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[15] Assay of Rab13 in Regulating Epithelial Tight Junction Assembly

By Anne-Marie Marzesco and Ahmed Zahraoui

Abstract

Rab13 is recruited to tight junctions from a cytosolic pool after cell–cell contact formation. Tight junctions are intercellular junctions that separate apical from basolateral domains and are required for the establishment/maintenance of polarized transport in epithelial cells. They form selective barriers regulating the diffusion of ions and solutes between cells. They also maintain the cell surface asymmetry by forming a "fence" that prevents apical/basolateral diffusion of membrane proteins and lipids in the outer leaflet of the plasma membrane. We generate stable MDCK cell lines

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expressing inactive (T22N mutant) and constitutively active (Q67L mutant) Rab13 as GFP-Rab13 chimeras. Expression of GFP-Rab13Q67L delays the formation of electrically tight epithelial monolayers, induces the leakage of small nonionic tracers from the apical domain, and disrupts the tight junction fence diffusion barrier. It also alters the tight junction strand structure and delays the localization of the tight junction transmembrane protein, claudin1. In contrast, the inactive Rab13T22N mutant does not disrupt tight junction functions, tight junction strand architecture, or claudin1 localization. Here we describe a set of assays that allows us to investigate the role of Rab13 in modulating tight junction structure and function.

Introduction

Rab13 regulates the assembly of tight junctions in epithelial cells (Marzesco *et al.*, 2002; Sheth *et al.*, 2000; Zahraoui *et al.*, 1994). Tight junctions (or *zonula occludens*) play a key role in the development and function of epithelia as well as endothelia. They are the most apical intercellular junctions and form a belt that completely circumvents the apex of the cells, separating apical from basolateral plasma membrane domains. Tight junctions establish a selective barrier regulating the diffusion of ions and solutes across the paracellular space (gate function). They also form a fence preventing the lateral diffusion of proteins and lipids from the apical to the lateral membrane and vice versa, thus contributing to the maintenance of epithelial cell surface asymmetry (Tsukita and Furuse, 1999; Tsukita *et al.*, 2001). In electron micrographs of thin sections, tight junctions appear as very close contacts between the outer leaflets of the plasma membranes of neighboring cells that often appear as focal hemifusions. In freeze fracture electron microscopy replicas, tight junctions appear as a network of intramembrane strands that encircle the apex of the cells. These intramembrane strands are thought to correspond to the focal contact seen in thin sections. Several tight junction proteins are identified. Occludin and claudins, two transmembrane proteins, are thought to seal the intercellular space and to generate series of regulated channels within tight junction membranes for the passage of ions and small molecules (Tsukita and Furuse, 1999, 2000; Zahraoui *et al.*, 2000).

Exactly how tight junctions assemble is still a matter of debate. Cytoplasmic proteins such as ZO-1 (zonula occludens 1), ZO-2, and ZO-3 link occludin and claudins to the underlying actin cytoskeleton. ZO proteins contain three PDZ (PSD95, Dlg, ZO-1) and an SH3 domain that may recruit and cluster proteins to tight junctions (Balda and Matter, 2000; Cordenonsi *et al.*, 1999; Matter and Balda, 2003; Wittchen *et al.*, 1999).

The expression of GFP-Rab13Q67L, but not the inactive GFP-Rab13T22N mutant, in stably transfected Madin–Darby canine kidney (MDCK) delays the formation of electrically tight epithelial monolayers as monitored by transepithelial electrical resistance (TER) and induces the leakage of small nonionic tracers from the apical domain. It also disrupts the tight junction fence diffusion barrier, induces the formation of aberrant strands along the lateral surface, and alters the distribution of the tight junction proteins, claudin1 and ZO-1. Our data reveal that Rab13 plays an important role in regulating both the structure and the function of tight junctions. We propose that Rab13, in its GTP-bound state, recruits an effector(s) that inhibits the recruitment of claudin1 to the cell surface. Conversely, Rab13T22N, which is unable to recruit the effector to the lateral membrane, may favor the assembly of tight junctions.

The methods described herein outline the different assays we used to analyze the activity of Rab13 on tight junction structure and function.

Methods

We investigate the role of Rab13 in tight junctions in the epithelial MDCK cell line grown on permeable filters. The filters are made of a porous membrane of nitrocellulose or polycarbonate, allowing these cells to grow and form a well-polarized monolayer. This enables the easy quantification of tight junction properties via the measurement of electric currents or tracer flux across monolayers. Investigation of the role of Rab13 on tight junction structure and function implies transfection of wild-type and mutant proteins. Because almost all functional tight junction assays measure properties of the entire monolayer and not of a single cell (Matter *et al.*, 2003), it is crucial that such cell populations express the transfected Rab13 proteins in a homogeneous manner. This is achieved by the selection of several clones of epithelial cells expressing Rab13 at similar levels of wild-type or mutant proteins (Fig. 1).

Cloning and Mutagenesis of Human Rab13

Rab13T22N and Q67L mutants are generated from Rab13 cDNA using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). For inactive (T22N) and constitutively active (Q67L) forms of Rab13, the oligonucleotides 5′TCGGGGGTGGGCAAGAATTGTCTGATCATTCGCTT-3′ and 5′-GGGACACGGCTGGCCTAGAGCGGTTCAAGACAATA-3′ are used, respectively. In addition, an *Eco*RI site is introduced upstream of the ATG codon of Rab13 by polymerase chain reaction (PCR) to facilitate Rab13 cDNA cloning. *Eco*R1–*Bam*H1 inserts encoding Rab13WT,

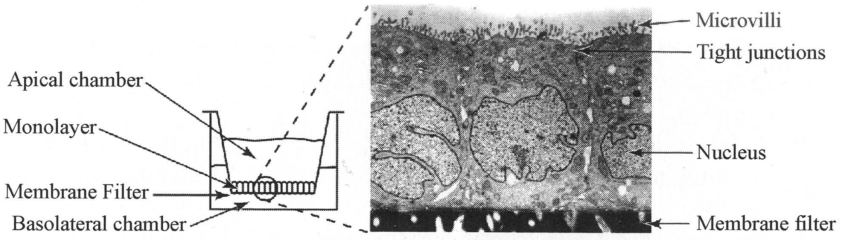

FIG. 1. Diagram of an epithelial cell monolayer grown on a permeable membrane. Electron micrograph of an MDCK cell monolayer. Arrows indicate basally located nuclei, apical microvilli, and tight junctions. The filter is seen at the base of the cells; the pores of the filter appear as white stripes.

Rab13T22N, and Rab13Q67L are fused to the C-terminus of the enhanced green fluorescent protein (GFP) and cloned into the pGFP-C3 vector (Clontech, Inc., Palo Alto, CA). All constructions are verified by sequencing.

Cell Culture and Generation of Stable MDCK Cells Expressing Wild-Type and Rab13 Mutants

MDCK strain II cells are grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 m*M* glutamine, 100 U/ml penicillin, and 10 mg/ml streptomycin. The cultures are incubated at 37° under a 10% CO_2 atmosphere.

Stable MDCK cell lines expressing GFP-Rab13WT, GFP-Rab13T22N, GFP-Rab13Q67L, and the mock MDCK expressing GFP are generated by transfection using the Superfect reagent kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany):

1. The day before transfection, seed 400,000 cells per 30-mm dish in 2 ml of DMEM, and incubate the cells at 37° under a 10% CO_2 atmosphere for 18 h.
2. Dilute 1 μg of plasmid DNA in 100 μl of DNA condensation buffer (EC buffer). Add 3.2 μl of Enhancer solution, mix by vortexing for 1 s, centrifuge a few seconds, and incubate for 10 min at room temperature.
3. Add 8 μl of Effectene reagent, mix by vortexing 10 s, centrifuge a few seconds, and incubate the mixture for 15 min at room temperature.
4. Wash the cells one time with DMEM and add 1.5 ml DMEM (with serum) to cells.

5. Add 0.5 ml of growth medium to the mixture, mix by pipeting up and down, and add the transfection reaction dropwise onto the cells in the 30-mm dishes. Swirl the dishes to distribute the transfection complex.
6. After incubation for 6 h, wash the cells two times with growth medium, and incubate the cells at 37° under a 10% CO_2 atmosphere for 18 h.
7. The cells are then trypsinized in trypsin/EDTA and plated, at a dilution of 1:10, in 10-cm dishes. Cell colonies are allowed to grow in the same medium supplemented with 1 mg/ml of G418 (Life Technologies, Inc.) for 15 days until they reach a size of 4–5 mm. Plates containing well-separated colonies are used to ensure easy isolation of individual colonies. Positive cell clones are selected using cloning rings. Colonies are grown in 48-well plates, then passed into 6-well plates, and finally cultured in 10-cm dishes. The expression of GFP, GFP-Rab13 WT, T22N, and Q67L mutants is checked by immunoblotting using a mouse monoclonal anti-GFP antibody (Roche Diagnostics GmbH, Mannheim, Germany). Stable transfected cells are maintained under selection in 500 μg/ml G418. The homogeneity of the clones expressing Rab13 wild-type T22N and Q67L mutants is checked by immunofluorescence. Two independent clones of GFP-Rab13T22N and GFP-Rab13Q67L are grown and subsequently analyzed.

Functional Analysis of Tight Junctions

In all our experiments, 600,000 cells/cm^2 are plated onto polycarbonate filters (0.4 μm pore size and 12 mm diameter; Transwell; Costar Corp., Cambridge, MA, see Fig. 1), and grown for 3–7 days. Immunoblot analysis using an anti-GFP antibody reveals that the expression of the ectopic GFP-Rab13 proteins is very weak, in particular for the GFP-Rab13T22N protein. This prompts us to treat the cells with 10 m*M* sodium butyrate for 15 h to stimulate the expression of the transfected cDNAs. Under these conditions, the sodium butyrate treatment does not cause changes in tight junction structure or function (Balda *et al.*, 1996; Marzesco *et al.*, 2002). Parental MDCK and MDCK expressing GFP alone are also treated with 10 m*M* sodium butyrate.

Localization of Tight Junction Proteins by Indirect Immunofluorescence. Analysis of tight junction assembly can easily be carried out by indirect immunofluorescence using confocal microscopy (Marzesco *et al.*, 2002). Immunofluorescence of a bona fide marker of tight junctions results in a honeycomb pattern in *x–y* optical sections (i.e., parallel to the monolayer),

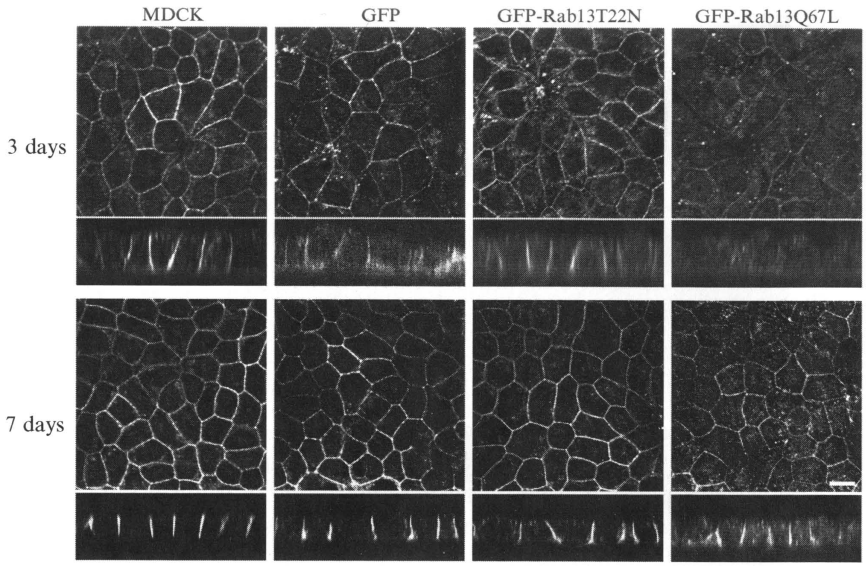

FIG. 2. Subcellular localization of claudin1 in cells expressing GFP-Rab13. Parental MDCK cells and cells stably expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L proteins are grown on Transwell filters for 3 or 7 days. Cells are immunostained for claudin1 and analyzed by confocal laser scanning microscopy. An *x–y* optical section taken at the tight junction level and an *x–z* optical section are shown. The confocal *x–y* sections show prominent ring-like structures of claudin1. Expression of GFP-Rab13Q67L delays the recruitment of claudin1 to cell–cell junctions. Bar = 10 μm.

whereas the most apical end of the lateral membrane appears labeled in *x–z* optical sections (i.e., perpendicular to the monolayer). Actually, a large number of tight junction-associated proteins have been identified and the corresponding antibodies are available. We usually localize several transmembrane (claudins, occludin) and cytosolic (ZO-1, ZO-2) proteins to examine the assembly of tight junctions. Staining of tight junction proteins requires extraction of proteins and/or lipids, particularly for cells grown on a filter. We perform immunofluorescence staining on parental or transfected MDCK cells (Fig. 2) using the following procedure:

1. Wash cells with phosphate-buffered saline (PBS) containing 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, and fix with 3% paraformaldhyde in PBS for 15 min.
2. The free aldehyde groups are quenched for 15 min with 50 m*M* NH_4Cl in PBS.

3. Excise the filter from the insert holder and cut a sector from the filter.
4. Permeabilize with 0.5% Triton X-100 in PBS for 15 min.
5. Block in PBS buffer containing 0.5% Triton X-100 and 0.2% bovine serum albumin (BSA). All subsequent incubations with antibodies and washes are performed with this buffer. The incubations were performed in a humid chamber, protected form light.
6. Incubate overnight at 4° with the polyclonal rabbit anti-claudin1 antibodies (Zymed Laboratories, Inc., San Francisco, CA).
7. Wash three times for 10 min each with the blocking buffer. Incubate for 45 min with affinity purified goat anti-rabbit IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, Inc.) and diluted 1:400.
8. Wash three times with blocking buffer and three times with PBS. The filter sector is mounted in 50% glycerol-PBS, covered with a coverslip and sealed with colorless nail polish. Analyze the staining (Fig. 2) using a Leica SP2 confocal laser scanning microscope (Leica Microscopy and Systems GmbH, Heidelberg, Germany).

Assessment of Monolayer Integrity

To monitor the quality of the epithelial cell monolayer tightness, which depends upon the establishment of functional tight junctions, various approaches have been developed. One method is to visualize the integrity of the monolayers by biotinylation of the apical plasma membrane (Fig. 3).

1. Wash the intact monolayers of cells grown on a filter (six-well plate) four times with ice-cold PBS^+ (PBS containing 1 m*M* $MgCl_2$ and 0.1 m*M* $CaCl_2$). All of the following steps are performed on ice, in order to avoid internalization of the biotin.
2. Stock solution of EZ-Link Sulfo-NHS-LC-biotin (MW 556.58; Pierce) is prepared in dimethyl sulfoxide (DMSO) at 200 mg/ml and stored at −20°. The biotinylation solution is freshly prepared by 1:250 dilution of the stock solution in PBS^+ (working concentration of 0.8 mg/ml).
3. Add 250 μl of biotinylation solution to the apical chamber and 1.5 ml of PBS^+ to the basal chamber. Incubate on ice at 4° for 20 min. Wash the filters two times with PBS^+ and incubate 30 min with 0.2 *M* glycine.
4. Fix with 2% paraformaldhyde in PBS for 15 min. The free aldehyde groups are then quenched for 15 min with 50 m*M* NH_4Cl in PBS.
5. Excise the filter from the insert, and block in PBS buffer containing 0.5% Triton X-100 and 0.2% BSA.

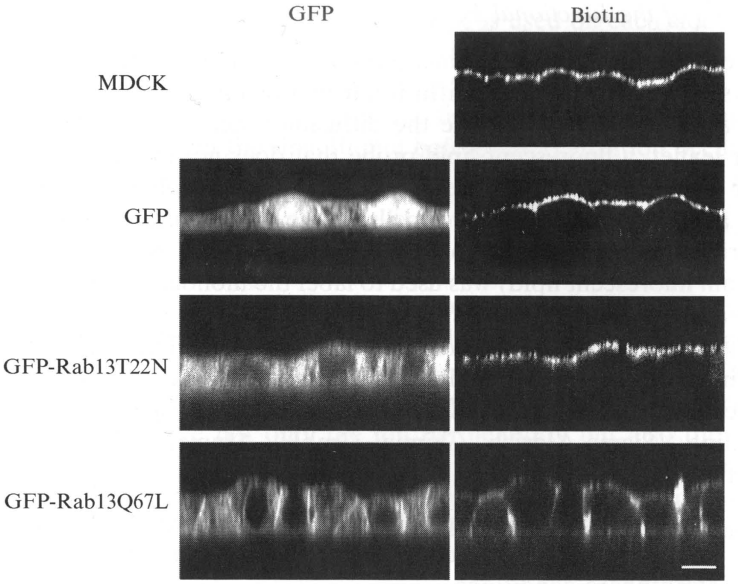

FIG. 3. Diffusion of fluorescent biotin from the apical to the lateral membrane in MDCK cells. Parental MDCK cells and cells stably expressing GFP, GFP-Rab13T22N, or GFP-Rab13Q67L proteins are grown on Transwell filters for 3 days. Biotin is loaded to the apical surface for 20 min at 0°. After washing, cells are labeled with streptavidin–Texas Red at 0°, and processed for confocal microscopy. An x–z optical section shows the distribution of fluorescent biotin. Note that biotin is restricted to the apical surface in MDCK and cells expressing GFP and GFP-Rab13T22N. However, in MDCK cells expressing GFP-Rab13Q67L, biotin is not restricted to the apical membrane but diffuses across the tight junction and stains the lateral membrane. This indicates that Rab13Q67L alters the tight junction barrier functions. Bar = 10 μm.

6. Incubate the filter for 45 min at 4° with streptavidin-Texas Red (ImmunoResearch Laboratories, Inc.) in blocking buffer.
7. After washing three times with blocking buffer and three times with PBS, the filters are mounted in 50% glycerol-PBS, covered with a coverslip, and sealed with colorless nail polish. Analyze the staining using a Leica SP2 confocal laser scanning microscope (Leica Microscopy and systems GmbH, Heidelberg, Germany).

It should be mentioned that the restriction of biotinylated proteins to the apical plasma membrane involves both the fence and gate functions of tight junctions. Therefore, to investigate the fence and the gate functions independently, other assays should be performed.

Analysis of the Junctional Fence

To investigate the tight junction fence function, we use a method that allows visualization of lipid diffusion from the apical plasma membrane to the basolateral domain. Since the diffusion fence is only efficient in the outer leaflet, a fluorescent lipid probe that does not flip-flop between the inner and outer membrane leaflet has to be used. Sphingomyelin derivatives allow efficient labeling of cells on ice, give reliable results, and are generally used. Therefore, for our studies, BODIPY R6G-sphingomyelin (550 nm fluorescent lipid) was used to label the monolayer according to the procedure described below.

Synthesis of BODIPY R6G-Sphingomyelin. BODIPY R6G-sphingomyelin is generated by coupling BODIPY R6G reactive dye (Molecular Probes, Inc., Eugene, OR) to amine-reactive sphingosylphosphorylcholine (Sigma-Aldrich Chimie GmbH, Munich, Germany) using dimethylformamide.

1. A suspension of 10.6 mg (0.0228 mmol) of sphingosylphosphorylcholine in 5 ml anhydrous dimethylformamide is stirred under inert atmosphere conditions.
2. Then 10 mg (0.0228 mmol) of BODIPY R6G (the succinimidyl ester of the 4,4-difluoro-5-phenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid) is added to the mixture and incubated further for 48 h at room temperature.
3. Removal of the solvent at 50° under reduced pressure provides the wanted *N*-(4,4-difluoro-5-phenyl)-4-bora-3a,4a-diaza-*s*-indacene-3 propionyl) sphingosylphosphorylcholine as a dark powder. The purity of the fluorescent lipid is checked by thin layer chromatography.
4. BODIPY R6G-sphingomyelin/BSA complexes are obtained by adding 400 μl of BODIPY R6G-sphingomyelin stock solution (1 m*M* in DMSO) to 10 ml of BSA solution (0.8 mg/ml defatted BSA in 10 m*M* HEPES, pH 7.4, 145 m*M* NaCl) under vigorous vortexing.

For visualization of diffusion by confocal microscopy:

1. Wash filter-grown MDCK cells twice with cold P buffer (10 m*M* HEPES, pH 7.4, 145 m*M* NaCl, 1 m*M* Na-pyruvate, 10 m*M* glucose, 3 m*M* $CaCl_2$).
2. Add 250 μl of BODIPY R6G-sphingomyelin/BSA complexes to the apical chamber and incubate the cells for 10 min on ice. After washing four times with ice–cold P buffer, the cells are either left on ice for 1 h or directly mounted in P buffer.
3. Samples are prepared by cutting out the filter from the insert holder.

4. For mounting, double-sided Scotch tape is used on each side of the microscope slide to support the coverslip and avoid placing pressure on the monolayer.
5. The lateral diffusion of fluorescent lipids is analyzed by confocal microscopy within the first 10 min before internalization occurs.

Analysis of the Paracellular Gate

Tight junctions also act as a selective diffusion barrier for ions and hydrophilic nonionic molecules. The selectivity of the paracellular diffusion barrier is based on the charge and size of hydrophilic nonionic molecules and is regulated by distinct physiological and pathological stimuli. For complete characterization of the paracellular diffusion barrier, it is necessary to analyze not only the ion permeability but also the paracellular permeability to hydrophilic tracers. Measurement of the transepithelial resistance and tracer flux across the cell monolayer is used to monitor the formation of the tight junction barrier during tight junction assembly.

Measurement of Transepithelial Electrical Resistance (TER). The measurement of TER is indicative of the ion permeability of the tight junctions. Since the measurement of TER of filter-grown epithelial cells is performed in the presence of culture medium, it mostly reflects primarily Na^+ permeability. TER of filter-grown epithelial cells is originally determined in Ussing chambers and requires a complicated setup. Actually, TER measurements are performed with a voltmeter.

1. MDCK cells are plated on filters (12 mm diameter) as instant confluent monolayers and grown for 5 days.
2. The same volume of medium is added to the upper and lower chambers. The TER is temperature sensitive, so special care should be taken to avoid cooling the samples.
3. The TER value of filter–grown MDCK cells is determined by applying an AC square–wave current of 20 μA at 12.5 Hz and measuring the voltage deflection with a Ag/AgCl electrode using an Epithelial VoltOhmMeter (EVOM, World Precision Instruments).
4. The TER value is obtained by subtracting the resistance value of an empty filter with culture medium.

Paracellular Flux Assay. Paracellular permeability diffusion of hydrophilic tracers can be measured using fluorescent compounds such as dextrans or radioactively labeled compounds such as mannitol. In our laboratory, we used the following tracers: [^{3}H]mannitol (182 Da), 4 kDa FITC-dextran, 40 kDa FITC-dextran, and 400 kDa FITC-dextran.

1. Cells are grown on filters to confluency for 3 days.
2. The stock solution of FITC-dextran (20 mg/ml) (Sigma-Aldrich Chimie GmbH, Munich, Germany) is dialyzed against P buffer (10 m*M* HEPES pH 7.4, 1 m*M* sodium pyruvate, 10 m*M* glucose, 3 m*M* $CaCl_2$, 145 m*M* NaCl) and diluted to 2 mg/ml in P buffer before the assay.
3. Replace the basolateral medium with 500 μl of P buffer, and the apical culture medium with either 250 μl of solution containing 2 mg/ml of 4K, 40K, or 400K FITC-dextran or 500 μl of culture medium containing 1 m*M* mannitol and 4 μCi/ml [^{3}H]mannitol.
4. Monolayers are incubated at 37° for either 3 h for FITC-dextran or 1 h for [^{3}H]mannitol.
5. Collect the basal chamber media. FITC-dextran is measured with a fluorometer (excitation: 392 nm; emission: 520 nm) (Perkin Elmer Applied Biosystems, Inc.). Radioactivity is counted in a liquid scintillation counter (Wallac Oy, Furky, Finland).

It should be mentioned that the TER and tracer permeability measurements are composites of the paracellular and transcellular pathways. Therefore, ultrastructural morphological studies should be undertaken to understand defects in TER and tracer diffusion.

Freeze-Fracture Electron Microscopy and Immunolabeling

This method allows visualization of tight junction strands organization and provides a measure of tight junction integrity. MDCK cells were plated in 10-cm-diameter tissue culture dishes and grown for 3 days postconfluency. The monolayers can be analyzed either by conventional freeze-fracture or by freeze-fracture immunolabeling.

Conventional Freeze-Fracture. MDCK monolayers are fixed in 2% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 30 min at room temperature. Cells are scraped from the substrate with a plastic cell scraper and infiltrated with 30% glycerol for 2 h at 4°. Cell pellets are frozen by quick immersion in liquid propane (Balzers, Lichtenstein) and stored in liquid nitrogen until replicated. Freeze-fracture is performed at −130° in a Balzers freeze-fracture 301 or 400 unit (Balzers, Lichtenstein). Replicas are examined using a Philips CM12 electron microscope.

Freeze-Fracture Immunolabeling (FL). In contrast to conventional freeze-fracture, the cells are processed without fixation after a rapid wash using the cell culture medium. SDS digestion and immunolabeling of replicas with polyclonal anti-claudin1 (1:200) and polyclonal anti-occludin (1:100) antibodies followed by protein A gold (10 nm) are performed as

previously described (Dunia *et al.*, 2001). Replicas are examined using a Philips CM12 electron microscope.

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