Active Rab11 and functional recycling endosome are required for E-cadherin trafficking and lumen formation during epithelial morphogenesis

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Submitted 17 February 2008; accepted in final form 21 June 2008

Desclozeaux M, Venturato J, Wylie FG, Kay JG, Joseph SR, Le HT, Stow JL. Active Rab11 and functional recycling endosome are required for E-cadherin trafficking and lumen formation during epithelial morphogenesis. Am J Physiol Cell Physiol 295: C545–C556, 2008. First published June 25, 2008; doi:10.1152/ajpcell.00097.2008.—The correct targeting and trafficking of the adherens junction protein epithelial cadherin (E-cadherin) is a major determinant for the acquisition of epithelial cell polarity and for the maintenance of epithelial integrity. The compartments and trafficking components required to sort and transport E-cadherin to the basolateral cell surface remain to be fully defined. On the basis of previous data, we know that E-cadherin is trafficked via the recycling endosome (RE) in nonpolarized and newly polarized cells. Here we explore the role of the RE throughout epithelial morphogenesis in MDCK monolayers and cysts. Time-lapse microscopy in live cells, altering RE function biochemically, and expressing a dominant-negative form of Rab11 (DN-Rab11), each showed that the RE is always requisite for E-cadherin sorting and trafficking. The RE remained important for E-cadherin trafficking in MDCK cells from a nonpolarized state through to fully formed, polarized epithelial monolayers. During the development of epithelial cysts, DN-Rab11 disrupted E-cadherin targeting and trafficking, the subapical localization of pERK and actin, and cyst lumen formation. This final effect demonstrated an early and critical interdependence of Rab11 and the RE for E-cadherin targeting, apical membrane formation, and cell polarity in cysts.

cyst; epithelia; cadherin; MDCK

EPITHELIAL BIOGENESIS REQUIRES the organized assembly of cell-cell contacts and establishment of apical-basal polarity. In polarized cells, the apical and basolateral membranes are segregated, each having different lipid and membrane protein compositions (14, 26, 44). Acquisition of apical-basolateral polarity follows the assembly of specialized epithelial junctions including tight junctions at the apical-basolateral boundary and adherens junctions on the lateral cell membrane. Epithelial cadherin (E-cadherin) is a major cell-cell adhesion protein that forms dynamic adherens junctions during epithelial morphogenesis and polarization and helps to maintain adhesion in mature epithelia (43). E-cadherin is incorporated into adherens junctions along with other proteins, including β-catenin, p120-catenin [p120(ctn)], and α-catenin, to form the cadherin-catenin complex (43), which associates with the actin cytoskeleton and with signaling proteins (35). Continual trafficking of newly synthesized E-cadherin to and from the cell surface is essential to ensure the dynamic formation of adherens junctions and to modulate cadherin-based adhesion during morphogenesis and wound healing (5).

The correct delivery of E-cadherin specifically to the lateral cell membrane is a critical step in its trafficking in polarized cells (28). Previous studies from our laboratory revealed that E-cadherin in HeLa and Madin-Darby canine kidney (MDCK) cells is trafficked from the trans-Golgi network (TGN) to the recycling endosome (RE) before its delivery to the basolateral membrane (24). The RE compartment was initially designated for recycling proteins, such as transferrin-loaded transferrin receptor (TfR), back to the cell surface following endocytosis (31, 42). More recently, the RE has been increasingly recognized as a way station for post-Golgi exocytosis and sorting of membrane and soluble cargo proteins destined for the cell surface (2, 6, 12, 17, 25, 29). In nonpolarized or early-polarized cells, basolateral proteins travel from the Golgi complex to the cell surface via the RE (2, 24), where, according to recent studies, these proteins are also sorted (12). Some apical proteins also appear to traverse the RE, although this route remains more controversial (10). Studies tracking basolateral proteins including newly synthesized TfR and vesicular stomatitis virus G (VSV-G) protein suggest that the RE may only be transiently used as a basolateral exocytic route before epithelial polarization, after which a more direct route to the cell surface is favored (17). Whether E-cadherin trafficking via the RE occurs throughout the whole process of epithelial cell polarization and morphogenesis remains to be tested, and this issue formed the focus for the current studies.

The small GTPase, Rab11a (referred to here as simply Rab11), is a well-known marker of the apical RE (15), and it operates to regulate epithelial polarity and membrane traffic into and out of the RE (11, 37). Rab11 acts through a variety of effectors including members of the Rab11-interacting proteins family, Rab11-FIPs (36), and myosin Vb (20). Experiments in nonpolarized mammalian cells showed perturbed E-cadherin trafficking in the presence of Rab11 mutants (24). Rab11 also interacts with components of the exocyst complex as part of the trafficking machinery at the RE (32), and loss of function of exocyst in Drosophila epithelial tissues implicated Rab11 in Drosophila epithelial-cadherin (DE-cadherin) trafficking throughout morphogenesis (19).

In the current study, we have used Rab11 mutants to investigate the further requirement for Rab11 and the RE in E-cadherin trafficking throughout the development of polarity and cyst formation in epithelia. The results revealed a continued dependence on an intact and functioning RE and on normal Rab11 GTP cycling for correct basolateral delivery of E-cadherin. Additionally, we showed that the RE is more globally

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required for the critical early stage of lumen formation during epithelia cyst morphogenesis.

**MATERIALS AND METHODS**

**Plasmids and cell culture.** Plasmids encoding full-length, wild-type Rab11 tagged with green fluorescent protein (GFP; Rab11-GFP), a dominant-negative (DN)-Rab11-GFP mutant (GDP-locked form with an S25N substitution), human E-cadherin (hE-cad) untagged or tagged with GFP, human transferrin receptor (hTIR), and dileucine mutants of hE-cad (substitutions LS87A and LS88A) tagged with GFP or mCherry (ΔS1-hE-cad-GFP or ΔS1-hE-cad-Cherry) have been described previously (24, 27). The hE-cad and ΔS1-hE-cad were tagged in the hE-cad-GFP or ΔS1-hE-cad-GFP plasmids by replacing the GFP-encoding sequence with a PCR-generated mCherry fragment from the pRSETB-Cherry vector (a generous gift from Dr. R. Tsien, University of California, San Diego).

Monolayers of wild-type MDCK strain II cells and MDCK cells stably expressing Rab11-GFP or DN-Rab11-GFP were grown and passaged in Dulbecco’s modified Eagle’s medium (GIBCO Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 4 mM L-glutamine at 37°C, in an atmosphere of 5% CO₂ and 95% air as previously described (28). Cells were plated on glass coverslips or on Transwell polycarbonate filters (Corning Costar, Cambridge, MA) at various densities and were allowed to polarize for up to 7 days.

**Transfection and microinjection.** MDCK cells were plated at subconfluent densities 24 h before being transfected with the appropriate plasmid(s) using the LipofectAMINE 2000 system (Invitrogen) following the manufacturer’s instructions. Cells were typically left to recover for 4–36 h posttransfection before use. For stable expression, transfected cells were passaged and maintained in medium containing Geneticin (G418 Sulphate; Invitrogen) for 10–14 days. Surviving cells were ring-cloned, and clonal lines with varying expression levels were grown. Clones with relatively low expression levels were selected by immunofluorescence microscopy and immunoblotting for experiments here; two or more clonal lines were used for most experiments.

Microinjection was carried out on confluent monolayers grown on glass coverslips of either MDCK cells transiently expressing hTIR and left to recover in fresh medium for 18 h, or MDCK cells stably expressing Rab11-GFP. Individual coverslips were transferred to a 35-mm dish with CO₂-independent medium and placed under an Axiovert 200 inverted microscope (Carl Zeiss) to select target cells for microinjection. Purified hE-cad-GFP or hE-cad-Cherry (at 0.1 µg/µl) were microinjected into the cytoplasm using an AIS-2 Micromanipulator System (Sutter Instrument). Cells stably expressing Rab11-GFP were microinjected with hE-cad-Cherry and left to recover for 6 h. Microinjected cells transiently expressing hTIR were left to recover for 4 h to allow expression of hE-cad-GFP, before labeling of the RE using human transferrin (hTfn) as described in *Recycling endosome labeling with transferrin and endosomal inactivation assay*. Cells were then fixed and assayed.

**Cystogenesis in three-dimensional cultures.** Untransfected MDCK cells and lines stably transfected with Rab11-GFP, DN-Rab11-GFP, or ΔS1-hE-cad-GFP were grown in three-dimensional (3D) cultures by plating them as an overlay on a thin base of extracellular matrix using a glass chamber slide support (26). Matrigel HGF (BD Biosciences, San Jose, CA) extracellular matrix was spread as a thin layer on precoated eight-well glass chamber slides (Nunc LabTek II, In Vitro Technologies) and allowed to gel at 37°C. Cell monolayers were trypsinized and resuspended at 2 × 10⁴ cells/ml in standard MDCK growth media. The single-cell suspension was mixed 1:1 with normal media containing 4% Matrigel (2% final concentration), and 200 µl of this was overlaid onto the set Matrigel base. The cells were fed every 3 days with 2% Matrigel in fresh media and grown for up to 10 days before being fixed and assayed.

**Recycling endosome labeling with transferrin and endosomal inactivation assay.** To specifically label the RE with hTfn, MDCK cells were transfected with hTIR and left to recover in fresh media for 18 h, and 10 µg/ml Texas Red-labeled hTfn (TR-hTfn) was then added for 1.5 h and chased for 45 min with fresh media before fixation or live imaging recording.

A horseradish peroxidase (HRP) inactivation assay was modified from the protocol of Ang et al. (2) and used here as previously described (29). In brief, hTIR-transfected MDCK cells were incubated with 10 µg/ml HRP-hTfn in media plus 1 µg/ml TR-hTfn (for visualization of the uptake) for 30 min in the dark at 37°C. Cells were washed twice in ice-cold PBS, and surface-bound HRP-hTfn and TR-hTfn were removed by two 5-min washes with 0.15 M NaCl and 20 mM citric acid, pH 5. Cells were then washed twice with ice-cold PBS and incubated in the dark for 1 h with PBS containing 0.1 mg/ml dianisomaminobenzidin (DAB), and 0.025% H₂O₂ was added to the inactivation sample (the control contained DAB but no H₂O₂). Cells were finally washed twice in PBS containing 1% BSA to stop the reaction and were incubated in prewarmed media for up to 4 days.

**Immunofluorescence and fluorescence imaging.** Cells grown on coverslips were fixed in 4% paraformaldehyde (PFA) in PBS for 60 min, permeabilized using 0.1% Triton X-100 for 10 min, and then stained as previously described (28). Fixation and immunostaining of cells grown in 3D cultures used a modification of published methods (26). Briefly, cells on glass chamber slides were fixed in 4% PFA/PBS for 30 min, permeabilized in 0.5% Triton X-100 for 10 min, blocked in PBS/0.5% fish gelatin/Saponin, then stained with primary antibodies and fluorescently tagged secondary antibodies for microscopic investigations. For antibodies requiring methanol fixation, incubation with −20°C cold methanol for 10 min was used.

Confocal imaging of fixed cells and live cell imaging were performed using an LSM 510 META confocal microscope (Carl Zeiss) using optical spectral separation. Single images were captured with an optical thickness of 0.7–1.7 µm. For Z series, a 0.34- to 0.5-µm step interval was used. The thresholds for the fluorescence intensity of each channel were carefully adjusted to most closely represent the signal strength of the original 2D images collected. Analysis was performed using LSM510 META software (Carl Zeiss Microlmaging) and Photoshop CS2 (Adobe).

**Quantification of colocalization between hTfn and biosynthetic hE-cad, based on the Pearson’s correlation coefficient (R), was performed on 3D cell reconstructions generated in Volocity v3.7 (Improvision). The thresholds of intensities for each fluorophore were predetermined for each cell using ImageJ v1.37p (National Institutes of Health, Bethesda, MD). For specifically measuring the degree of colocalization of hE-cad with hTfn in the RE, staining of E-cadherin at the membrane was removed from the equation by cropping the image before calculation.

Live cell imaging was performed on individual live cells grown on 35-mm dishes (MatTek). During imaging, the cells were immersed in CO₂–independent medium at 37°C using a heated microscope stage mount. The selective photobleaching of E-cadherin at the plasma membrane was performed using the Zeiss LSM 510 software. On a still image of the cell to be recorded, an area of interest was manually drawn around the plasma membrane and was specifically bleached by illumination in the green channel with maximum laser power reiter- ated 50 times. Recording was performed on the confocal microscope, with excitation in the green and red channels and with frames recorded every 8 s. Videos were analyzed, cropped, and constructed using ImageJ v1.37p, Volocity v3.7, and Photoshop CS2 and were exported as Quick-Time videos (Apple) with a playback speed of 10 frames/s. In the supplemental data (available online at *American Journal of Physiology-Cell Physiology* website), Video 1 shows trafficking of vesicles containing newly synthesized hE-cad-GFP (green).
in transfected MDCK cells, from the Golgi to the RE labeled with TR-hTfn (red), and towards the cell surface to make contact with the cell membrane.

**Antibodies and reagents.** Canine E-cadherin was recognized using the monoclonal antibody 3B8, obtained from Dr. W. Gallin (University of Alberta, Edmonton, Canada). Anti-human E-cadherin is a kind gift from Dr. A. Yap (University of Queensland, Brisbane, Australia). Antibody against Rab11 was obtained from BD Biosciences Pharmingen, and known markers of apical polarity and tight junction formation included rabbit anti-phosphorylated ezrin/radixin/moesin (pERM) polyclonal antibody (Chemicon) and rabbit anti-zonula occludens-1 (ZO-1) (N-term)-polyclonal antibody (Zymed). Secondary antibodies included Cy3-conjugated sheep anti-mouse and goat anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa 647-conjugated goat anti-rabbit and goat anti-mouse (Molecular Probes), and Alexa 488-conjugated goat anti-rabbit and goat anti-mouse IgGs (Molecular Probes). Alexa 488-conjugated phalloidin and Texas Red-conjugated phalloidin were used to label F-actin, and 4,6-diamidino-2-phenylindole (DAPI) was used to label nuclei (Molecular Probes, Eugene, OR). HRP-hTfn was purchased from Jomar Diagnostics (Stepney, Australia). Alexa 488-conjugated goat anti-rabbit and Alexa 647-conjugated goat anti-rabbit and goat anti-mouse antibodies included Cy3-conjugated sheep anti-mouse and goat anti-ingen, and known markers of apical polarity and tight junction formation included rabbit anti-phosphorylated ezrin/radixin/moesin (pERM) polyclonal antibody (Chemicon) and rabbit anti-zonula occludens-1 (ZO-1) (N-term)-polyclonal antibody (Zymed). Secondary antibodies included Cy3-conjugated sheep anti-mouse and goat anti-rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa 647-conjugated goat anti-rabbit and goat anti-mouse (Molecular Probes), and Alexa 488-conjugated goat anti-rabbit and goat anti-mouse IgGs (Molecular Probes). Alexa 488-conjugated phalloidin and Texas Red-conjugated phalloidin were used to label F-actin, and 4,6-diamidino-2-phenylindole (DAPI) was used to label nuclei (Molecular Probes, Eugene, OR). HRP-hTfn was purchased from Jomar Diagnostics (Stepney, Australia), and TR-hTfn was purchased from Molecular Probes.

**RESULTS**

**E-cadherin traffic through the recycling endosome.** E-cadherin is expressed primarily on the basolateral membrane of polarized MDCK cells, with small amounts of newly synthesized E-cadherin present around the perinuclear Golgi complex (Fig. 1A). We previously showed newly synthesized E-cadherin at the RE in MDCK cells where it colocalizes with recombinant Rab11 (24). Here we used additional, characteristic markers of the RE, namely, human transferrin receptor (hTfR) bound to its ligand transferrin (hTfn), in fixed and live MDCK cells to follow E-cadherin post-Golgi trafficking. Cells were cotransfected with untagged hE-cad, Rab11-GFP, and hTfR and were then incubated with TR-hTfn under conditions that concentrate the majority of label in the RE (2). The transfected cells were analyzed within 6 h to focus on hE-cad in the biosynthetic pathway. A significant proportion of hE-cad staining appeared in prominent, ring-shaped organelles positive for Rab11-GFP and hTfn, corresponding to REs (Fig. 1B).

To demonstrate E-cadherin moving through the RE on its way to the cell surface, we performed time-lapse microscopy on live MDCK cells transfected with hE-cad-GFP and hTfR. TR-hTfn marked the cell surface and internalized TR-hTfn served to label the RE in these cells (Fig. 1C and supplemental data). There was typically strong labeling of newly synthesized hE-cad-GFP in the Golgi area, with some faint signal already at the membrane (data not shown). Figure 1C shows images of these cells after photobleaching in the green channel to remove GFP fluorescence from the cell membrane and cytoplasm, leaving hE-cad-GFP signal only in the Golgi area. Under these conditions, membrane carriers positive for hE-cad-GFP were visualized moving from the Golgi region toward the periphery of the cell, where many fused with larger, TR-hTfn-positive REs (Fig. 1C). Carriers positive for both TR-hTfn and hE-cad-GFP subsequently moved from the REs toward the cell surface to make contact with the plasma membrane (Fig. 1C).

Overlap between staining of TR-Tfn and hE-cad-GFP was also studied in fixed cells (Fig. 1D). Preconfluent, nonpolarized transfected cells were examined after TR-Tfn uptake, and, to compare this to E-cadherin trafficking in fully polarized cells, individual cells in polarized monolayers were microinjected with hE-cad-GFP and allowed to take up TR-Tfn. In both cases we see significant colocalization of hE-cad-GFP with TR-Tfn in REs. Furthermore, the degree of overlap between hE-cad-GFP and the TR-hTfn-labeled RE was assessed on 3D reconstructions of preconfluent or fully polarized MDCK cells to account for the total pools of each marker in cells (Fig. 1D).

Overlap of the two fluorescent signals was quantified as Pearson’s correlation coefficient (R). Preconfluent cells showed significant colocalization of hE-cad-GFP and TR-hTfn with an average Pearson’s coefficient of R = 0.354 ± 0.089. Other structures in the cells were labeled individually for hE-cad-GFP or TR-hTfn representing nonoverlapping transport steps. In polarized MDCK cells, a correlation coefficient of R = 0.268 ± 0.025 also indicated a significant overlap of these markers as evidence that trafficking of E-cadherin via the RE persists in fully polarized cells.

Thus, in both live and fixed MDCK cells, the transient overlap of hE-cad-GFP with TR-hTfn is new evidence that the RE is a way station for E-cadherin en route to the cell surface. Tracking microinjected hE-cad-GFP in fully polarized monolayers also shows that this route is maintained after the cells have become polarized.

**E-cadherin surface delivery is dependent on the functional integrity of the recycling endosome.** We next asked whether the RE is a requisite compartment for E-cadherin surface delivery and E-cadherin-based adhesion during the formation of cell monolayers. Preconfluent MDCK cells were cotransfected with hE-cad-GFP and hTfR and allowed to recover for 12 h post-transfection. The RE compartment was chemically inactivated by allowing the cells to take up HRP-hTfn, along with lower amounts of TR-hTfn (to visualize the uptake), and then treating with DAB and peroxide to inactivate the endosomes, as previously described (2, 29). Control cells were treated in the absence of peroxide, and in these cells, the RE remained functional and the cells grew to form a patent monolayer of adhesive cells, showing the typical pattern of endogenous E-cadherin staining with cobblestone boundaries and some intracellular staining (Fig. 2). Exogenous hE-cad-GFP was colocalized with endogenous E-cadherin at the cell membranes and inside the cells, and both proteins showed some overlap with TR-hTfn in the cytoplasm. In contrast, the inactivated cells showed mislocalization of both hE-cad-GFP and endogenous E-cadherin, with neither delivered to the cell membrane. Instead, the majority of hE-cad-GFP and depleted endogenous E-cadherin was concentrated intracellularly in a tightly defined TR-hTfn-positive compartment. The cells themselves were dysmorphic, with disrupted cell-cell contacts concomitant with reduced or absent E-cadherin staining at the surface. Cells expressing only endogenous E-cadherin retained their surface staining but showed increased intracellular accumulation and overgrowth of neighboring cells. Noticeably, individual cells that had not ingested HRP-hTfn, and therefore had functional REs, still had prominent surface staining of recombinant and endogenous E-cadherin.

These results show a correlation between RE function, E-cadherin surface delivery, and the maintenance of polarized, adhesive monolayers. From this we conclude that a functional RE is required for the surface delivery of both endogenous E-cadherin and overexpressed hE-cad-GFP.
Recycling endosome regulates E-cadherin trafficking during the maturation of MDCK monolayers. Recent studies suggested that the RE is bypassed during the trafficking of some basolateral cargo, with a more direct route to the surface used in the later stages of cell polarization (17). We therefore investigated the role of the RE in E-cadherin trafficking during the establishment and maturation of polarized monolayers using Rab11 mutants to disrupt RE trafficking. Stable MDCK cells expressing low levels of either Rab11-GFP or dominant-negative DN-Rab11-GFP (GDP-locked form) were evaluated daily as they developed from sparse cells to a polarized monolayer. Endogenous E-cadherin and Rab11-GFP were localized at each time point (Fig. 3A). Preconfluent cells showed Rab11-GFP-labeled vesicles dispersed throughout the cytoplasm. E-cadherin staining in these cells was apparent in perinuclear vesicles, some of which were also positive for Rab11-GFP. By day 4, the cells were polarized and Rab11-GFP was increasingly expressed at the apical pole, emulating the very tight subapical location of the (apical) RE previously described in polarized cells (15). E-cadherin first appeared on lateral membranes in regions of cell-cell contact and then progressed over time to mark the entire basolateral cell borders in more confluent monolayers (Fig. 3A). Both E-cadherin and Rab11-GFP staining resembled those for endogenous E-cadherin and Rab11, respectively, in untransfected MDCK cells (data not shown).

DN-Rab11-GFP was observed throughout the cytoplasm in preconfluent cells; however, unlike the wild-type protein, it did not redistribute to a subapical position during cell polarization (Fig. 3B). In addition, the monolayer itself exhibited cells with much more diverse cell heights and shapes—overall, lacking the regular cobblestone appearance of normal monolayers on

Fig. 1. Newly synthesized epithelial (E) cadherin (E-cad)-green fluorescent protein (GFP) colocalizes with and traffics via the recycling endosome (RE) on its way to the cell surface. A: immunofluorescence to visualize endogenous E-cad using a canine-specific anti-E-cad antibody and actin with Alexa 488-phalloidin in MDCK cell monolayers. As expected, confocal microscopy analysis shows E-cad staining at cell junctions, on the basolateral membrane (see XZ reconstruction), with some intracellular staining of trafficking E-cad. DAPI, 4,6-diamidino-2-phenylindole. B: MDCK cells were cotransfected with human E-cad (hE-cad), Rab11-GFP, and human transferrin receptor (hTfR) and were then allowed to take up Texas Red-human transferrin (TR-hTfn) to label the RE. Staining for newly synthesized hE-cad and confocal microscopy analysis show the three proteins overlapping in ring-shaped organelles (arrows) in the cytoplasm, corresponding to REs. Nucleus (N) is indicated (dotted line). C: MDCK cells were cotransfected with hE-cad-GFP and hTfR, then allowed to take up TR-hTfn to label the RE, and imaged live using confocal microscopy. At the beginning of the time-lapse recording, the cells were photobleached outside the Golgi area in the green channel. Position of the vesicle of interest is indicated in each frame by an arrow. Top: a vesicle positive for hE-cad-GFP fuses with the RE, positive for TR-hTfn (frame 160 s to 192 s). Bottom: a vesicle positive for both TR-hTfn and hE-cad-GFP moves toward the cell surface and makes contact with the plasma membrane (frame 688 s to 736 s). D: Pearson’s correlation coefficient for colocalization between hE-cad-GFP and TR-hTfn in preconfluent and polarized cells was determined on three-dimensional reconstructions of independent cells (n = 30), and the average values are shown as a graph (error bars correspond to SD). Single-slice views of representative nonpolarized and polarized cells are shown on the left. R = 0.3548 ± 0.089 in preconfluent cells and R = 0.268 ± 0.025 in polarized cells, showing that biosynthetic E-cad colocalizes with the RE in both nonpolarized and polarized cells.

Fig. 2. RE inactivation and E-cad trafficking in polarized MDCKs. MDCK cells were cotransfected with hE-cad-GFP and hTfR and allowed to take up horseradish peroxidase (HRP)-hTfn (endosomal ablation) and TR-hTfn (to visualize the uptake). Chemical blockage of the RE was performed using diaminobenzidine (DAB) and H2O2, and the cells were then incubated for up to 4 days before analysis. Immunofluorescence was performed to visualize endogenous E-cad. Effects of the RE inactivation on both endogenous E-cad and hE-cad-GFP was visualized by confocal microscopy. Top: control cells (no H2O2) showed both endogenous E-cad and hE-cad-GFP at the plasma membrane in cells containing TR-hTfn (arrows). Bottom: inactivated cells (+ H2O2) in the monolayer (indicated by white dotted line) displayed internalized endogenous E-cad and hE-cad-GFP (see top arrows). Some cells without inactivated REs displayed a normal localization of endogenous E-cad and hE-cad-GFP (see bottom arrows). Scale bar is 10 μm.
E-cadherin was located at the basolateral membrane in the mutant-expressing cells, but it was found in an aberrant, discontinuous pattern, with notable “breaks” in the lateral membrane staining (see Fig. 3B, day 7, boxed area). E-cadherin was also present inside the cells, near the apical pole, and some of this staining colocalized with DN-Rab11-GFP. Western blot analysis of total E-cadherin protein showed similar amounts in the two Rab11-expressing cell lines (wild-
type and DN) during polarization (data not shown). Thus, mutation of Rab11 impaired uniform monolayer development, and it disrupted cell polarity and E-cadherin trafficking to the basolateral membrane. The abnormal appearance of E-cadherin at the apical membrane in the Rab11-DN-GFP cells suggested that a functional RE is required to correctly sort E-cadherin to the basolateral membrane.

We next examined whether Rab11 plays a role in E-cadherin transport and sorting once the cells are already polarized in a monolayer. Fully polarized Rab11-GFP stable cells were microinjected with hE-cad-Cherry and allowed to recover for 6 h before analysis (Fig. 3C). Confocal microscopy of the monolayer in the XZ plane revealed colocalization of the apical Rab11-GFP compartment with vesicles positive for hE-cad-Cherry, which was also present at the basolateral plasma membrane. This suggested that in polarized MDCK cells, biosynthetic hE-cad-Cherry traffics through the apical RE visualized by Rab11 on its way to the basolateral membrane. Wild-type MDCK cells transiently expressing hE-cad-Cherry with and without Rab11-GFP, dominant-negative DN-Rab11-GFP, or ΔS1-E-cad-Cherry were then examined (Fig. 3D). The recombinant hE-cad appeared correctly on the basolateral cell membrane together with endogenous E-cadherin. Some hE-cad-Cherry was also observed in the apical cytoplasm, consistent with our previous observation of it being in the Golgi or REs, en route to the plasma membrane. Cotransfection of Rab11-GFP did not perturb the basolateral expression of hE-cad-Cherry, as previously observed in Fig. 3C. However, coexpression of DN-Rab11-GFP in the subapical RE caused the missorting of newly synthesized hE-cad-Cherry to the apical membrane of polarized cells (Fig. 3D). The effects of DN-Rab11 on the trafficking of E-cadherin in fully polarized cells implicates the RE as an exocytic destination for this cargo, even in polarized cells. Finally, as further evidence that DN-Rab11 is causing missorting of E-cadherin during its exocytosis, we compared the fate of hE-cad in cells expressing DN-Rab11 with that of a targeting mutant of E-cadherin. The ΔS1-E-cad mutant has a critical dileucine motif ablated, and, as a result, it is missorted in MDCK cells, appearing on the apical membrane (28). Expression of ΔS1-E-cad-Cherry in MDCK cells here gives apical staining in pattern reminiscent of that caused by expression of DN-Rab11.

Rab11 is essential for E-cadherin trafficking and lumen formation during cyst morphogenesis. Many features of cells undergoing morphogenesis into polarized epithelia are revealed optimally during the growth of epithelial cysts (18). To examine the ability of Rab11-GFP (at low expression levels) and DN-Rab11-GFP MDCK cell lines to form cysts, cells were plated in Matrigel according to established protocols (26). Cells were grown for up to 10 days and were then fixed, stained, and examined by confocal imaging at various intervening times.

At 3 days, the Rab11-GFP cells began to form spherical structures with appearance of a lumen, delineated by strong actin staining (Fig. 4A). Rab11-GFP was expressed in the cytoplasm, in an increasing gradient from the basal to apical pole. By day 10, mature, patent Rab11-GFP cysts were formed with a single layer of cells organized spherically around a central hollow lumen. Rab11-GFP was localized in a tight band at the apical pole immediately underneath the actin staining (Fig. 4A), similar to the location observed for endogenous Rab11 in wild-type MDCK cysts (Fig. 4B). Endogenous E-cadherin localized at the basolateral membrane from the earliest time point and throughout cyst development (Fig. 4C). As further markers of polarity, we stained for ezrin-radixin-moesin in its phosphorylated form (pERM) and the tight junction-associated protein zonula occludens-1 ZO-1 (16). The pERM proteins are restricted to the subapical compartment of polarized epithelia where they link the plasma membrane and the cytoskeleton (for review, see Ref. 13). Accordingly, pERM staining appeared in a subapical pattern colocalized with the actin band (Fig. 4C). ZO-1 staining appeared in typical, single puncta on the apicolateral membranes between adjacent cells (Fig. 4D). Thus, cell polarity and cyst morphogenesis were unchanged in cells overexpressing modest levels of Rab11-GFP compared with cysts formed from untransfected cells examined over the same time course (Fig. 4B). In addition, the expression of Rab11-GFP did not perturb E-cadherin targeting to the basolateral membrane, one of the earliest events seen in cell polarization.

In contrast, expression of the DN-Rab11-GFP severely affected cyst formation. Most of the presumptive cysts we examined were dysmorphic from early stages (<3 days; Fig. 5A). The first notable difference was the lack of cuboidal cells in the forming cysts, which, instead, contained cells of diverse shapes and sizes. Second, the process of lumen formation was consistently disrupted by DN-Rab11-GFP expression, although to varying degrees, whereby some cysts formed no lumen at all, while others showed cells encroaching on malformed lumens (Fig. 5A). Accompanying this, the characteristic single-cell layer of cysts often gave way to regions of multilayered cells. Notably, DN-Rab11-GFP localized to the cytoplasm in a very reticulated pattern and never redistributed to the subapical position, typical of the RE (Fig. 5A). Actin and pERM no longer concentrated apically in the mutant-expressing cells but instead appeared over the entire cell surface (Fig. 5, A and B). Importantly, E-cadherin was also delivered to the entire cell surface in some cells, consistent with disrupted basolateral traffic (Fig. 5C). The degree of E-cadherin missorting varied with the dose of DN-Rab11-GFP in the forming cysts (Fig. 5C). Cysts expressing a low dose of DN-Rab11-GFP showed E-cadherin on the basolateral membrane of some cells, whereas other cells had E-cadherin over the total cell membrane. In cysts expressing DN-Rab11-GFP at higher levels, E-cadherin was always missorted to the entire surface of the cells. Intact staining of ZO-1 at tight junctions indicated that some aspects of cell polarity remained unchanged in the DN-Rab11-GFP cysts over a range of expression levels (Fig. 5D).

Thus, an excess of GDP-locked Rab11 in the form of DN-Rab11-GFP disrupts cyst morphogenesis. The disruption of lumen formation and the apical placement of actin and pERM implicates Rab11 and the RE in these processes, and our findings here in cysts recapitulate the aberrant targeting of E-cadherin seen in DN-Rab11-GFP-expressing MDCK monolayers.

Mistargeting of E-cadherin results in dysmorphic cysts. Since lateral cell surface placement of E-cadherin is an early event during cyst formation as shown above, E-cadherin missorting is likely to be a factor responsible for the abortion of cyst formation observed with the DN-Rab11-GFP MDCK cells. To address this, the ΔS1-E-cad sorting mutant (see Fig. 3D) was used to investigate the link between E-cadherin targeting and cyst formation. We generated a stable cell line
Fig. 4. Cystogenesis of MDCK cells expressing wild-type Rab11-GFP. Stable MDCK cells expressing Rab11-GFP were allowed to grow in Matrigel for up to 10 days before fixation, immunostaining, and confocal microscopy. L, cyst lumen. A: Rab11-GFP and actin staining in 3- and 10-day-old Rab11-GFP MDCK cysts shows Rab11-GFP in the subapical compartment of the cells early in the process, underneath the luminal actin staining (arrows). B: endogenous Rab11 and actin were stained in 10-day-old untransfected MDCK cysts. Similar to Rab11-GFP, endogenous Rab11 staining appeared in the apical compartment as a tight band, directly underneath the actin band. C: endogenous E-cad and phosphorylated ezrin/radixin/moesin (pERM) were stained in 3- and 10-day-old Rab11-GFP cysts. E-cad was observed at the basolateral membrane, whereas pERM localized to the subapical compartment of the cells. D: tight junction-associated protein zonula occludens-1 (ZO-1) staining in 10-day-old Rab11-GFP MDCK cysts appeared as single puncta at apicolateral cell membranes. Scale bar is 10 μm.
Fig. 5. Effects of dominant-negative DN-Rab11-GFP on cystogenesis. Stable MDCK cells expressing DN-Rab11-GFP were allowed to grow in Matrigel for up to 10 days before fixation, immunostaining, and confocal microscopy. A: expression of mutant DN-Rab11-GFP in the cells prevented normal development of the cysts, with disrupted patent lumen formation and altered morphology. The DN-Rab11-GFP (arrow) was expressed in a reticulated intracellular pattern. B: pERM and actin immunostaining were distributed over the entire cell surface in DN-Rab11-GFP cysts. C: E-cad location was assessed in cysts expressing increasing amounts of DN-Rab11-GFP (indicated as + to +++). E-cad was mistargeted to the entire cell in a dose-dependent manner with the DN-Rab11-GFP abundance in the cysts. D: tight junction-associated protein ZO-1 (arrow) immunostaining in 10-day-old DN-Rab11-GFP (++) cysts localized normally in tight puncta at the tight junctions. Scale bar is 10 μm.
expressing low amounts of ΔS1-E-cadherin-GFP and cultured them as cysts for up to 10 days (Fig. 6). Compared with untransfected cells, cyst formation in ΔS1-E-cadherin-GFP-expressing cells was impaired and resulted in dysmorphic cysts with, again, disrupted lumen formation. Indeed, most cysts remained as a ball of unordered cells with actin and pERM randomly localized around cell surfaces. Figure 6 demonstrates that within a cyst harboring a pseudolumen, ΔS1-E-cadherin-GFP was found on the apical side of cells facing the lumen.

Therefore, deliberate placement of E-cadherin on the apical membrane using the ΔS1-E-cad-GFP mutant also leads to loss of lumen formation and loss of cyst cell polarity. This further suggests that the correct sorting and trafficking of E-cadherin, via the RE, is critical for epithelial polarity and cyst formation. In addition, we have newly identified E-cadherin targeting via the RE as a critical event in epithelial lumen formation.

DISCUSSION

E-cadherin plays a pivotal role in adherens junction formation and in establishing and maintaining epithelial cell structure. E-cadherin is essential during development, from early embryogenesis through the later stages of organogenesis, as shown by genetic analysis in Drosophila and in knockdown mouse models (21, 38, 41). Although the involvement of E-cadherin in the formation of the adherens junction has been previously examined (34), the route and trafficking components required for E-cadherin exocytosis have not been fully documented. This work here studied the role of the RE and Rab11 in the delivery of E-cadherin to the basolateral membrane in monolayers at various stages of polarization and during morphogenesis in epithelial cyst formation. We showed in live and fixed cells that newly synthesized E-cadherin traffics through the RE and that inactivation of this organelle with HRP-Tfn effectively blocked surface delivery of E-cadherin, affecting long-term cell growth, cell polarity and cell-cell adhesion. Similar to findings in nonpolarized cells, E-cadherin trafficked via the Rab11-positive RE on its way to the plasma membrane in our polarized cells. Further experiments using a GDP-locked Rab11 mutant expressed in polarizing monolayers and cyst cultures demonstrated a role for Rab11 during cell polarization and epithelial morphogenesis. In both cases, expression of Rab11-DN-GFP correlated with mis-sorting of E-cadherin to the apical cell surface, as observed with a previously characterized sorting mutant of E-cadherin. Cell polarity was also compromised by the dual actions of a dysfunctional RE and the misplacement of E-cadherin. Notably, lumen formation was particularly compromised in Rab11-DN-GFP-expressing cysts, implicating the RE in this early and critical determinant of cyst formation. Taken together our findings show that Rab11 and the RE play ongoing roles in the exocytosis and sorting of E-cadherin during formation of polarized epithelia and thereafter in established, polarized monolayers and cysts.

Our findings support the growing concept of the RE as an important compartment for exocytosis of multiple proteins; the RE is often associated with the polarized delivery of proteins to cell surface domains in epithelial cells and in macrophages (2, 25, 29). In MDCK cells, the RE mediates the exocytic delivery of other basolateral proteins, VSV-G and TfR (2, 6, 17), although Gravotta et al. (17) showed TfR bypassing the RE on its direct journey to the cell surface in fully polarized epithelia. In the current study, newly synthesized E-cadherin still appears to traverse the RE throughout polarity. E-cadherin expressed in already polarized cells in monolayers colocalized transiently with the RE on its way to the cell surface. E-cadherin also maintained this dynamic overlap with the RE in cells forming cysts. Taken together our results suggest that Rab11 and the RE play ongoing roles in the exocytosis and sorting of E-cadherin during formation of polarized epithelia and thereafter in established, polarized monolayers and cysts.
polarized cells. Such a difference may depend on cargo sorting into different exit carriers leaving the TGN, where E-cadherin is loaded into golgin-97 carriers for transport to the RE (23); the carriers for other basolateral cargo are unknown. Further studies on the post-TGN carriers and the TGN or RE-based sorting requirements for different basolateral cargo proteins may reveal different mechanisms for their routes of cell surface delivery.

Increasing evidence suggests that some sorting does indeed occur within the RE. Sorting adaptors have been localized to the RE, including subunits of the AP1B complex which have been linked to the RE sorting of VSV-G protein and Tfr (2, 6). Basolateral sorting of E-cadherin by a membrane proximal dileucine motif (28), or by a type Iγ phosphatidylinositol phosphate kinase (22), also appears to occur at the RE. Other trafficking components such as the VAMP3 SNARE protein, also implicated in adaptor-mediated sorting and trafficking from the RE, might additionally regulate E-cadherin trafficking (12).

Although there is significant overlap between E-cadherin and RE markers, this represents only a small proportion of total cellular E-cadherin and is suggestive of a transient residence in the RE for this newly synthesized protein. Indeed, a requisite role for the RE in E-cadherin trafficking has been described in Drosophila, despite a rather limited colocalization of E-cadherin with Rab11 in pupal epithelial cells of the dorsal thorax (19). Similarly, E-cadherin was rarely found colocalized with Rab11 in germline stem cells of the Drosophila ovary, although Rab11-null animals exhibited less E-cadherin at the membrane than controls (4). These studies all point to a crucial but fleeting functional relationship between E-cadherin and RE proteins, during the relatively rapid transit of new proteins, as described previously for transport of cargos between the TGN and the RE (6). There may be different routes through the RE itself, with different exocytic cargo accessing different RE compartments and machinery. Thus, VSV-G is regulated by and colocalizes with Rab8 in the RE (1) but E-cadherin traffic is unaffected by Rab8 mutants (data not shown) and is instead linked here with Rab11 in the RE. In macrophages, we have previously demonstrated that exocytic cargo, in the form of different cytokines, is seemingly segregated within the RE (25). In MDCK cells, the RE has subcompartments or even adjacent organelles typically defined as the apical RE classically attributed to Rab11 localization and a more basal common RE marked by Tfn (40). E-cadherin appears to localize transiently with both Rab11 and Tfn and further high-resolution imaging will be needed to determine whether this is sequential movement through distinct RE domains or whether it involves dynamic or joint subdomains of the RE. Mapping other RE markers will eventually help to define the precise role of this compartment and its physical relationship with sorting and exocytic trafficking. Thus, specific marker(s) of the RE can be attributed to specific trafficking events, as recently illustrated by the Arf6-dependent, and Rab11- or Rab27-independent pathways identified for recycling lipid rafts out of the RE to the plasma membrane during re-adhesion (3).

Disrupting RE function mistargeted E-cadherin to the apical membrane both in MDCK cells monolayers and in cysts. In assessing other markers of cell polarity, we found that ZO-1 at tight junctions was not disrupted in DN-Rab11 cells, suggesting that ZO-1 location is independent of the RE and is preserved in the presence of mistrafficked E-cadherin. Since ZO-1 itself is a cytoplasmic protein, it is unlikely to need the RE for trafficking to the plasma membrane. Despite the disrupted trafficking of E-cadherin in DN-Rab11 cysts, it appears that sufficient E-cadherin was still delivered to lateral membranes to support adherens junction formation and the subsequent assembly of tight junctions. Parenthetically, DN-Rab11 cells did have aberrant localization of normally apical pERM and F-actin, consistent with the novel finding here that apical membrane development is hindered by disruption of the RE and by E-cadherin mistrafficking. Of note, Rab11 is required for vesicular insertion along the lateral surface and for membrane growth during Drosophila cellularization (33). Reinforcing this notion, our mutant DN-Rab11 cells never formed a normal cyst structure, instead displaying cells of diverse sizes and heights consistent with disrupted membrane insertion. The aberrant apical delivery of E-cadherin in DN-Rab11 cells, emulated the missorting induced by the ΔS1-E-cad sorting mutant. Because this mutant still binds β-catenin, which also gets missorted in MDCK monolayers (27), we speculate that the Rab11 mutation is disrupting cystogenesis by having potential adhesive E-cadherin all around the cell perimeter instead of confined to lateral membranes.

Nonetheless, the aberrant delivery of E-cadherin to the apical membrane with DN-Rab11-GFP or with the sorting mutant ΔS1-E-cadherin has serious consequences for cyst formation, in particular the early, critical process of lumen formation. Thus, one important consequence of sorting in the RE is the exclusive targeting of E-cadherin to the basolateral membrane to engage epithelial polarity from early stages and to ensure morphogenesis is on track thereafter. Recent studies suggest an essential role for E-cadherin early on in establishing cell polarity (7, 30), and one that can be independent of cell-cell adhesion (9). Cells in these situations often retained cell-cell contacts. Our findings are consistent with earlier suggestions (9) that lateral membrane E-cadherin mediates a targeting patch for establishing cell polarity and lumen formation. During cyst morphogenesis, lumen formation is an early and critical step as it denotes the acquisition of the polarized phenotype of the cells. In epithelial cells, PTEN induces the apical segregation of phosphoinositides, apical membrane proteins, and Cdc42, for lumen formation (26). Our results suggest that the RE’s critical role in lumen formation probably involves the trafficking of some of these proteins in addition to E-cadherin. The current study of MDCK cyst morphogenesis served to link lumen formation to Rab11 and RE function, and both of these aspects to E-cadherin targeting. Thus, this study provided new evidence in a mammalian system for the roles of the RE in E-cadherin trafficking and epithelial morphogenesis that were previously shown in Drosophila (8, 19, 39).

In conclusion, our study expands the function of the RE as a necessary compartment for the correct trafficking and sorting of E-cadherin to the plasma membrane during epithelial polarization and in mature epithelia. During morphogenesis, Rab11 is required for cyst formation including normal trafficking of E-cadherin, for development of polarity and for lumen formation.

**ACKNOWLEDGMENTS**

We thank members of the Stow lab and colleagues as stated for kindly providing reagents. Confocal microscopy was...
performed at the Australian Cancer Research Council (ACRF)/Institute for Molecular Bioscience Dynamic Imaging Facility for Cancer Biology, which was established with the support of the ACRF.

GRANTS

This work was supported by a fellowship and grants from the National Health and Medical Research Council of Australia (to J. L. Stow).

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