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Regulation of Tight Junction Assembly and Epithelial Polarity by a Resident Protein of Apical Endosomes

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Abstract

The establishment of tight junctions and cell polarity is an essential process in all epithelia. Endotubin is an integral membrane protein found in apical endosomes of developing epithelia when tight junctions and epithelial polarity first arise. We found that the disruption of endotubin function in cells in culture by siRNA or overexpression of the C-terminal cytoplasmic domain of endotubin causes defects in organization and function of tight junctions. We observe defects in localization of tight junction proteins, reduced transepithelial resistance, increased lanthanum penetration between cells and reduced ability of cells to form cysts in three-dimensional culture. In addition, in cells overexpressing the C-terminal domain of endotubin, we observe a delay in re-establishing the normal distribution of endosomes after calcium switch. These results suggest that endotubin regulates trafficking of polarity proteins and tight junction components out of the endosomal compartment, thereby providing a critical link between a resident protein of apical endosomes and tight junctions.

Keywords

endosomes; endotubin; epithelia; polarity; tight junctions

Epithelial cells serve as an adaptable and selective barrier to the diffusion of macromolecules between the epithelial lumen and the serum; in mammalian cells, this barrier function is maintained by precise regulation of the tight junctional complexes at the apical pole of the cell (1–4). Furthermore, because the apical domain is the interface between the ‘outside world’ and the bloodstream, the ability of the tight junction to selectively exclude antigens or pathogens is critical to normal function, and increased epithelial permeability is correlated with infection and development of inflammatory disease (5–7). However, tight junction proteins undergo remodeling under normal conditions, and this remodeling is regulated by controlled internalization and recycling of tight junction proteins. The small GTPases Rab13/Rab8 and the effector JRAB/MICAL as well as the cdc42GAP/scaffolding protein complex Amot/Rich1 regulate this process, but the mechanism by which junctional proteins are sorted and recycled is poorly understood (8–11).

The assembly and maintenance of tight junctions is inextricably linked to the preservation of polarity of epithelial cells. Thus, not only do tight junctions serve to preserve the distinct protein and lipid compositions of the apical and basolateral plasma membrane domains (12–

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14), but assembly of tight junctions is highly coordinated by proteins that regulate epithelial cell polarity. For example, the Par/aPKC complex, together with adhesion molecules and scaffolding proteins such as crumbs and junctional adhesion molecule (JAM), designate the site of tight junction assembly that define the apical and basolateral membrane domains (15–20). Also, maintenance of tight junction structure relies on polarity proteins, which may modulate targeted insertion of newly synthesized proteins to the junctional complex and recycling of endocytosed junctional proteins to the junctional region.

Endotubin (ET, also referred to as apical endosomal glycoprotein, or AEGP in the NCBI database, Swiss-Prot: Q6UXC1.2) is an integral membrane protein that was first identified as a resident of apical endosomes in developing intestinal epithelial cells (21,22). ET is found in the endosomes of epithelial tissues and is first expressed when these tissues develop epithelial polarity [(23,24), Wilson et al., unpublished data]. ET interacts with the small GTPase Rab14, which has been shown to control trafficking of someapical membrane proteins (25).

To analyze the function of ET, and in particular its role in epithelial integrity and polarity, we have used two tools. First, we have used siRNA to generate an ET knockdown in MDCK cells. Second, we have generated a construct that contains a fusion between green fluorescent protein (GFP) and the C-terminal cytoplasmic domain (CD) of ET. This domain of ET has been implicated in apical sorting (25–27), and we hypothesized that overexpression of this domain, as part of a GFP-fusion protein, might interfere with the function of endogenous ET, i.e have a dominant-negative effect on the cell. By interfering with the function of ET by either of these two methods, we find that ET has a role in tight junction assembly and cell polarity. We suggest that ET may provide an integral membrane scaffolding in the apical endosomes for polarity and tight junction proteins, and that it could modulate targeted recycling of tight junction components and ultimately cell polarity.

Results

The domain structure and intracellular localization of endotubin in epithelial cells

ET is a 140-kD protein encoded by a gene that was previously cloned and sequenced (28). It is a transmembrane (TM), endosomal glycoprotein that contains extracellular MAM repeat domains (MAM), two LDLa domains containing cysteine-rich repeats and putative calcium-binding sites (LDLa), a TM domain and a C-terminal CD, as shown in Figure 1A.

Previous studies showed that ET is present in the apical region of the epithelial cells of the developing ileum [(22); Figure 1B] and is associated with Rab14, a protein involved in apical sorting (27). ET is present in apical endosomes (Figure 1C, D), and colocalizes with ricin, an endosomal marker (Figure 1E). To further understand the role of ET in this process, we used immunoelectron microscopy to identify the precise subcellular localization of ET in the epithelium of the ileum. In particular, we were interested in determining whether ET is localized in the regions of the junctions. As shown in Figure 1C, although ET is present in the apical endosomes [as previously reported, (22)], it is absent from the tight junctional region and lateral membranes of the cells.

Use of endotubin siRNA to identify the role of endotubin in MDCK cells

We used siRNA to reduce the levels of ET present in MDCK cells to study the role of this protein in epithelial cell function. MDCK cells containing either ET-siRNA or scrambled (control) siRNA were generated (see Materials and Methods). The effect of these siRNAs on levels of ET in the cells was examined. As shown in Figure 2A, the level of ET is drastically reduced in cells containing ET-siRNA, compared with cells containing scrambled siRNA. This reduced level of ET is specific and not because of lower levels of sample loaded on the

gel, as the levels of actin are the same in cells containing either ET or scrambled siRNA (Figure 2A). Furthermore, knockdown of ET did not affect the levels of the tight junction proteins occludin and claudin-1. These findings indicate that this siRNA specifically reduces the levels of ET in MDCK cells.

To test the effect of reduced levels of ET on epithelial integrity, we grew MDCK cells on filters for3 days, and measured the transepithelial resistance (TER) of the cells (see Materials and Methods). As shown in Figure 2B, the TER is significantly reduced (from 204 ± 5 to 175 ± 4 Ωcm2) in cells containing ET-siRNA compared with cells containing scrambled siRNA. While this effect is modest, it is important to note that knockdown of some components of the tight junctions, such as claudin-1 and occludin, do not result in changes in tight junction structure or TER (29,30). This finding suggests a role for ET in the structural integrity of the epithelium.

To determine whether the ET knockdown-induced reduction in TER is caused by defective tight junctions, we examined three proteins, claudin-1, occludin and ZO-1, which are normally found in tight junctions. Immunoblotting showed that the protein levels of all of these proteins remained the same after ET knockdown (Figure 2A). However, confocal immunofluorescence microscopy of cells transfected with control or ET-siRNA together with a GFP transfection marker showed that, although the junctional distribution of ZO-1 is not affected by the reduction in levels of ET (Figure 2C, bottom panel), both claudin-1 and occludin are present at greatly reduced levels at the lateral membranes (Figure 2C, top and middle panels). This finding suggests that the reduced TER of these cells may be because of a defect in the structure of the tight junctions between the cells. This does not appear to be because of off-target effects of ET knockdown, as cotransfection with ET-siRNA together with full-length ET that is not susceptible to knockdown restored the lateral cell labeling of claudin-1 (Figure 2D).

Disruption of endotubin function by overexpression of the cytoplasmic domain of endotubin

To further test the role of ET in apical sorting and junction formation, we made a construct encoding a GFP-fusion protein and the ET-CD. DNA encoding the C-terminal CD of ET was fused to the 3 end of DNA encoding GFP (Figure 3A). This fusion construct (GFP-CD) was transfected into MDCK cells, and these cells were then compared with MDCK cells containing a construct with GFP alone (see Materials and Methods).

Effect of GFP-CD on localization of expressed endotubin

To examine theeffect of GFP-CD on the localization of ET, we expressed GFP-CD in MDCK cells overexpressing full-length ET, as ET is normally expressed at levels too low to be imaged (26). We then used antibodies against the lumenal domain of ET to detect the full-length ET (Figure 1A). As shown in Figure 3B, the distribution of full-length ET is disrupted in cells coexpressing GFP-CD fusion protein, compared with cells expressing GFP. In particular, in cells containing GFP-CD, full-length ET is no longer localized to just the apical regions of the cells. Thus, the fusion construct has a dominant-negative effect on the targeting of ET.

To determine whether ET isaberrantly localized because of a direct effect of the GFP-CD fusion protein at the apical endosomes, we asked whether GFP-CD is localized to the apical endosomes of these cells. We used Rab11 as a marker for apical endosomes, and determined whether Rab11and GFP-CD colocalize. As shown in Figure 3C, these two proteins colocalize, indicating that GFP-CD is exerting its effect at the normal cellular location of ET.

Effect of GFP-CD on tight junctions in MDCK cells

To test the effect of GFP-CD on the cells, we next asked whether there was any effect of the GFP-CD fusion protein on the TER of MDCK cells grown in culture. As shown in Figure 3D, cells expressing GFP-CD have a significant reduction in TER (221–178 Ωcm2), compared with cells expressing GFP alone. This finding indicates that the GFP-CD fusion protein interferes with both the function of ET and in the integrity of the epithelium. This result agrees with our findings from the knockdown experiments with ET-siRNA (see above).

To learn more about the mechanism by which GFP-CD fusion protein interferes with the TER of MDCK cells, and in particular whether this is because of defects in the tight junctions, we fixed cells in the presence of lanthanum nitrate and processed them for transmission electron microscopy. Penetration oflanthanum into the intercellular space between cells occurs more when tight junctions are open (permeable) than when they are closed (less permeable). Therefore, the junctions between cells stain more effectively with lanthanum in the presence of open junctions. The strain of MDCK cells used in this study (MDCK II) is a relatively low resistance cell line (31,32), and some penetration of lanthanum between the cells is expected at steady state. However, as shown in Figure 3D, there is a significant increase (from 50 to 73%) in lanthanum-positive (open) junctions in cells containing the GFP-CD construct, compared with cells containing just GFP. This finding indicates that disruption of ET by overexpression of the CD leads to defective tight junctions.

We expectedthat the defects in the tight junctions might reflect a defect in localization of various junction proteins. We therefore examined the distribution of various junction proteins in MDCK cells expressing GFP-CD compared with those expressing GFP. We found that the distributions of occludin, ZO-1 and claudin-1 were all similar in epithelia formed from cells containing either GFP or GFP-CD (Figure 3E). Furthermore, immunoblots showed indistinguishable levels of occludin, ZO-1 and claudin-1 in extracts from cells expressing either GFP-CD or GFP (Figure 3F). Thus, the defective junctions do not appear to be caused by alterations in either distributions or levels of any of these three proteins. However, the finding that the localizations of occludin and claudin-1 are not affected by GFP-CD is in contrast to our finding of the effect of siRNA on the distribution of occludin and claudin-1 (see Discussion).

The experiments described earlier suggested a role for ET in tight junction organization and function. ET could have a role in establishment and/or maintenance of tight junctions. To determine whether ET is important in establishing junctions between adjacent cells, we treated MDCK cells containing GFP-CD or GFP with low concentrations of calcium. Under these conditions, junctions open due to the loss of calcium-dependent adhesion. We then re-exposed cells to normal concentrations of calcium to allow junctions to reform (calcium switch), and measured the TER at various times after addition of normal calcium. As shown in Figure 4A, cells containing GFP-CD re-establish TER at a slower rate than cells containing GFP alone. Indeed, 4 h after the addition of normal calcium, cellscontaining GFP-CD have less than 50% of the TER of cells containing GFP. This result suggests that overexpression of the CD of ET interferes with the ability of endogenous ET to re-establish tight junctions.

To determine whether delayed re-establishmentof TER is associated with delayed localization of junction proteins in cells containing GFP-CD, we examined the localization of occludin and ZO-1 at various times following the addition of normal calcium. In cells expressing GFP-CD, compared with cells expressing GFP alone, there was a marked reduction in rate of reappearance of both occludin and ZO-1 in the regions between adjacent

cells (Figure 4B). In particular, by 2 h, cells containing GFP showed occludin and ZO-1 at the lateral membranes between adjacent cells, but cells containing GFP-CD did not (Figure 4B). Cells overexpressing the CD of ET do eventually establish normal localization of occludin and ZO-1, as the patterns seen at 4 h are indistinguishable from those seen in cells with GFP (Figure 4B). This finding suggests that overexpression of GFP-CD interferes with normal localization of the junction proteins occludin and ZO-1, and suggests the delayed re-establishment of TER in cells that are reforming junctions is because of delayed delivery of proteins to these sites.

Effect of GFP-CD-mediated disruption of endotubin function on endosomal organization

Low concentrations of calcium not only disrupt tightjunctions but also affect the organization of apical endosomes. For example, in control MDCK cells (containing GFP alone) Rab11, which is a marker for apical endosomes, has an altered distribution immediately after the addition of normal calcium, comparedwith that seen 4 h later (Figure 4C). To test whether GFP-CD affects the re-establishment of normal endosomal organization, we compared the Rab11 distribution in MDCK cells expressing GFP-CD with that in cells expressing GFP alone. As shown in Figure 4C, endosomal reorganization is delayed in cells containing GFP-CD compared with that in cells containing GFP alone. This result suggests that ET has a role not only in re-establishing normal junctions but also in re-establishing normal endosomal distributions. Interestingly, GFP-CD is observed at the lateral membranes 2 h after calcium switch (Figure 4D). This may indicate that, although ET is concentrated in apical endosomes at steady state, cycling of ET to lateral membranes could be an important component of junction assembly.

Effect of disruption of endotubin function upon cell spreading

The delay of junction formation seen in cells expressing GFP-CD could be because of delayed spreading of the cells, which would result in failure of the cells to establish intercellular contacts. To test if cells expressing GFP-CD were not in contact with each other after calcium switch, we performed calcium switch followed by live-cell imaging of the same region of the epithelium (see Materials and Methods). As shown in Figure 5, at time 0, before addition of normal calcium-containing media, the cells are rounded but densely packed. After 30 min in normal calcium, cells expressing GFP are nearly fully spread and are in contact with adjacent cells. In cells expressing GFP-CD, the cells are not as well spread, but most appear to be in contact with adjacent cells. After 90 min in normal calcium medium, cells expressing GFP-CD still remain somewhat rounded, but all the cells are in contact with adjacent cells. Furthermore, as shown in Figure 4D, at 2 h after calcium switch, we observe that the lateral membranes of cells expressing GFP-CD are in contact with GFP-CD enriched at these domains. Together, these results suggest that the failure of the GFP-CD cells to assemble tight junctions after calcium switch is not because of failure of the cells to appose their lateral membranes.

Effect of disruption of endotubin function, by siRNA or overexpression of GFP-CD, on epithelial cyst formation

When MDCK cells are grown in three-dimensional culture, they develop into cysts containing lumens with the apical domain on the luminal surface (33). To achieve this, the cells must organize themselves such that the apical membranes of the cells in the cyst face inwards, and the basal regions are in contact with extracellular matrix outside the cyst. Cysts were labeled with antibodies against the apical protein gp135 (podocalyxin) and were quantified as normal when there was a single, central lumen, and abnormal when there was more than one large gp135-labeled structure. As shown in Figure 6A, cells containing control (scrambled) siRNA form cysts with appropriately oriented cells and lumens at high frequency (68%). However, only 22% of cells containing ET-siRNA form normal cysts and

instead establish multiple lumens. Immunofluorescent labeling showed that cysts containing ET-siRNA had multiple lumens of various sizes (Figure 6A, bottom panel). We obtained similar results with cells stably expressing GFP-CD (Figure 6B). Cells expressing GFP alone form cysts with normal lumens more than 80% of the time, whereas cells containing GFP-CD form cysts with normal lumens only about 40% of the time. Interestingly, the lateral marker E-cadherin was present on the lateral membranes in cells expressing both GFP and GFP-CD, although there may be decreased expression of E-cadherin in cells that have grown within the cyst lumen (Figure 6B, bottom panel). As in the cysts transfected with ET-siRNA, GFP-CD-expressing cysts contained multiple lumens that labeled with gp135. These results indicate that the disruption of ET function affects the organization of cellsin relation to each other, and it seems likely that the defective junction formation described earlier leads to defective cell polarity in three-dimensional culture.

Discussion

Tight junction integrity is essential to the maintenance of barrier function and polarity of epithelia. Here, we show that an integral membrane protein of the apical endosomes (ET) plays a role in the assembly of tight junctions and epithelial polarity, presumably through regulation of the recycling of tight junction components after internalization into the apical endosomal compartment. Our data support the model depicted in Figure 7, where ET forms a complex, in theendosome, with Rab14 and cargo to be delivered to the plasma membrane. After budding of the transport vesicle, this complex is delivered to the junctional region. ET then recycles back to the apical endosomal compartment.

In this study, we describe the use of two tools (GFP-CD and ET-siRNA) to analyze the function of ET in cells. We show that both of these tools are effective at disrupting the function of ET. In the case of ET-siRNA, cells show lower TER and reduced localization of the tight junction proteins occludin and claudin-1 to the tight junction. These findings suggest that the reduced levels of ET lead to a reduction in localization of junction proteins, which in turn leads to compromised ability of the tight junctions to form (as measured by TER). While displacement of occludin or claudin-1 per se may not cause lower TER (29,34), loss of these molecules could indicate disruption of other tight junction components that leads to the observed phenotype.

The second tool, GFP-CD, is also effective at disrupting the function of ET. Overexpression of GFP-CD leads to aberrant localization of expressed full-length ET, suggesting that GFP-CD interferes with the ability of ET to interact with a cellular component required for correct localization. We also found that overexpression of GFP-CD leads to reduced TER and increased tight junction permeability. The CD of ET is known tohave a role in apical sorting (26,27), and it is likely that overexpression of this domain interferes with the ability of endogenous ET to successfully carry out this function. So, in this case, GFP-CD acts in a dominant-negative manner. For example, the overexpressed CD may interact with one or more proteins that normally bind to endogenous ET such as Rab14(25), preventinginteraction with full-length ET.

Interestingly, unlike ET-siRNA, GFP-CD does not clearly affect the steady-state distribution of occludin or claudin-1, but does affect the reassembly of tight junctions after calcium switch. In the case of siRNA, it is likely that reduced availability of ET to carry out apical sorting leads to reduced localization of junction proteins. However, in the case of GFP-CD, as endogenous ET is still present, the GFP-CD in apical endosomes may only partially prevent functions thatrely on full-length ET. While we do not know if ET interacts directly with tight junction proteins, it could modulate recycling of integral membrane proteins

through either direct binding or by providing a membrane domain that organizes junctional components.

Tight junctions control the barrier function of epithelia by providing an adaptable structural block to paracellular movement (1,3,35). Tight junction permeability can be modulated by bacterial toxins, viral proteins and proinflammatory cytokines (1,3,35–41). Importantly, some of these processes result in endocytosis of tight junction proteins, which likely provides an important regulatory pathway for junction assembly and disassembly (2,9,42–44).

The membrane trafficking pathways that modulate the internalization and recycling of junctional proteins remain incompletely understood. For example, both clathrin- and caveolin-dependent pathways have been shown to mediate occludin uptake (2,45), and both apical and basolateral endosomal compartments have been implicated in recycling back to the tight junction (10,41). Like other basolaterally targeted proteins (46), some tight junction proteins harbor basolateral sorting signals, and are recycled to the junction via an AP1B-mediated sorting event (47). In contrast, the adhesion protein E-cadherin has been shown to traffic through Rab11-positive endosomes in a μ1B-independent manner (48). Furthermore, apical endocytosis has been shown to stabilize polarity proteins and adherens junctions in Drosophila (15), suggesting that the apical endocytic pathway may serve both to regulate tight junction structure and polarity. Recent work has implicated trafficking from endosomes as an important regulator of epithelial polarity (11,49,50), and polarity complexes, including the aPKC/Par3/Par6 complex, together with other polarity mediators, such as crumbs and PALS1-associated tight junction protein (PATJ), promote assembly of the tight junction (16,18,51–53). Our finding that interfering with ET function both impairs tight junction structure and disrupts the ability of the cells to form single lumen cysts in three-dimensional culture further supports the connection between tight junction assembly and epithelial polarity. As ET contains protein:protein adhesion domains, is present in apical endosomes and binds the regulator of apical targeting Rab14, we propose that ET's role is to act as a scaffolding to generate a membrane domain in the apical early endosomes for assembly of complexes that contain tight junction proteins as well as polarity regulators.

Materials and Methods

Culture media and reagents

Cell culture media were obtained from Invitrogen, FBS from Gemini Bioproducts. Transfection reagents were from Amaxa Biosystems. Rabbit anti-occludin, anti-claudin-1 and anti-ZO-1 were from Zymed Laboratories. Rat anti-E-cadherin (DECMA) was from Sigma-Aldrich. Mouse anti-gp135 was a gift from Karl Matlin (University of Chicago). Anti-ET (5F11) has been described (22,54). Secondary antibodies were from Jackson ImmunoResearch Laboratories. Hoechst dye was used at 300 nm and TOPRO-3 was used at 1 μm and were from Molecular Probes. Anti-Rab11 antibody was a gift from Dr Jim Goldenring at Vanderbilt University. Cytosine arabinoside was from Calbiochem. All other reagents used were from Sigma Chemical Company.

Cell culture and transfection

MDCK type II cells were maintained as described (26). Cells were transfected with full-length ET, GFP-CD or GFP control vectors as indicated. Stably transfected cells were maintained in media supplemented with 400 μg/mL G418. The cDNA coding sequence for the 38 amino acid cytoplasmic tail of ET was amplified by polymerase chain reaction (PCR) and ligated into the TOPO vector (Invitrogen). The insert was isolated by digestion with EcoR1 and BamH1 and ligation into pEGFP-C2 mammalian expression vector(BD

Biosciences Clontech). The construct was sequenced to ensure in-frame insertion into the coding region. These plasmids or the empty vector as control were transfected into MDCK cells using Amaxa nucleofection program P29. Two clones of each transfected cell line were analyzed to ensure similar phenotype and eliminate the possibility of a random insertion event being responsible for observed differences. All experiments were also conducted in untransfected MDCK cells to verify similarity with control MDCK-GFP.

For polarity studies, cells were seeded at confluence and grown on Costar 0.4-μm pore Transwell filters (Corning Life Sciences) for 4–5 days. For studies on non-polarized cells, cells were either imaged directly on the culture plate or plated onto sterilized coverslips.

MDCK cysts were grown by plating cells in a single cell suspension in multichamber slides coated with Matrigel and grown for 4–7 days in media containing 2% Matrigel.

siRNA knockdown of endotubin

siRNA knockdown of ET was accomplished using the following sequences: sense 5′-GGAGUUCCAGAUCGUGUUU-3′ and antisense 5′-AA ACAGCUGGAACUCC-3′ and controls consisted of scrambled siRNA (Ambion). siRNA against ET or scramble was transfected into MDCK cells using Amaxa electroporation system (Amaxa), as per manufacturer's instructions and knockdown was confirmed by western blotting. Following transfection, cells were immediately plated on plates or in Matrigel, as necessary for subsequent experiments.

Transmission electron microscopy

For lanthanum permeability studies, stably transfected MDCK-GFP-CD and MDCK-GFP cells were fixed in 3% glutaraldehyde in 0.1 m cacodylate buffer containing 1% lanthanum nitrate from the apical side and fixative lacking lanthanum on the basolateral side. Further processing was as described (55). Sections were examined using a Philips 410STEM at 80 kV and images were acquired using an AMT-XR40 (Advanced Microscopy Techniques) digital camera and processed using Adobe Photoshop software. For electron microscopic immunocytochemistry, neonatal rat ileum was fixed and embedded as described (22). Sections were treated with anti-ET for 2 h at room temperature, washed and incubated with unconjugated rabbit anti-mouse antibody (5 mg/mL) for 30 min, followed by protein A conjugated to 5 nm gold for 30 min.

Immunofluorescent labeling and confocal microscopy

Cells were fixed in 4% paraformaldehyde, washed and blocked in buffer containing 0.05% saponin and 10% FBS in PBS. Cells were incubated with primary antibodies, washed in PBS and incubated with secondary antibodies for 30 min. Nuclei were labeled by TOPRO-3 or DAPI and mounted with Aqua Poly/Mount (Polysciences Inc.). Cells were imaged using a 100× oil immersion objective, numerical aperture (NA) 1.4. Simultaneous two-orthree-channel recording was performed using excitation wavelengths of 488, 533 and/or 633 nm through a z-stack of 10–50 μm in thickness. Images were obtained using Zeiss LSM Laser Scanning System and Image Browser software packages and processed using Adobe Photoshop software.

For ricin uptake experiments, cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-labeled ricin (Sigma) for 30 min at 4°C in serum-free media followed by warming to 37°C for 10 min. Cells were then fixed and processed for indirect immunofluorescence as described earlier.

Immunoblotting

Samples were solubilized by homogenization in buffer containing 20 mm Tris–HCl, pH 7.4, 1% TX-100, 1% sodium deoxycholate, 0.1% SDS, 100 mm NaCl plus the protease inhibitors aprotinin and leupeptin. Equal protein amounts (20 mg) were boiled in sample buffer and separated by SDS–PAGE. After electrophoresis, proteins were transferred to nitrocellulose, blocked with 5% milk/0.1% Tween-20 in TBS and incubated with specific antibodies followed by secondary antibody conjugated to horseradish peroxidase (1:10 000). Blots were developed using Pierce Supersignal West Pico and Dura-Extended chemiluminescence kits (Pierce). Blots were stripped using Pierce Restore Western blot stripping buffer and reprobed using monoclonal antibody against β-actin.

Calcium switch

Cells were seeded at confluence and grown on Costar Transwell filters for 4 days. Media were replaced with low-calcium media (S-MEM with 2% dialyzed FBS) (56,57)for 18–20 h. After replacement of calcium-containing media, cells were fixed at intervals for immunofluorescent labeling. TER was measured on separate filters using a Millipore MiliCell ERS voltohmmeter every 15 min after restoration of calcium-containing media. To inhibit proliferation, these filters were treated with 5 μm cytosine arabinoside.

For imaging of monolayers after calcium switch, cells were seeded at confluence in a 6-well plate and grown for 3 days. Cells were placed in calcium-free media for 30 min at 37°C and then placed in normal calcium media for the times indicated in the figure. The plates were oriented so that the same field was imaged at each time-point, and images were acquired using an inverted Olympus light microscope using Hoffman modulated optics.

Statistical analysis

Statistical comparisons were made using a simple t-test, and significance considered as $p < 0.05$. All data areexpressed as mean ± SEM.

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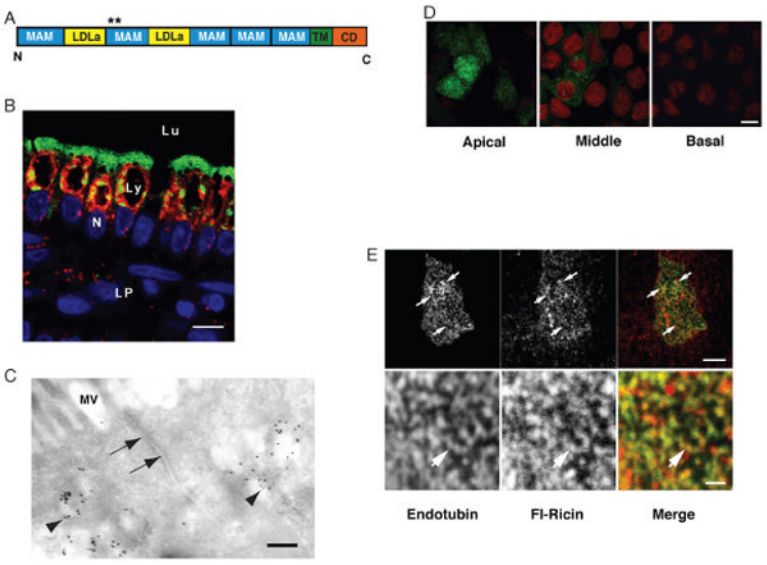

Figure 1.

Structure (A) and localization (B–E) of endotubin in cells. A) Domain structure of the endotubin protein, showing extracellular MAM repeat domains, LDLa domain-containing cysteine-rich repeats and putative calcium-binding sites, the TM domain and a C-terminal CD. Asterisks (**) denote epitope recognized by antibody against full-length endotubin. B) Localization of endogenous endotubin in apical region of the ileum. Green, endotubin; red, lysosomes; blue, nuclei. Lu, lumen of ileum; Ly, lysosome; N, nucleus; LP, lamina propria. Bar, 10 μm. C) Immunoelectron microscopy of neonatal rat ileum enterocytes, showing localization of endogenous endotubin in apical endosomes. Cells were labeled by indirect immunoelectron microscopy with mouse anti-endotubin antibodies followed by protein A conjugated to 5 nm gold. Gold particles are concentrated in apical regions associated with endosomes (arrowheads), but not in microvilli (MV), the junctional region or lateral membranes between two adjacent cells (arrows). Bar, 1 μm. D) Confocal microscopy of MDCK cells stably overexpressing endotubin showing apical localization of endotubin (green). Cells expressing endotubin were labeled with mouse anti-endotubin antibodies, and fluorescein-labeled goat anti-mouse antibody, with the nuclei labeled with propidium iodide (red stain). Labeling of endotubin is restricted to the apical cytoplasm. Bar, 10 μm. E) Confocal microscopy of MDCK cells stably overexpressing endotubin, showing localization of endotubin in endosomes. Indirect immunofluorescence of endotubin (green, left panel) and fluorescence of the endosomal marker fluorescent ricin (red, Fl-ricin, middle panel) internalized for 10 min. Merged image shows colocalization (right panel), and arrows point to obvious patterns of colocalization. Lower panel: high magnification of apical endosomes containing endotubin (green) and fluorescent ricin (red). Arrows indicate colocalization. Top panel, bar, 5 μm; bottom panel, bar, 1 μm.

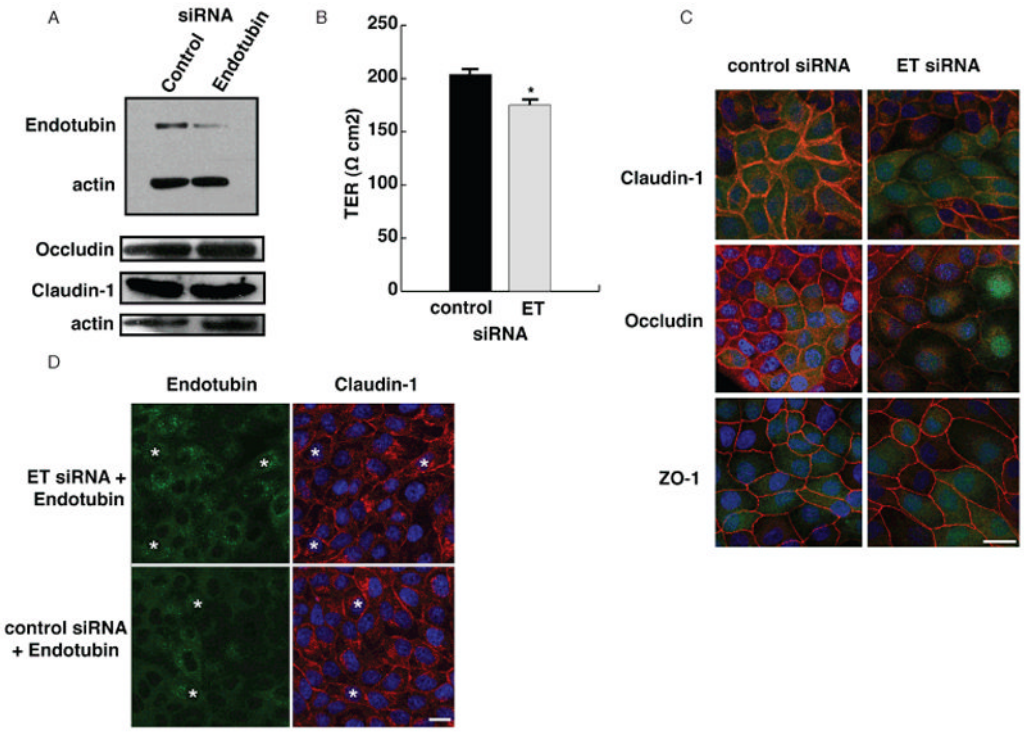

Figure 2.

Effect of ET siRNA on mdck cell tight junctions. A) Western blotting shows levels of ET and actin in cells treated with ET or control siRNA indicating effective knockdown of ET expression. The expression levels of the tight junction proteins occludin and claudin-1 are unaffected by ET knockdown. B) Effect of ET knockdown on TER of MDCK cells. Cells containing ET or control siRNA were grown on filters for 3 days, and the TER was then measured. The TER of ET knockdown cells is significantly less than that of controls (*$p < 0.05$). C) Effect of ET knockdown on localization of tight junction proteins. MDCK cells containing ET or scramble siRNA together with a GFP transfection marker were labeled for immunofluorescence microscopy of claudin-1 (red), occludin (red) and ZO-1 (red), and viewed by confocal microscopy. ET knockdown disrupts lateral labeling of claudin-1 and occludin, but does not affect ZO-1 distribution. D) Cells transfected with full-length ET (green, left panel) together with ET or control siRNA were labeled for claudin-1 distribution (red, right panel). Expression of full-length ET that is not susceptible to siRNA knockdown results in normal claudin-1 distribution at the lateral membranes, indicating that junctional effects are not because of off-target effects of the siRNA. Bar, 10 μm.

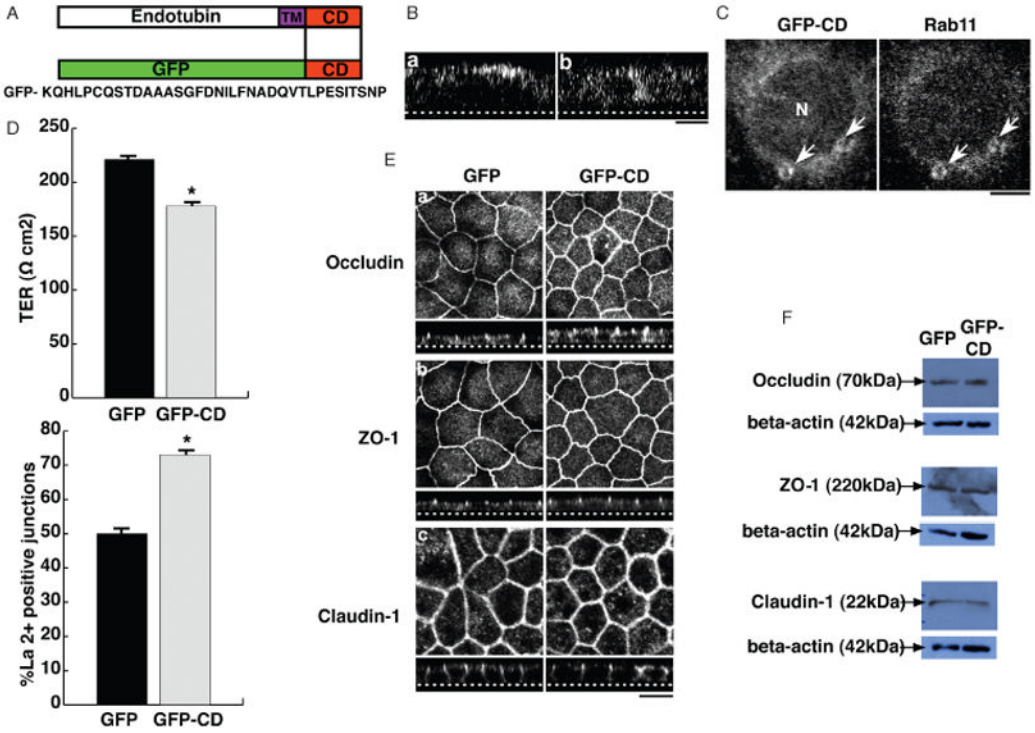

Figure 3.

Effect of expression of GFP-CD on tight junction structure and function. A) Construction of a fusion protein containing GFP fused to theN-terminal end of the cytoplasmic tail of endotubin. B) Confocal immunofluorescence microscopy showing a z-section of MDCK cells expressing full-length endotubin, in the presence of GFP (a) or GFP-CD (b), labeled with antibodies that recognize only full-length endotubin (see Figure 1A). Endotubin is normally targeted to apical endosomes (a), but is randomly distributed in the presence of GFP-CD (b). Bar, 10 μm. C) Confocal microscopy of stably transfected MDCK cells, showing colocalization (arrows) of GFP-CD (visualized by GFP fluorescence) with Rab11. Bar, 1 μm. D) TER (top) and lanthanum permeability (bottom) of MDCK cells stably expressingeither GFP or GFP-CD. Cells expressing GFP-CD have decreased TER and increased permeability to lanthanum. E) Indirect immunofluorescence localization of occludin, ZO-1 and claudin-1 in cells stably expressing GFP or GFP-CD. Bar, 10 μm. F) Levels of occludin, ZO-1 and claudin, detected by western blotting, in cells containing GFP or GFP-CD fusion protein. For each protein, the actin loading control was performed on the same blot.

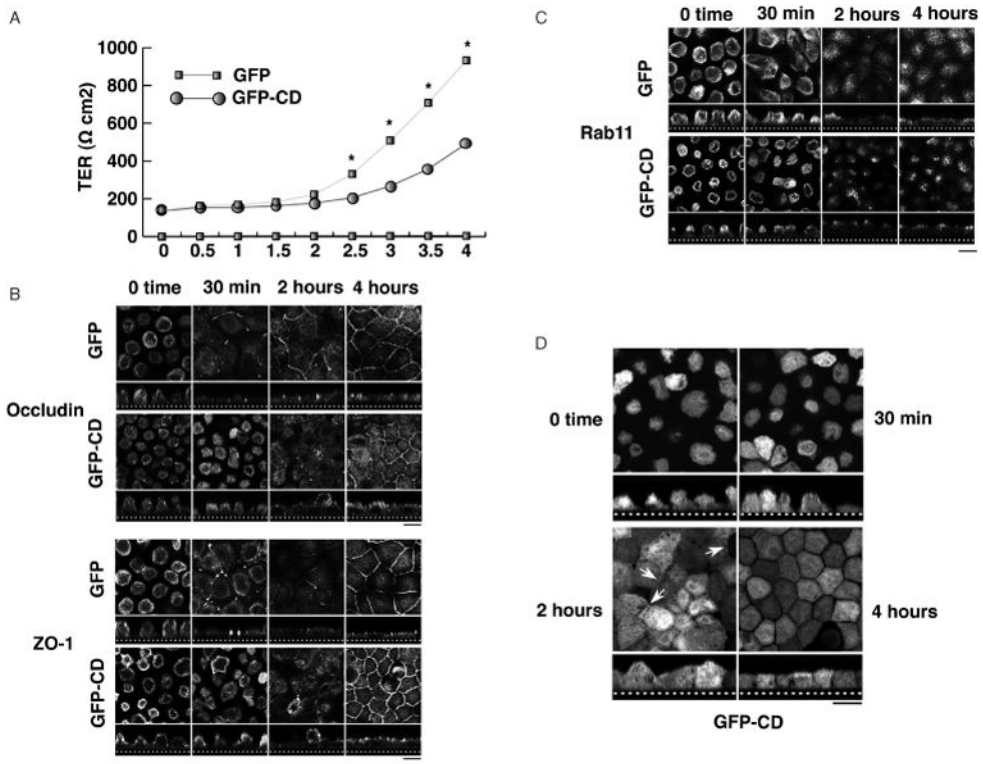

Figure 4.

Time–course of recovery of TER and localization of occludin, ZO-1, Rab11 and GFP-CD during reassembly of junctions in the presence of GFP or GFP-CD. MDCK cells stably expressing GFP-CD or GFP were exposed to low calcium for 18 h and then placed in normal calcium-containing media to stimulate reassembly of junctions. A) TER in cells containing GFP (squares) or GFP-CD (circles) at intervals following addition of calcium. Cells expressing GFP-CD are delayed in the reestablishment of TER. B) Indirect immunofluorescence localization of occludin and Z0–1 in cells containing GFPor GFP-CD, at intervals following addition of calcium. Occludin and ZO-1 reassemble at the lateral membranes more slowly in cells expressing GFP-CD. C) Indirect immunofluorescence localization of Rab11 and GFP-CD in cells at intervals following addition of calcium. D) GFP-CD localizes transiently to the lateral membranes after calcium switch. Two hours after replacement of calcium-containing media, GFP-CD (imaged by GFP fluorescence) is present at the lateral membranes (arrows). Bars, 10 μm.

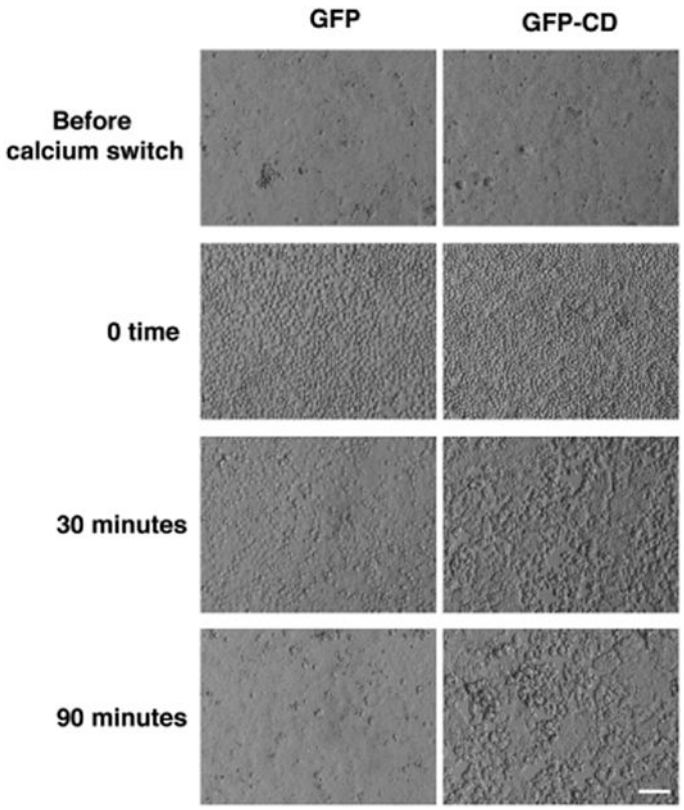

Figure 5.
Effects of expression of GFP-CD upon cell spreading after calcium switch. Cells expressing GFP (left panel) or GFP-CD (right panel) were incubated in low calcium medium for 30 min and then placed in normal calcium-containing medium and the same field of cellswas imaged using Hoffman modulated optics at the intervals indicated. By 30 min in normal calcium-containing media, nearly all the cells are in contact with each other, and all cells are in contact after 90 min in normal calcium. Bar, 100 μm.

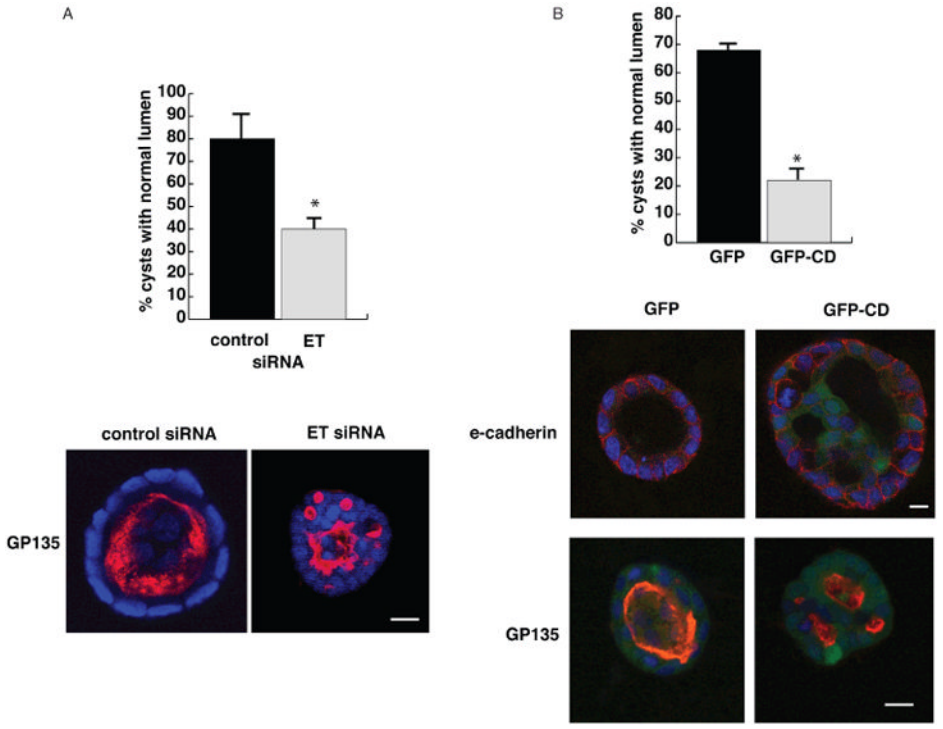

Figure 6.

Effect of ET knockdown or GFP-CD expression on epithelial cyst formation. MDCK cells were grown in three-dimensional culture for 4 days and then labeled with antibodies against the apical plasma membrane marker gp135 (red) (A, B, bottom panels) or E-cadherin (red, B, top panel) and with TOPRO-3 to stain nuclei (blue). Cysts with normal lumens were defined as a single large lumen as visualized by labeling with the apical protein gp135. At least 100 cysts were counted for each experimental condition. A) Top panel. Quantification of cysts formed by cells containing ET-siRNA or control (scrambled) siRNA. ET knockdown results in a significant decrease in the number of normal cysts (*$p < 0.01$). Bottom panel. Indirect immunofluorescence of cysts formed after transfection with ET-siRNA or control siRNA using anti-gp135 (red) and stained with TOPRO-3 to label nuclei (blue). ET knockdown cysts exhibited multiple small lumens. B) Top panel. Quantification of cysts formed after expression of GFP or GFP-CD. Expression of GFP-CD results in a significant decrease in cysts with normal lumens. Bottom panel. Indirect immunofluorescence of cysts formed by cells containing GFP-CD of GFP alone. The lateral marker E-cadherin (red) is localized to the lateral membranes in both GFP-and GFP-CD-expressing cells. However, cells that have grown within lumen appear to have lower levels of E-cadherin expression. Cysts of cells expressing GFP-CD form multiple lumens, as seen by labeling with gp135 (red). Bar, 20 μm.

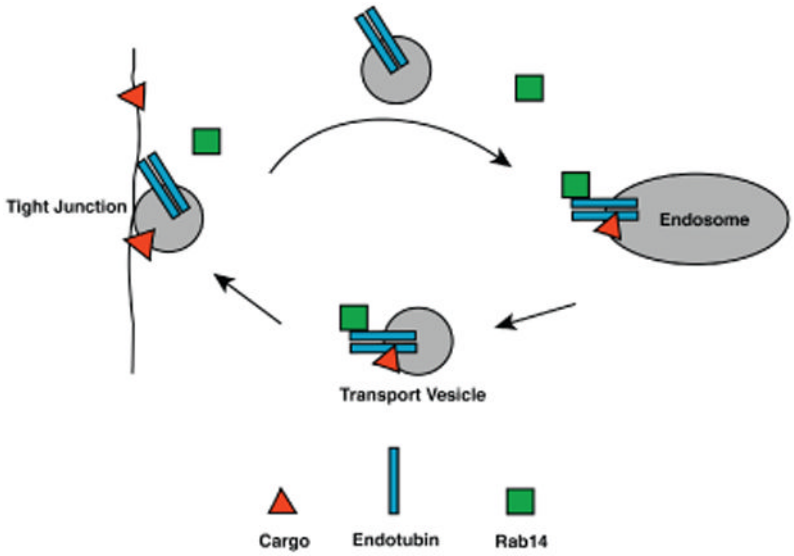

Figure 7.

Model for the role of endotubin in junction assembly. Endotubin forms a complex in the endosome with cargo to be delivered to the plasma membrane, together with Rab14 (27). After budding of the transport vesicle, this complex is delivered to the junctional region where the cargo is released. Endotubin then recycles back to the apical endosomal compartment for another cycle.