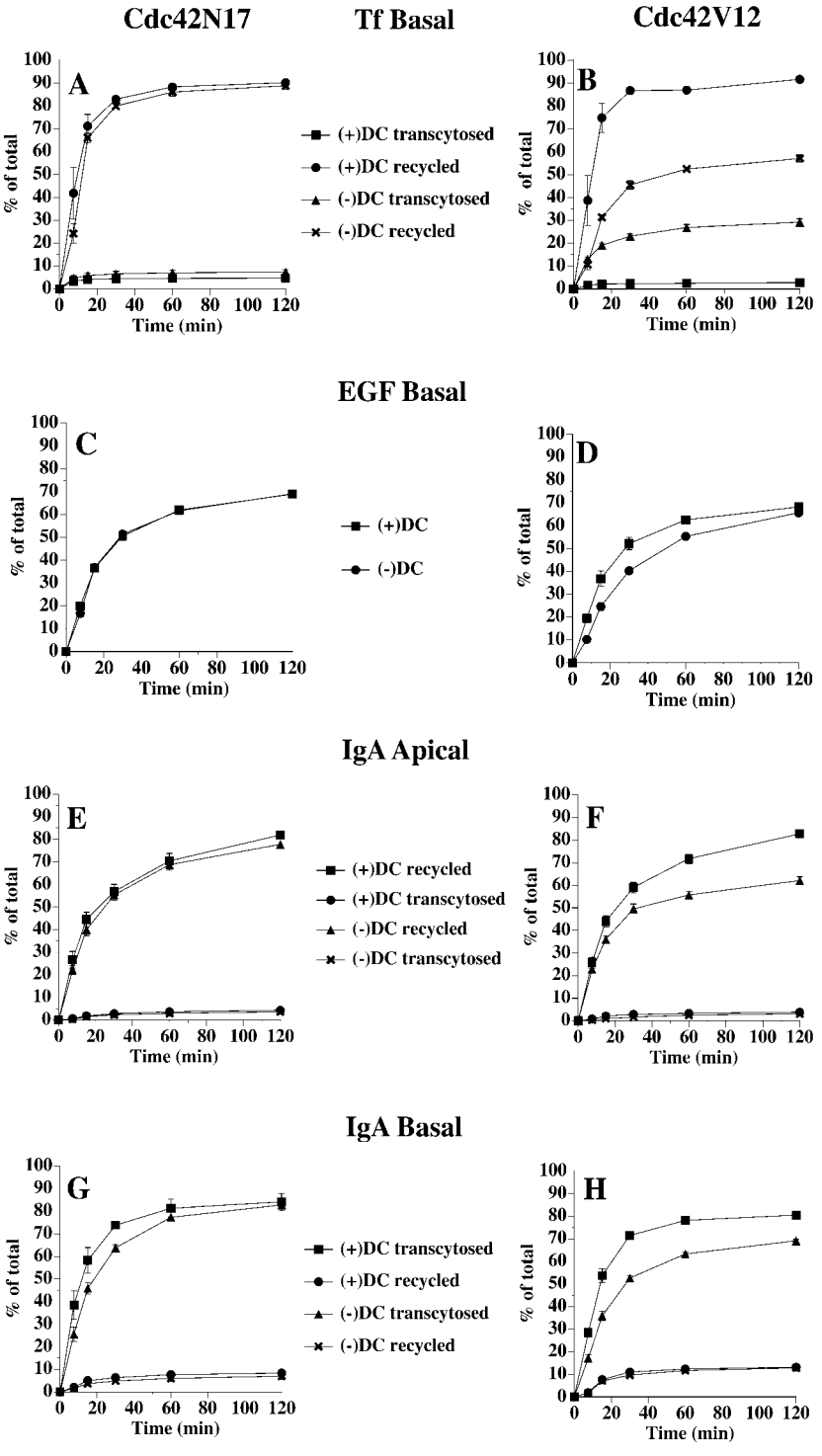

Figure 7. Postendocytic fate of internalized Tf, EGF, or IgA in cells expressing Cdc42N17 or Cdc42V12. Cdc42N17 (A, C, E, and G) or Cdc42V12 (B, D, F, and H) cells were cultured on Transwells in the presence (+) or absence (−) of DC for 48 h. (A and B) ^{125}I-Tf was internalized from the basolateral surface of the cells for 45 min at 37°C, and the cells were washed and then chased for 120 min. The percentage of total ligand released apically (transcytosed) or basolaterally (recycled) is shown. (C and D) ^{125}I-EGF was internalized from the basolateral surface of the cells for 10 min at 37°C, and the cells were washed and then chased for 120 min at 37°C. The percentage of total ligand degraded is shown. (E and F) ^{125}I-IgA was internalized from the apical surface of the cells for 10 min at 37°C, and the cells were washed and then chased for 120 min. The percentage of total ligand released apically (recycled) or basolaterally (transcytosed) is shown. (G and H) ^{125}I-IgA was internalized from the basolateral surface of the cells for 10 min at 37°C, and the cells were washed and then chased for 120 min. The percentage of total ligand released apically (transcytosed) or basolaterally (recycled) is shown. In A–H, experiments were repeated four times in triplicate. Values are averages of the means from each of the four trials ± SEM

by expression of these mutant proteins. To test this possibility we analyzed the effect of Cdc42V12 or Cdc42N17 expression on delivery of newly synthesized pIgR to the basolateral surface of the cells. In control Cdc42N17 cells ~70% of the pIgR was directly delivered to the basal pole of the cells and ~10% was delivered directly to the apical pole of the cell (Figure 9A). The balance remained in the cell. Expression of Cdc42N17 resulted in a small but significant ($p < 0.05$ with the use of Student's *t* test) increase of ~20% in basolateral delivery of the pIgR but had no effect on apical delivery (Figure 9A). In cells expressing Cdc42V12 basolateral delivery of the pIgR was dimin-

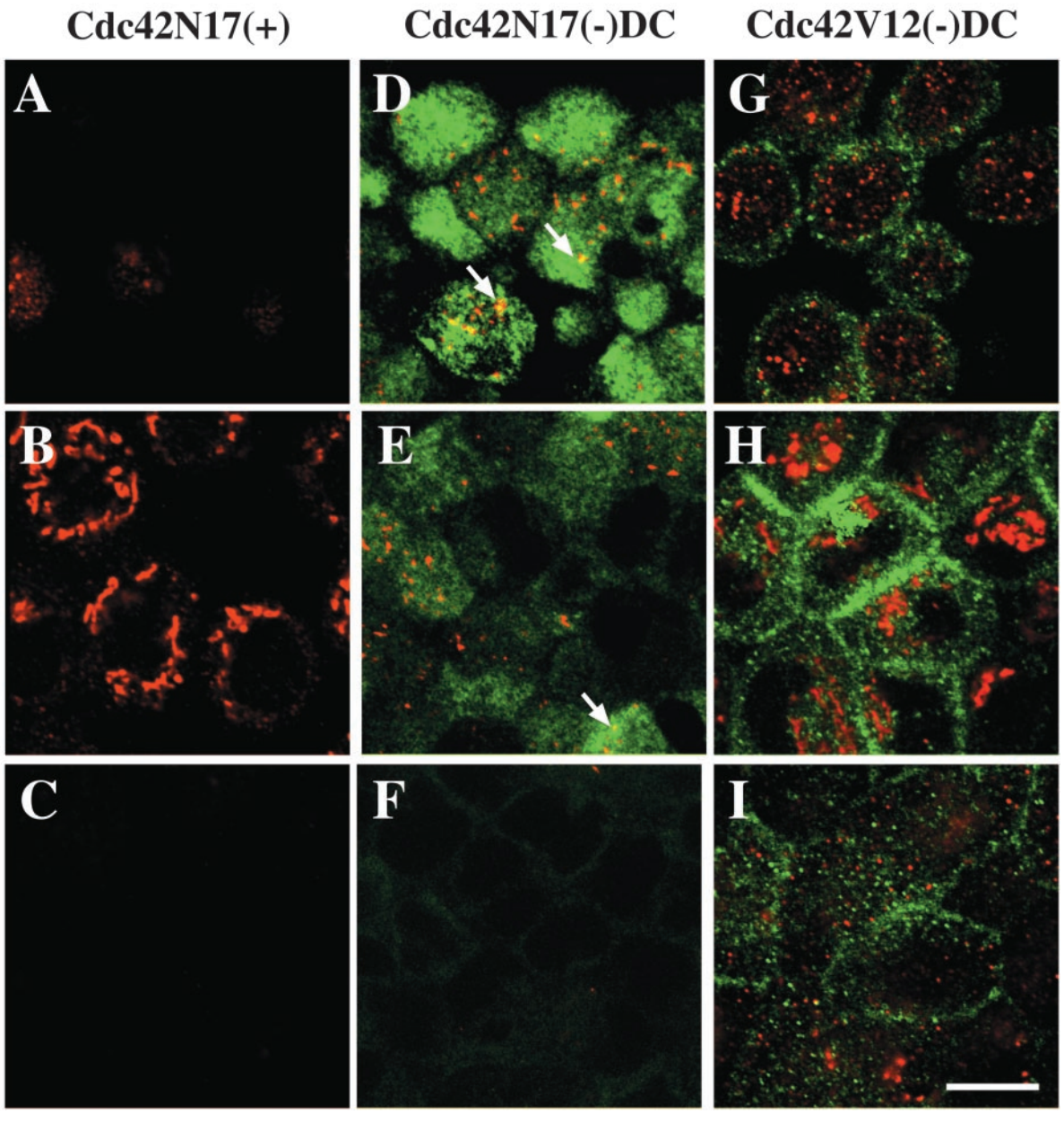

Figure 8. Distribution of the Golgi-associated protein giantin in cells expressing Cdc42N17 or Cdc42V12. Shown are Cdc42N17 cells cultured in the presence of DC (A–C), Cdc42N17 cells grown in the absence of DC (D-F), and Cdc42V12 cells cultured in the absence of DC (G–I). The distribution of giantin in Cdc42V12 cells grown in the presence of DC was identical to A–C and is not shown. Cells were fixed with the use of a pH-shift protocol, incubated with giantin and myc tag-specific antibodies, and then incubated with appropriate secondary antibodies conjugated to FITC or Cy5. Images were captured with the use of a scanning-laser confocal microscope and the FITC and Cy5 signals were merged. The staining for giantin appears red and that of the myc tag-labeled Cdc42V12 or Cdc42N17 appears green in this figure. Shown are single optical sections from the apical pole of the cell (A, D, and G), at the level of the nucleus (B, E, and H), or along the lateral surfaces of the cell (C, F, and I). Representative regions of colocalization are marked with arrows. Bar, 10 μm.

ished by ~30%, whereas apical delivery was stimulated by ~50% (Figure 9A).

Next, we examined the effects of Cdc42V12 and Cdc42N17 expression on secretion of the gp80 complex. This endogenous protein is secreted predominantly at the apical pole of the cell with an apical to basolateral ratio of ~3–4:1 (Urban *et al.*, 1987). Expression of Cdc42N17 resulted in an increase in both the kinetics and the amount of gp80 secreted at the apical pole of the cell (Figure 9B). No effect was observed on basolateral secretion of gp80. In contrast, expression of Cdc42V12 slowed apical secretion of gp80 (Figure 9C) but had little affect on basolateral secretion of gp80. To rule out

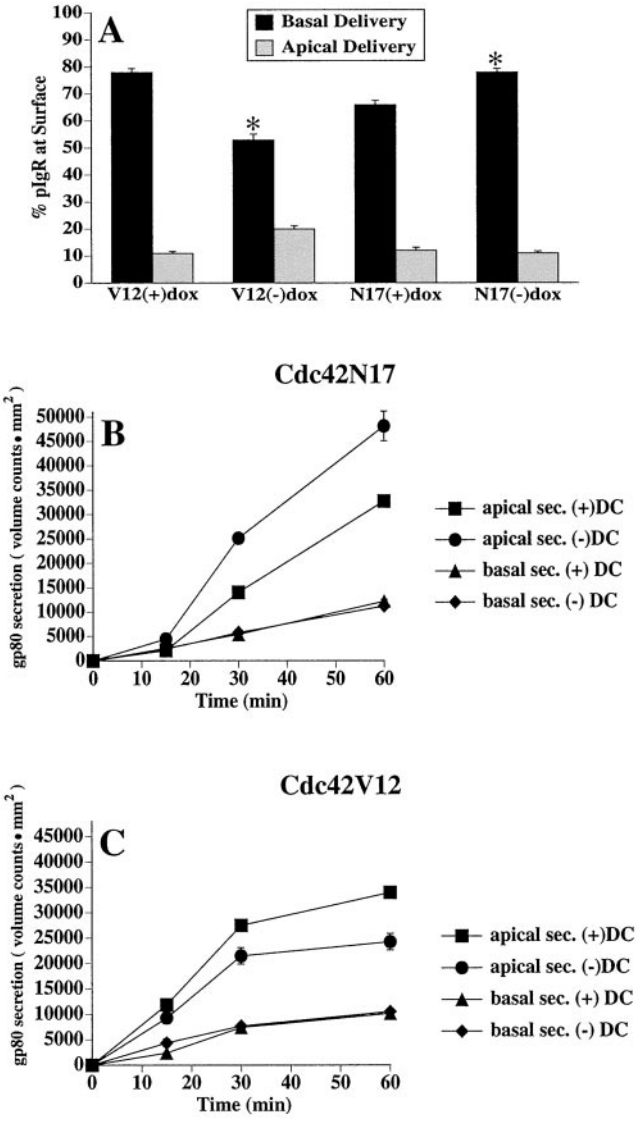

Figure 9. Biosynthetic transport in cells expressing mutants of Cdc42. (A) Cdc42N17 or Cdc42V12 cells were grown in the presence (+) or absence (−) of DC on Transwell filters for 48 h. Cells were metabolically labeled with [^{35}S]cysteine for 15 min at 37°C and then chased in the presence or absence of basolateral trypsin for 60 min at 37°C as described previously (Apodaca *et al.*, 1993; Aroeti and Mostov, 1994). In the presence of trypsin newly synthesized pIgR that is delivered to the basolateral cell surface is rapidly proteolyzed. By comparing the amount of immunoprecipitable pIgR in non trypsin-treated cells and those treated with trypsin it is possible to quantify the extent of pIgR delivery to the basolateral or apical cell surface. The percentage of apical and basolateral delivery is shown (mean ± SEM; n ≥ 5). Cdc42N17 (B) or Cdc42V12 (C) cells were grown in the presence (+) or absence (−) of DC on Transwell filters for 48 h. Cells were metabolically labeled for 15 min with [^{35}S]methionine/cysteine and then chased for 15–60 min at 37°C. An aliquot of the apical or basolateral medium was separated by SDS-PAGE and the amount of apical or basolateral gp80 secretion was quantified with the use of a PhosphorImager. Values are means ± SEM, n ≥ 3.

the possibility that these observations were simply the result of Cdc42-mediated changes in protein synthesis, we confirmed by ^{35}S-protein labeling that the total amount of newly synthesized proteins was unaltered by expression of Cdc42N17 (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations). In cells expressing Cdc42V12 the amount of total protein synthesis was doubled relative to control cells at this time point (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations). However, this could not explain the decreased levels of gp80 secretion.

DISCUSSION

Cdc42-dependent Regulation of Tight Junctions

Cdc42 is an important regulator of cellular polarity in multiple cell types including the budding yeast *Saccharomyces cerevisiae* and T-lymphocytes (Adams *et al.*, 1990; Stowers *et al.*, 1995). Our data and that of Kroschewski *et al.* (1999) indicate that Cdc42 may also be important in the maintenance of cellular polarity in polarized MDCK cells. The tight junction is crucial for the maintenance of cell polarity and regulates paracellular flux of ions and solutes (the so-called gate function), as well as impedes the movement of lipids and proteins in the extracellular leaflet of the plasma membrane (fence function; reviewed by Cereijido *et al.*, 1998). It was recently reported that RhoA and Rac1 may be important modulators of tight junction function (Jou *et al.*, 1998). Our data indicate that Cdc42 may also play a role in this process. We observed that the morphology of the tight junctions was significantly altered by expression of Cdc42V12, consistent with previous reports (Kroschewski *et al.*, 1999; Joberty *et al.*, 2000). In contrast, only minimal changes were observed in the tight junction morphology of cells expressing Cdc42N17. Both Kroschewski *et al.* (1999) and Takaishi *et al.* (1997) also observed no effect of mutant Cdc42N17 expression on tight junction morphology and suggested that tight junction function may be unaltered. However, these investigators used individually microinjected cells or cells cultured on plastic, which hindered their ability to measure changes in solute and ion flux across the monolayer.

Functionally, the gate and fence function of tight junctions was perturbed by mutants of Cdc42. We observed that at early time points after cell-cell contact (18 h) TER was decreased by 40–60% in cells expressing either Cdc42V12 or Cdc42N17. We confirmed that the gate function of tight junctions was altered by measuring the paracellular flux of small and large solutes. Consistent with the measurements of TER, the flux of inulin and of Tf and multimeric IgA complexes were increased by expression of mutant Cdc42. These effects were especially pronounced at 18 h but were less prominent at the 48-h time point. These results indicate that expression of Cdc42N17 or Cdc42V12 may alter early steps in the formation of tight junctions; however, with time tight junctions (of variable functionality) are able to assemble. Finally, we observed that the fence function of cells expressing Cdc42V12 was perturbed, because BODIPY-sphingomyelin was able to diffuse between the apical and basolateral domains. In contrast, we observed that pIgR, labeled at the apical or basolateral surface with IgA, did not diffuse under identical conditions. Our data indicate that expression of Cdc42V12 may selectively alter the diffusion of lipids across the tight junction. Interestingly, expression of a

truncated mutant of occludin similarly alters diffusion of a labeled lipid but not of a membrane protein (Balda *et al.*, 1996).

Mutants of Cdc42 may modulate tight junction morphology and function by several different mechanisms. We observed that expression of either Cdc42N17 or Cdc42V12 affected the cortical actin cytoskeleton. Actin is associated with tight junctions and tight junction function can be altered by disruption of the actin cytoskeleton (Madara, 2000). Alternatively, the effects we observed may reflect Cdc42-dependent alterations in the Par6/Par3/protein kinase C-ζ protein complex (Joberty *et al.*, 2000). In *Caenorhabditis elegans* the Par proteins are required for asymmetric cell division and polarized growth. Proteins homologous to Par proteins are found in mammalian cells, interact with Cdc42 and Rac1 (Joberty *et al.*, 2000; Qiu *et al.*, 2000), and are thought to play some role, as yet undefined, in the formation of tight junctions at sites of cell-cell contact (Joberty *et al.*, 2000). In this regard, we observed that Cdc42V12 was localized along the lateral membranes of the cells, and this localization correlated with the extended distribution of tight junction proteins along the lateral surfaces.

Modulation of Endocytic Traffic by Cdc42

In addition to a potential role in modulating tight junction function, we also observed that mutants of Cdc42 also impaired the normal function of endocytic pathways. These pathways are known to be crucial in the maintenance of cell polarity (reviewed by Yeaman *et al.*, 1999). Expression of Cdc42V12 slowed the rate and extent of apical and basolateral endocytosis, whereas Cdc42N17 expression selectively slowed apical endocytosis. The slowing of apical endocytosis by both Cdc42V12 and Cdc42N17 indicates that cycling of Cdc42 between GDP- and GTP-bound states may be necessary for normal endocytic activity. Our results are consistent with recent observations that Cdc42 developmentally regulates endocytosis in dendritic cells (Garrett *et al.*, 2000). Moreover, they indicate that, in addition to Rho and Rac (Jou *et al.*, 2000; Leung *et al.*, 1999), Cdc42 may be important in the regulation of endocytosis in polarized epithelial cells.

Expression of Cdc42V12 also altered the postendocytic traffic of several ligands. The largest effect was on the postendocytic traffic of basolaterally internalized Tf. We observed that Tf recycling was slowed, and there was a significant increase in the amount released at the apical pole of the cell and the Tf-receptor was now expressed at the apical cell surface. These observations are consistent with the finding by Kroschewski *et al.* (1999) that proteins that normally recycle at the basolateral pole of the cell are observed at the apical surface of cells expressing Cdc42V12. Whereas apical release of Tf was stimulated in cells expressing Cdc42V12, transcytosis of basolaterally internalized IgA was slowed in Cdc42V12-expressing cells. These contrasting effects indicate that Cdc42V12 expression might impact multiple compartments or sorting events. For example, by impairing the function of the Rab11-positive elements of the apical recycling endosome (which are involved in IgA transcytosis but not Tf recycling; Wang *et al.*, 2000), expression of Cdc42V12 would impair basolateral to apical transcytosis. And, by altering the normally efficient basolateral sorting/targeting machinery in basolateral or common endosomes, expression of Cdc42V12 would result in apical release of Tf ligand. In addition to effects on Tf recycling and IgA transcytosis, expression of Cdc42V12 slowed both EGF degradation and recycling of apically internalized IgA.

In contrast, expression of Cdc42N17 had little effect on many of these postendocytic trafficking pathways. The only demonstrable effect was the significant slowing of basolateral to apical transcytosis. Other dominant-negative mutants of small GTPases (e.g., rab25, RhoA, Rac1) have little affect on postendocytic traffic (Casanova *et al.*, 1999; Leung *et al.*, 1999; Jou *et al.*, 2000), and a dominant-negative mutant of the early endosome-associated GTPase RhoD has little affect on endosome function (Murphy *et al.*, 1996). Perhaps some endocytic traffic is modulated only by activation of these small GTPases. This would be similar to the effects of protein kinase C and protein kinase A on postendocytic traffic. When activated, these kinases modulate traffic, whereas inactivation of these kinases apparently has no affect (Cardone *et al.*, 1994; Hansen and Casanova, 1994).

Cdc42 and Golgi Function

Unlike previous studies (Erickson *et al.*, 1996; Kroschewski *et al.*, 1999), we did not observe any significant association of the Golgi with Cdc42V12 and only limited association of Cdc42N17 with this organelle. This was true of cells that were plated at low density on plastic, when cells were cultured on filters and allowed to polarize for 1–3 d, or when protein expression was attenuated by the addition of various concentrations of DC (Rojas, Ruiz, Leung, Jou, and Apodaca, unpublished observations). However, we did observe that in cells expressing Cdc42V12 the Golgi appeared fragmented and in cells expressing Cdc42N17 the Golgi ribbons were dispersed throughout the cytoplasm. The underlying cause of this effect is unknown but could reflect alterations in the cytoskeleton. As described in Figure 2, expression of Cdc42N17 or Cdc42V12 had significant effects on the distribution of the actin cytoskeleton. Although Cdc42 is associated with the Golgi of many cell types, such is not always the case. In yeast, Cdc42 is normally localized to the site of bud formation (Adams *et al.*, 1990), in the kidney cortex Cdc42 is primarily associated with the plasma membrane and cytosol (Boivin and Béliveau, 1995), and in adrenal chromaffin cells Cdc42 is exclusively localized in a subplasmalemmal region in both resting and nicotine-stimulated cells (Gasman *et al.*, 1999). The difference between our observations and those previously made in MDCK cells (Kroschewski *et al.*, 1999) may reflect the different mechanisms of achieving mutant Cdc42 gene expression or the differences in the level of protein expression.

Cdc42V12 expression diminished the amount of newly synthesized pIgR delivered to the basolateral surface of the cells (coupled with a small increase in direct TGN to apical delivery), consistent with previous observations that basolateral delivery of membrane proteins is altered in cells expressing Cdc42V12 (Kroschewski *et al.*, 1999). In addition, we observed that Cdc42V12 impaired the apical secretion of the endogenous gp80 protein. In contrast, expression of Cdc42N17 stimulated basolateral delivery of the pIgR and apical secretion of gp80. Our results are consistent with observations made in yeast Cdc42–1^{ts} mutants; at the restrictive temperature they cannot establish a single bud site; yet they grow isotropically. This continued growth indicates that secretion is unimpeded, even in the absence of Cdc42

expression (Adams *et al.*, 1990). Our results are in contrast to observations that basolateral delivery of vesicular-stomatitis virus G-protein is impaired in cells expressing Cdc42N17, whereas apical delivery is apparently unaffected (Kroschewski *et al.*, 1999).

As described above this could reflect differences in the methods used to obtain mutant Cdc42 expression, the levels of Cdc42N17 expression, or the techniques used to quantify transport defects across the whole monolayer. Alternatively, it may reflect differences in the pathways used to deliver proteins to the cell surface. The basolateral vesicular-stomatitis virus G-protein used by Kroschewski *et al.* (1999) contains a tyrosine-dependent basolateral sorting determinant (Thomas and Roth, 1994), whereas the pIgR does not (Aroeti *et al.*, 1993). Perhaps there is more than one mechanism/pathway for delivery of basolateral proteins and these are differentially sensitive to expression of Cdc42V12. Even yeast are thought to have more than one pathway to the cell surface (Finger and Novick, 1998), and these pathways may be differentially regulated by Cdc42.

The opposite effects of Cdc42V12 and Cdc42N17 expression on gp80 secretion indicate that Cdc42 may be an important regulator of biosynthetic cargo delivery. In its GTP-bound state Cdc42 may slow traffic, and in its inactive GDP-bound state Cdc42 may stimulate biosynthetic traffic. These effects could reflect alterations in cargo delivery to or exit from the Golgi or targeting of newly synthesized proteins from the Golgi to the cell surface. Alternatively, they may be the result of Cdc42-dependent alterations in the actin cytoskeleton. Recently, it was observed that active Cdc42 interacts with γ-coat-protein complex I and may modulate early steps in biosynthetic traffic (Wu *et al.*, 2000). Because newly synthesized pIgR is transported from the Golgi to an apical endosomal compartment (likely to include elements of the apical recycling endosome and/or elements of the common recycling endosome) before its arrival at the basolateral cell surface (Orzech *et al.*, 2000), Cdc42 may also act through modulation of endocytic pathways.

Cdc42 Effector Pathways

Several membrane trafficking steps including phagocytosis, exocytosis, endocytosis, receptor recycling, and basal to apical transcytosis are thought to require the actin cytoskeleton (Mukherjee *et al.*, 1997; reviewed by Apodaca, 2001). By altering the actin cytoskeleton, Cdc42 could modulate these pathways. In addition, Cdc42 is known to interact with several other downstream activator/effector proteins including Wiskoff-Aldrich syndrome protein (WASP), phopholipase D, phosphatidylinositol 3-kinase (PI-3-K), and IQGAP (Zheng *et al.*, 1994; Nobes and Hall, 1995; Singer *et al.*, 1995; Aspenström *et al.*, 1996; Hart *et al.*, 1996; Symons *et al.*, 1996; McCallum *et al.*, 1998). WASP is known to be important in the nucleation of the actin cytoskeleton (Rohatgi *et al.*, 1999). Through modifications of the lipid bilayer, both phospholipase D and phosphoinositol-4-phosphate 5-OH kinase are known to affect the ability of coat proteins to bind to organellar membranes (De Camilli *et al.*, 1996; Liskovitch, 1996). Activation of PI-3-K is especially intriguing because endocytic traffic is altered in cells treated with wortmannin, a potent inhibitor of PI-3-K (Hansen *et al.*, 1995). Uncontrolled activation of PI-3-K and its effectors may have dramatic effects on postendocytic traffic. Finally, IQGAP interacts with Cdc42, actin, and calmodulin and is localized to the Golgi where it may play a role in Golgi localization and function (McCallum *et al.*, 1998). Acting through these effectors, Cdc42 may control multiple pathways that are used to generate and maintain epithelial cell polarity.

In summary, our results indicate that Cdc42 may control multiple aspects of epithelial polarity by modulating tight junction morphology and function and endocytic and biosynthetic traffic directed to the apical and basolateral surfaces of these cells.

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Note added in proof: During final acceptance of this manuscript, Müsch A., Cohen, D., Kreitzer, G., and Rodriguez-Boulan, E. published an article in EMBO J. (*20*: 2171–2179; 2001), detailing cDC42-dependent regulation of apical and basolateral protein exit from the TGN.

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