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

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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. 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
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 (hTfR), 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 clones with relatively low expression levels were selectively grown. Clones with high expression levels were passaged and maintained in medium containing Geneticin (G418 Sulphate; Invitrogen) for 10–14 days. Surviving clones 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 expression of hE-cad-GFP, before labeling of the RE using human transferrin (hTfn) as described in experiments here; two or more clonal lines were used for most investigations. For antibodies requiring methanol fixation, incubation with methanol was performed for 30 min, permeabilized in 0.5% 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 in PBS for 30 min, permeabilized in 0.5% Triton X-100 for 10 min, blocked in PBS/0.3% 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 reiterated 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 toward 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), and TR-hTfn was purchased from Molecular Probes.

RESULTS

E-cadherin traffics 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.
C548 RECYCLING ENDOosome IN E-CADHERIN TRAFFICKING

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The image contains several panels labeled A, B, C, and D, each depicting microscopic images of cellular structures.

**Panel A:**
- **E-cad** shows an image of cellular junctions.
- **Actin** highlights the cytoskeletal network.
- **Merge** combines both E-cad and Actin images.
- **E-cad + dapi** shows cell nuclei labeled with DAPI.

**Panel B:**
- **hE-cad** and **Rab11-GFP** images are overlaid with **TR-hTfn** and their **Merge**.

**Panel C:**
- Time-lapse images showing cellular processes over 688 seconds with **hE-cad-GFP** and **TR-hTfn**.

**Panel D:**
- **Non polarized** and **Polarized** conditions are compared with **hE-cad-GFP/ TR-hTfn**

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Pearson's correlation coefficient (R) graph is shown with non-polarized and polarized conditions.
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
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-

Fig. 3. Trafficking and sorting of E-cad requires functional RE and Rab11. A and B: MDCK cells were stably transfected with either Rab11-GFP (A) or dominant-negative (DN)-Rab11-GFP (B). E-cad (in red) location in relation to Rab11-GFP and DN-Rab11-GFP was assessed (arrows) in polarized cells by confocal microscopy in the XY and XZ planes. A: at days 1 and 2, E-cad was found at the cell membrane and in the cytoplasm (arrow). After day 4, the majority of E-cad was at the cell membrane. Rab11-GFP staining moved increasingly toward the apical pole as the cells became polarized (XZ-axes reconstructions). B: in the DN-Rab11-GFP cells, cell morphology was affected, with cells lacking the cobblestone shape of normal cells and DN-Rab11-GFP dispersed throughout the cytoplasm. E-cad was present at the basolateral plasma membrane but also persisted intracellularly (arrows at days 2 and 4) and appeared at the apical pole (asterisks at days 2 and 4). Staining at the plasma membrane was distributed in a discontinuous pattern (boxed areas at day 7). C: polarized MDCK cells stably expressing Rab11-GFP were microinjected with hE-cad-Cherry, and the protein was expressed for 6 h before analysis. A representative cell is shown in the XY plane. Three different Z sections of the cell are indicated in dotted lines (1–3) and are shown beneath. E-cad vesicles can be observed together in the Rab11-positive compartment and at the plasma membrane. D: fully polarized MDCK cells were transfected with hE-cad-Cherry with or without DN-Rab11-GFP, or with sorting mutant ΔS1-hE-cad-Cherry. Endogenous E-cad was immunostained. Result shown is representative of 4 independent experiments. DN-Rab11-GFP caused the mistargeting of hE-cad-Cherry to the subapical compartment. Similarly mistargeted ΔS1-hE-cad-Cherry was also found at the apical poles of cells. Scale bar is 10 μm.
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 implicate 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.

Mismatching 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 aberration 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 missorting 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 and then moves to the cell surface. E-cadherin also maintained this dynamic overlap with the RE in cells forming cysts. Taken together our results suggest that E-cadherin needs a functional RE compartment and GDP-GTP cycling Rab11 for its correct targeting to the basolateral membrane even in polarized cells. Our results reveal distinct differences in the dependence of different cargo on the RE for trafficking in
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 1γ 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 cellular E-cadherin and is suggestive of a transient residence in the plasma membrane during re-adhesion (3).

Disrupting RE function misdirected E-cadherin to the apical membrane in DN-Rab11 cysts with the sorting mutant A51-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 that 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.

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REFERENCES


