Analysis of Membrane Traffic in Polarized Epithelial Cells

Spatial asymmetry is fundamental to the structure and function of most eukaryotic cells (Drubin and Nelson, 1996). A basic aspect of this polarity is that the cell's plasma membrane is divided into discrete domains. The best studied and simplest example of this occurs in epithelial cells, which line exposed body surfaces (Matter and Mellman, 1994; Mostov et al., 1999, 2000). Epithelial cells have an apical surface facing the outside world and a basolateral surface contacting adjacent cells and the underlying connective tissue. These surfaces have completely different compositions and are separated by tight junctions, which block movement of plasma membrane proteins between the apical and basolateral surfaces and also prevent diffusion of extracellular material between cells. Epithelial cells use two pathways to send proteins to the cell surface. Newly made proteins can travel directly from the trans-Golgi network (TGN) to either the apical or basolateral surface. Alternatively, proteins can be sent to the basolateral surface and then endocytosed and transcytosed to the apical surface. A schematic view of a simple polarized epithelial cell is given in Figure 15.5.1.

Studies on membrane traffic in polarized epithelial cells have been greatly facilitated by the use of cell lines grown as a well-polarized monolayer. The most widely utilized of these is the Madin-Darby canine kidney (MDCK) cell line. Other cell lines that have been used for studying polarized membrane traffic include Caco-2 (human intestinal cancer) and FRTL (Fischer rat thyroid; Weimbs et al., 1997). Table 15.5.1 summarizes the cell lines most commonly used in studies of membrane traffic in polarized epithelial cells. Generally these cell lines are maintained by growing adherent cells in standard cell culture plastic dishes or flasks; however, for the actual experiments in which polarized traffic is assessed, the cells are usually grown on porous filters. Such filters are available mounted in holders sold by several manufacturers, including Corning, Falcon, and Nunc. These devices come in several sizes and are available with pores of different diameters. Most of the work done by the authors has been with 12-mm Corning Transwells with filters containing pores of 0.4-μm diameter. Table 15.5.2 lists the main types of units that are commercially available.

The usual type of study is to examine the biosynthetic delivery of a membrane or secreted protein to the apical or basolateral surface (see Basic Protocol 2). This sort of experiment is quite similar to studying the delivery of proteins to the surface of nonpolarized cells, as described in *UNIT 15.4*, with the principal difference being that delivery to either the apical or basolateral surface is measured, as opposed to the plasma membrane as a whole. Although MDCK cells contain numerous endogenous apical and basolateral plasma membrane proteins, these are usually expressed at very low levels, making it difficult to study the trafficking of any individual protein. Additionally, one often wishes to examine the polarized localization or trafficking of a protein that may not be expressed endogenously in MDCK cells; therefore, one typically begins a project by transfecting cDNA for the protein of interest into MDCK cells in order to overexpress the protein for study (see Basic Protocol 1). Most of the authors' experience is with the calcium phosphate-based transfection system (see Basic Protocol 1; Breitfeld et al., 1989). Cells can be transfected by other methods, including various commercially available lipid-based transfection reagents (e.g., Lipofectamine 2000) or by electroporation. Although transient transfection and expression systems can be used, in many cases the fraction of cells expressing the protein of interest is very low, and the cells that express the transfected protein tend to be less well polarized in part because the process of polarization takes 4

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to 7 days, whereas transient protein expression generally peaks at 48 hr and is often gone by 72 hr; therefore, in most cases it is advisable to generate stable clones (see Support Protocol 1). These clones are then expanded for further use by growing on standard tissue culture dishes. For studies of membrane traffic the cells must be cultured on filter units (see Support Protocol 2). It is important to check that the cells form a tight, well-polarized monolayer on the filter. Several methods are available to do this, and a particularly easy method is presented in this unit (see Support Protocol 3). A cell surface biotinylation assay to measure delivery of proteins is presented (Basic Protocol 3; Luton and Mostov, 1999). As the study of polarized membrane traffic is fundamentally the study of the localization of proteins, it is extremely useful to visualize the location of proteins in the epithelial cell. In addition, a fixation method to use for laser scanning confocal immunofluorescence

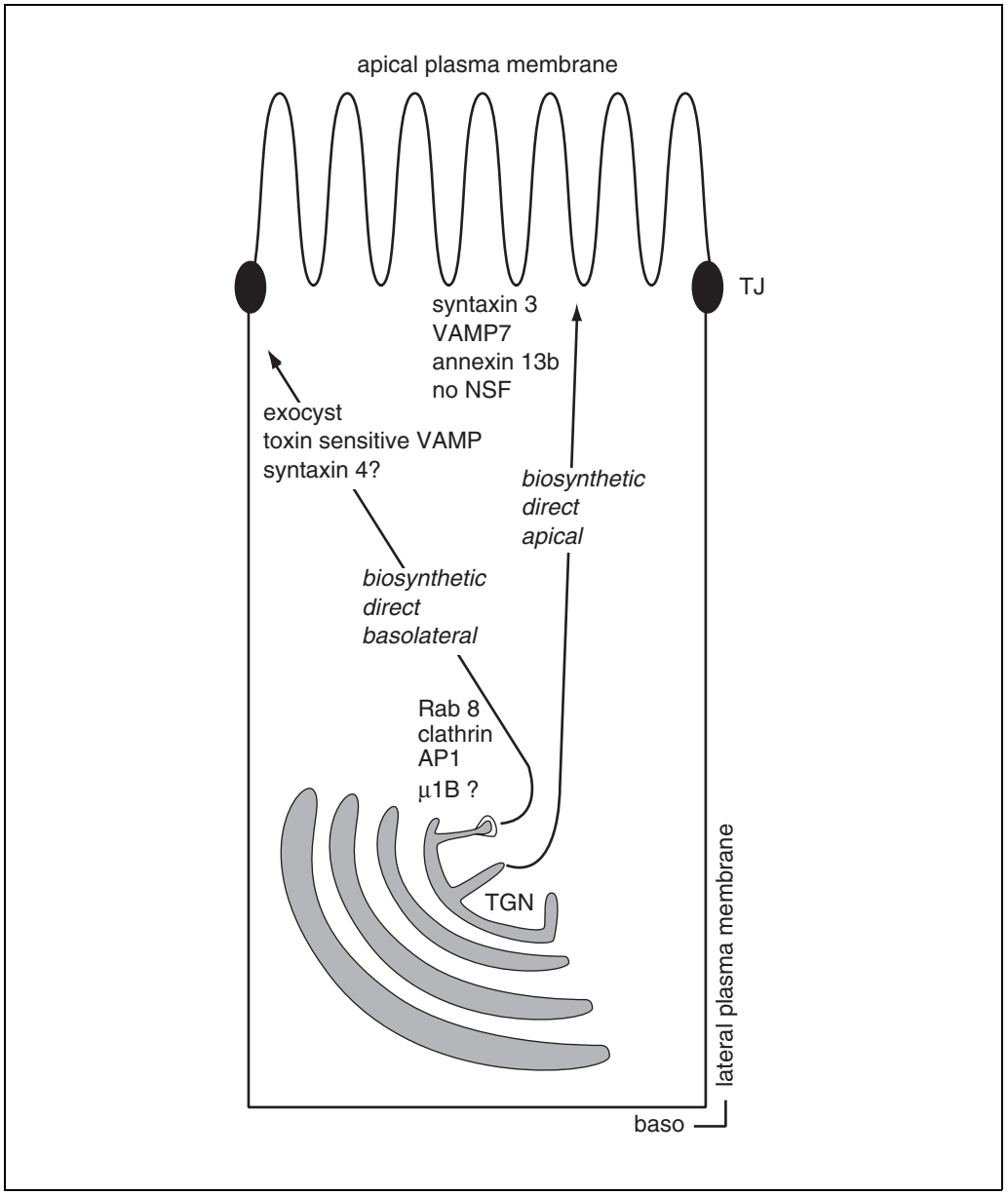

Figure 15.5.1 Schematic diagram of a polarized epithelial cell. Only the major biosynthetic pathways leading directly from the TGN to the apical and basolateral surfaces are shown. Components of the membrane traffic machinery that are likely to be involved in each of these two pathways are also indicated. For further details, see Mostov et al. (2000). Abbreviations: TGN, trans-Golgi network; TJ, tight junction.

Table 15.5.1 Cell Lines Commonly Used to Study Epithelial Membrane Traffic in Polarized Epithelial Cells

Cell line	Origin	Comments	ATCC#[a]
MDCK	Canine kidney	Most widely used. Two major categories, types I and II, though many clones with specific phenotypes.	CCL-34
Caco-2	Human intestine	Although derived from human colon cancer, more closely resembles small intestine. Slow growing.	HTB-37
FRTL	Rat thyroid	Some glycolipids and GPI-anchored proteins are found at the basolateral surface, unlike other epithelial cells.	CRL-1468

[a]The ATCC home page can be reached at *http://www.atcc.org.*

Table 15.5.2 Commercially Available Filters for Growth of Epithelial Cells

Material	Manufacturer	Comments
Polycarbonate	Corning Millipore BD Biosciences Labware Nalge Nunc International	Most widely used
PTFE/FP	Millipore Corning BD Biosciences Labware	Good optics. Coating with extracellular matrix (ECM) is usually required; can be purchased precoated
PET	BD Biosciences Labware	Available in different pore densities, with low density having better optical properties, but lower porosity. Available precoated with several types of ECM
Anopore	Nalge Nunc International	Very low intrinsic fluorescence, well-suited for immunofluorescence microscopy
Cellulose esters	Millipore	High protein binding
Polyester	Corning	Good optics

microscopy of proteins in polarized epithelial cells is also presented (see Basic Protocol 4; Luton and Mostov, 1999).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly (e.g., use of a sterile hood).

NOTE: All culture incubations should be performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified. Some media (e.g., DMEM) require altered levels of CO_2 to maintain pH.

BASIC PROTOCOL 1

TRANSFECTION OF POLARIZED EPITHELIAL CELLS IN SUSPENSION AND SELECTION OF RESISTANT CLONES

The goal of this protocol is to transfect a plasmid containing the gene of interest into mammalian epithelial cells under a suitable promoter (i.e., a CMV promoter). This protocol provides the basis for the other experimental protocols described.

Materials

Confluent cells grown on 10-cm tissue culture dish
HEPES buffered saline (HeBS), pH 7.05 (see recipe; also see *APPENDIX 2A*)
20 µg plasmid DNA in 1 to 20 µl H_2O
2 M $CaCl_2$: filter sterilize and store up to 6 months at 4°C
CMF-DPBS (*APPENDIX 2A*)
Trypsin/EDTA solution—e.g., 0.25% (w/v) trypsin/0.2% (w/v) EDTA (*UNIT 1.1*)
MEM medium (*APPENDIX 2B*) with and without 5% (v/v) FBS (*APPENDIX 2A*)
20 mM chloroquine in water: filter sterilize and store up to 6 months at 4°C
15% (w/v) glycerol in HeBS: filter sterilize and store up to 6 months at 4°C
Selection medium: MEM/5% FBS containing eukaryotic antibiotic (e.g., G418; see recipe)
Nontransfected cells
10-cm dishes

Additional reagents and equipment for picking clones (see Support Protocol 1)

Transfect cells

1. Split cells grown on a 10-cm dish 1:10 on the day prior to transfection in order to yield ~30% confluency on the day of transfection.
2. To 0.5 ml HEPES buffered saline (HeBS), pH 7.05, add 20 µg plasmid DNA in 1 to 20 µl water, and 31 µl of 2.0 M $CaCl_2$. Flick the tube for 20 sec.

   The pH of the HeBS has to be exact for a good precipitate to form.

   This step should be done in a sterile hood.
3. Incubate the plasmid at room temperature for 30 min, avoiding any unnecessary shaking of the sample.
4. Trypsinize one subconfluent (i.e., ~30% confluent or ~1×10^7 cells) 10-cm dish of cells by adding 1 to 2 ml of trypsin/EDTA solution after washing with CMF-DPBS.

   For trypsinization of MDCK cells, use 0.25% (w/v) trypsin/0.2% (w/v) EDTA. The desired concentration of cells is ~0.5 to 1 × 10^6 cells/ml.
5. Centrifuge cells for 3 min at 300 × *g*, room temperature. Resuspend in 5 ml MEM medium with 5% FBS.
6. Transfer 1 ml of the cell suspension into a 10-cm dish.
7. Slowly add 500 µl DNA-Ca^{2+} coprecipitate (step 3) while simultaneously agitating the 10-cm dish. Incubate at room temperature for 15 to 20 min.
8. Add 50 µl of 20 mM chloroquine (200 µM final) to 3.5 ml MEM/5% FBS. Add the MEM/FBS/chloroquine mixture to the dish and agitate to spread cells.
9. Incubate 6 to 18 hr (e.g., overnight) at 37°C in order to allow the cells to attach to the dish.

Plate transfected cells

10. Remove the medium. Wash once with chloroquine-containing medium (see step 8).
11. Add 2 ml of 15% glycerol in HeBS gently. Incubate exactly 1 min at 37°C.
12. Remove glycerol by washing gently twice with MEM without FBS, and then adding 10 ml MEM/5% FBS.
13. Allow cells to grow 2 to 3 days at 37°C, until dish is confluent.

    Don't allow the cells to grow >3 days, even if the dish is not yet confluent as this will result in an overgrowth of nontransfected cells. The important thing is to allow the cells to divide several times.

14. Trypsinize the cells (step 4), and resuspend in 10 ml selection medium. As a control, use nontransfected cells.

Clone cells

15. Split the cells into 7 different dilutions in 10-cm dishes:

Cell suspension (ml)	0.025	0.05	0.1	0.2	0.4	0.8
Selection medium (ml)	9.975	9.95	9.9	9.8	9.6	9.2

    Shake to distribute cells evenly.

Select stably transfected cells

16. Incubate cells at 37°C for 16 to 21 days (i.e., until the colonies are big enough to pick), adding fresh selection medium every 4 to 6 days.

    Depending on the stability of the selection agent, it may not be necessary to change the medium.

    Cells will start to die after 3 to 5 days and colonies should be seen within 10 to 14 days, but will not be big enough to pick until after 16 to 21 days.

17. Clones should generally be picked from the dishes with the highest dilution possible (see Support Protocol 1).

    Dilutions in which 0.4 or 0.8 ml cells were used can usually serve as the pooled clones.

PICKING STABLY TRANSFECTED CLONES

SUPPORT PROTOCOL 1

The goal in this protocol is to identify and stably propagate epithelial cell clones that express the gene of interest.

Additional Materials *(also see Basic Protocol 1)*

~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium (see Basic Protocol 1)
Calcium- and magnesium-free DPBS (CMF-DPBS; *APPENDIX 2A*)
Medium-sized glass cloning ring, sterile (Bellco Glass)
0.5% (w/v) SDS lysis buffer
12% (w/v) slurry of CL-2B beads
12-well tissue culture plates

1. Examine ~16-day-old 10-cm dishes containing diluted transfected and nontransfected cells in selection medium, looking for single colonies. With a marker, draw a circle around the single colonies on the outside bottom of the dishes.

If the original 10-cm dishes (i.e., the one which was split into seven different dilutions; see Basic Protocol 1, step 15) was ~30% confluent, single colonies are generally found in the dilutions in which 0.025 to 0.2 ml cells were used.

2. Wash dishes with 10 ml CMF-DPBS.
3. Use a Pasteur pipet attached to a vacuum device to aspirate a "dry" ring (i.e., devoid of liquid) around a single colony.

   The "dry" ring will prevent trypsin from leaking under the cloning ring in step 4. Do this procedure for each colony individually, as the cells will dry out if more than one colony is harvested at a time.

4. Place a medium-sized sterile glass cloning ring around the colony. Add trypsin/EDTA solution until the cloning ring is filled. Repeat steps 3 and 4 for all colonies to be picked.

   For epithelial cells (e.g., MDCK), use 0.25% trypsin and 0.2% EDTA.

   At least 15 clones should be picked, though more is preferable (i.e., 50 if possible).

5. After all the cloning rings have been placed, incubate for ~20 min at room temperature in a sterile hood.
6. Remove the trypsin containing the loose cells and add to the well (i.e., 1 colony per well) of a 12-well tissue culture plate containing 2 ml selection medium. Repeat for all colonies.
7. Resuspend cells by pipetting up and down several times. Transfer 1 ml from each well to the corresponding well of a new 12-well plate (one plate will be for protein collection and the other for colony expansion).
8. Incubate to confluency (i.e., ~5 to 7 days) at 37°C.

   It is generally not necessary to change the medium if the cells are harvested as soon as they are confluent.

   The length of time to confluency depends on how many loose cells are transferred in good condition to the wells. This can vary significantly from well to well.

9. Add 500 µl 0.5% SDS lysis buffer, boil the samples 5 min at 100°C. Vortex samples 10 min. Preclear the samples by adding 20 µl of a 12% slurry of CL-2B beads. Mix several times. Microcentrifuge briefly at maximum speed. Collect supernatant and use an aliquot for immunoblotting (*UNIT 6.2*).
10. For the colonies that show expression, use the second identical well to expand the cells.

    The authors recommend keeping the cells under selection while expanding the colony size.

SUPPORT PROTOCOL 2

CULTURE OF EPITHELIAL CELLS ON FILTERS

The goal in this protocol is to allow epithelial cells to become fully polarized. This is accomplished by culturing epithelial cells on porous filters that allow the cells to access medium both apically and basolaterally.

Additional Materials *(also see Basic Protocol 1 and Support Protocol 1)*

10-cm dish of confluent epithelial cells (Table 15.5.1)
12-mm Transwell filters and appropriate dishes
IEC clinical centrifuge with 12 × 15 rotor

Additional reagents and equipment for determining the tightness of epithelial monolayers (see Support Protocol 3)

1. Wash a 10-cm dish of confluent epithelial cells with 10 ml CMF-PBS.
2. Add 3 ml trypsin/EDTA solution (e.g., 0.25% trypsin/0.2% EDTA for MDCK cells) and incubate at 37°C for 15 min.
3. Tap dish to agitate cells. When all cells are detached, add 8 ml MEM/5% FBS.

   This step is done in order to neutralize the trypsin.
4. Transfer to an appropriate tube and centrifuge cells 3 min at 300 × *g* (e.g., 1000 rpm in an IEC 12 × 15 rotor), room temperature. Discard the supernatant.
5. Resuspend cells in 10 ml MEM/5% FBS and mix thoroughly by pipetting up and down ~10 times. Add 0.5 ml of this solution to the apical chamber (i.e., inside) of a 12-mm Transwell filter. Then add an additional 0.4 ml MEM/5% FBS to the apical chamber for a total of 0.9 ml.

   This corresponds to ~2.5×10^5 cells/well.
6. Add 1 ml MEM/FBS to the basal chamber (i.e., area surrounding the Transwell filter).
7. Incubate at 37°C, replacing 1 ml apical and 1 ml basolateral medium every day for 3 to 5 days until cells form a tight monolayer (see Support Protocol 3).

   IMPORTANT NOTE: *It is important to first remove the basal medium and then remove the apical medium, and to replace in the opposite order. If the basal medium is present and the apical medium is absent then the cells can be pushed off the filter.*

   The cells are fully polarized and ready to examine after 4 to 7 days of growth on the filter.

DETERMINING THE LEAKINESS OF A MONOLAYER OF CELLS GROWN ON A FILTER

SUPPORT PROTOCOL 3

When grown on a Transwell or similar filter support, epithelial cells should form a confluent monolayer; however, with most types of filter materials, it is difficult or impossible to clearly visualize the cells themselves. Hence, it is not easy to determine if they have formed a confluent tight monolayer. Several methods have been devised to measure how intact the monolayer is. Classically, electrical resistance across the monolayer is measured; however, type II MDCK cells typically have a rather low electrical resistance, on the order of 100 ohms/cm^2, and so electrical resistance is rather insensitive to defects in the monolayer. Thus, the authors devised an extremely simple method to monitor the integrity of the monolayer. Although it is qualitative, it has been found to be a very reliable predictor of the integrity of the monolayer and the success of the experiment in the authors' laboratory.

Additional Materials *(also see Support Protocol 2)*

Pasteur pipet connected to a vacuum system

1. Culture cells on filters, as described (see Support Protocol 2).
2. The day before the experiment (i.e., typically after 3 to 6 days of growth on the filter), add normal cell culture medium to the apical well to fill it to overflowing.

   This support protocol is usually performed as part of the daily replacement of apical and basolateral media.

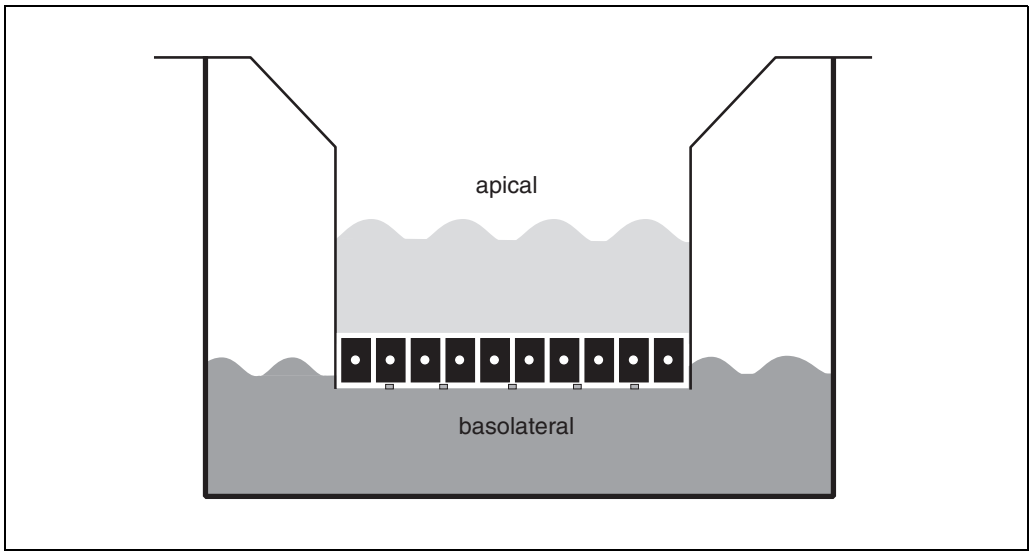

Figure 15.5.2 Schematic diagram of polarized epithelial cells growing in a Transwell. Cells are black rectangles with small white circles indicating nuclei. The apical and basolateral medium are gray. The fluid level of the apical medium is higher than the basolateral medium, as occurs during Support Protocol 3.

3. Use a sterile Pasteur pipet connected to a vacuum system to aspirate most of the medium from the basolateral chamber, leaving just enough medium to contact the entire basal surface of the filter.
4. Incubate the Transwell filter, still in the multiwell plate, at 37°C overnight (i.e., typically 8 to 16 hr).
5. Determine the tightness of the monolayer by assessing the fluid level of the apical chamber.

   If the cell monolayer is intact, the fluid level inside the apical chamber will not go down overnight—i.e., the apical chamber will remain filled to the brim (Figure 15.5.2). If, on the other hand, the fluid level leaks, the monolayer is not tight and the cells should be discarded.

BASIC PROTOCOL 2

PULSE-CHASE EXPERIMENTS IN POLARIZED EPITHELIAL CELLS

The goal of this protocol is to study the trafficking of apical and basolateral secreted proteins in transfected polarized mammalian epithelial cells. This is accomplished utilizing a radioactive amino acid as a tracer molecule (Lipshutz et al., 2000).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimentor and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters (see Support Protocol 2)
Dulbecco's phosphate-buffered saline (DPBS; *APPENDIX 2A*)
Starvation medium: MEM medium (*APPENDIX 2B*) lacking the radioactive tracer amino acid
Radioactive amino acid (e.g., 1175 Ci/mmol [^{35}S]methionine)
MEM medium
0.5% (w/v) SDS lysis buffer (see recipe)

Parafilm
Humid box: plastic box with 2 charcoal bags and a piece of wet Whatman filter paper
12-well Transwell tissue culture plate (Table 15.5.2)
Phosphorimager
Scalpel

Additional reagents and equipment for gel electrophoresis (Chapter 6)

Label cells

1. Obtain 4- to 7-day-old epithelial cell cultures growing on 12-mm Transwell filters.
2. Wash cells three times with 37°C Dulbecco's phosphate-buffered saline (DPBS).
3. Add 500 µl starvation medium apically and basolaterally. Incubate at 37°C, 5% CO_2 for 15 min.

   For example, if [^{35}S]methionine is to be used as the radioactive amino acid, then the starvation medium is medium lacking methionine.

4. Place 25-µl spots containing radioactive amino acid (e.g., 4 µl of 1175 Ci/mmol [^{35}S]methionine and 21 µl starvation medium) on a Parafilm sheet inside a humid box.

   The humid box is a plastic box to which two bags containing charcoal are attached to the top inside surface of the box in order to trap the aerosolized [^{35}S]methionine. A piece of Whatman filter paper is placed on the bottom of the plastic box and soaked with water in order to prevent the cells from drying out. Finally, a piece of Parafilm is placed on top of the damp Whatman filter paper.

5. Remove the basal medium while leaving the apical medium in place. Carefully, set one filter on top of each spot for basolateral exposure of cells to the radioactive amino acid.
6. Incubate (i.e., allow labeling to occur) for 15 to 20 min at 37°C. Analyze labeled protein.

   If one is interested in analyzing the synthesis of secretory proteins, carry out steps 11 and 12 at this point.

7. Prepare a new 12-well plate containing fresh MEM medium. Transfer transwells to the new plate and wash cells three times apically and basolaterally with 37°C DPBS.
8. Add 300 µl MEM medium apically and 500 µl basolaterally. Incubate for the desired amount of time (e.g., 1 hr) at 37°C.

   In the case of incubation in an environment without CO_2, 20 mM HEPES is added to the medium as a buffer.

9. Collect apical medium. Transfer transwells to a new 12-well plate, and collect the basolateral medium.
10. Analyze aliquots of the secreted apical and basolateral medium on an appropriate gel by electrophoresis (Chapter 6), and determine amounts of radioactivity using a phosphorimager.

    Immunoprecipitation (UNIT 7.2) of specific secretory proteins can be done before gel electrophoresis.

11. Cut out the filters with a scalpel and collect the cells. Lyse cells by placing the filters in 500 µl of 0.5% SDS-lysis buffer.
12. Determine the amounts of intracellular protein radioactivity by analyzing aliquots on an appropriate gel (*UNIT 6.1*) followed by phospoimaging (*UNIT 6.3*).

    Again, immunoprecipitation (UNIT 7.2) can be performed before gel electrophoresis.

BASIC PROTOCOL 3

BIOTINYLATION OF NEWLY SYNTHESIZED EPITHELIAL CELL SURFACE PROTEINS

The goal in this protocol is to study the trafficking of newly synthesized apical and basolateral epithelial cell surface proteins using a combination of a radioactive amino acid tracer molecule and sulfo-NHS-biotin, an exogenous cell surface marker. Sulfo-NHS-biotin is used to label all cell surface proteins. Individual surface proteins are then isolated by immunoprecipitation (*UNIT 7.2*). This method is adapted from one developed by E. Rodriguez-Boulan and colleagues (LeBivic et al., 1989).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimentor and surroundings. Carry out the experiment and dispose of wastes in appropriately designated area, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

Materials

Radioactively labeled amino acid
4-day-old epithelial cultures growing on 12-mm Transwell filters (see Support Protocol 2)
Hanks' balanced salt solution (HBSS; *APPENDIX 2A*), 4°C
20 mg/ml sulfo-NHS-biotin (Pierce) in anhydrous DMSO: prepare just before use
10 mM Tris buffered saline, pH 7.4 (TBS; *APPENDIX 2A*)
0.5% (v/v) SDS lysis buffer (see recipe)
2.5% (v/v) Triton dilution buffer (see recipe)
Protein A–Sepharose beads without and with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies
Streptavidin beads (e.g., Pierce)
Mixed micelle wash buffer (see recipe)
Final wash buffer (see recipe)
5% (w/v) SDS
2× loading buffer (see recipe)

Platform rocker
Orbital shaker (e.g., Bellco)
Phosphorimager

Additional equipment and reagents for radioactively labeling epithelial cultures (see Basic Protocol 2, steps 1 to 6) and denaturing (SDS) gel electrophoresis (*UNIT 6.1*)

Label cells

1. Add radioactively labeled amino acid to 4-day-old epithelial cultures growing on 12-mm Transwell filters as described for pulse-chase experiments (see Basic Protocol 2, steps 1 to 6), labeling for 15 min.
2. Remove medium and wash cells three times with cold HBSS.
3. Just before using prepare 20 mg/ml sulfo-NHS-biotin in anhydrous DMSO and then dilute it 1:100 with HBSS to a final concentration of 200 µg/ml. Add either 400 µl apically or 800 µl basolaterally to cells.

   Sulfo-NHS-biotin binds tightly to primary amines on cell surface proteins via the NHS ester.

4. Incubate 30 min at 4°C on a platform rocker.
5. Remove biotin solution and wash five times with 10 mM Tris buffered saline.

Prepare lysate

6. Cut out filters and place in 500 µl of 0.5% SDS lysis buffer in a microcentrifuge tube. Boil 5 min, vortex 15 min, and microcentrifuge 5 min at 13,000 rpm. Transfer supernatant to new microcentrifuge tube.

   The filters are most easily cut out of the plastic ring with a surgical scalpel (i.e., Bard-Parker no. 11 blade).

7. Add an equal volume of 2.5% Triton dilution buffer to give final concentrations of 0.25% SDS lysis buffer/1.25% Triton dilution buffer.

Immunoprecipitate target protein

8. Preclear the lysate by incubating with 4 µl protein A–Sepharose beads free of antibody on an orbital shaker for 30 min at 4°C. Microcentrifuge 20 min at 13,000 rpm, 4°C. Transfer supernatant to a new microcentrifuge tube.

9. Add protein A–Sepharose precoated with rabbit antimouse and the monoclonal antibody of choice, or rabbit polyclonal antibodies, to 10 mg/ml. Incubate 12 hr at 4°C.

10. Block streptavidin beads by incubating in equal amounts of 0.5% SDS and 2.5% Triton dilution buffer for 1 to 12 hr.

    This mixture of SDS and Triton buffers is also called lysis buffer.

11. Wash protein A–Sepharose-antibody complexes three times with mixed micelle wash buffer and one time with final wash buffer by diluting with buffer and then microcentrifuging.

12. To recover immunoprecipitated biotinylated antigens, boil the beads in 40 µl of 5% (w/v) SDS for 5 min.

    Boiling in 5% (w/v) SDS will separate the biotinylated antigens from the antibody-protein A complex. The biotin, however, will remain attached to the antigen (surface protein of interest).

13. Dilute with 460 µl of 0.5% SDS/2.5% Triton dilution buffer and microcentrifuge 1 min at 13,000 rpm. Transfer the supernatant to a fresh microcentrifuge tube.

14. Add 13 µl streptavidin beads (i.e., 13 µl per filter) and immunoprecipitate overnight on an orbital shaker at 4°C.

15. Wash streptavidin-bead-antibody complex three times with mixed micelle wash buffer and one time with final wash buffer as described above (step 11).

16. Add 8 µl of 2× loading buffer, boil 5 min, and analyze by denaturing SDS gel electrophoresis (*UNIT 6.1*).

    The DTT (dithiothreitol; present in the loading buffer) is a reducing agent that will disrupt the S-S bond in the sulfo-NHS-biotin allowing for recovery of the protein.

17. Determine phosphorimager counts according to the manufacturer's instructions.

    This procedure identifies newly synthesized proteins of interest that reach the apical or basolateral surface, depending on which was exposed to biotin.

BASIC PROTOCOL 4

INDIRECT IMMUNOFLUORESCENCE OF PROTEINS IN POLARIZED EPITHELIAL CELLS

The goal of this protocol is to identify the location of proteins in polarized epithelial cells. This is accomplished using a primary antibody directed against the protein of interest and a secondary antibody containing an immunofluorescent marker directed against the species in which the primary antibody was made.

Materials

5- to 7-day-old cells growing on 12-mm Transwell filters (see Support Protocol 2)
DPBS (*APPENDIX 2A*), ice cold
16% or 40% (v/v) paraformaldehyde
Quenching solution, fresh (see recipe)
Permeabilizing solution, fresh (see recipe)
Primary antibody
Secondary antibody coupled to fluorophore
DPBS/0.1% (v/v) Triton: add 250 µl of 20% (v/v) Triton X-100 to 49.75 ml DPBS (*APPENDIX 2A*), store up to 6 months at 4°C
Antifade mounting solution

Metal board
Orbital shaker (e.g., Bellco)
Humid box: plastic box with a piece of damp Whatman filter paper
Aluminum foil
Scalpel
Inverted fluorescent microscope with appropriate glass slide and cover slip

1. Cool 5- to 7-day-old cells growing on 12-mm Transwell filters to 4°C by placing on a metal board on ice.
2. Prepare 4% (v/v) paraformaldehyde in ice-cold DPBS from either a 16% or 40% paraformaldehyde stock.
3. Wash Transwell filters twice with 1 ml ice-cold DPBS, both apically and basally.
4. Fix cells with 4% paraformaldehyde in DPBS by adding 0.5 ml to the basal and apical surfaces. Allow cells to fix by incubating 20 min on a slowly rotating orbital shaker (e.g., setting 3 on a Bellco Glass orbital shaker) in a container on ice.
5. Wash Transwell filters three times with 1 ml DPBS to remove paraformaldehyde.
6. Add 1 ml fresh quenching solution both apically and basally, and rotate slowly for 10 min at room temperature.

   The glycine in the quenching solution will bind to the remaining paraformaldehyde.
7. Wash once with 1 ml DPBS.
8. Add 1 ml fresh permeabilizing solution both apically and basally. Incubate 15 min in a 37°C water bath.
9. Dilute primary antibody in permeabilizing solution as appropriate (i.e., 1:100) in 150 µl. Place 40 µl as a drop on a square of Parafilm inside a humid box and place the Transwell filter on the drop (i.e., add basally) and add 110 µl to the apical surface of the Transwell filter.

   The concentration of primary antibody depends on the properties of each individual antibody. A rough rule is that this technique will require five to ten times the amount required for immunoblotting.

10. Incubate 1 hr at 37°C on an orbital shaker.
11. Transfer Transwell filter back to the plate. Wash four times with 1 ml permeabilizing solution, incubating on an orbital shaker for 5 min each time.
12. Dilute secondary antibody coupled to fluorophore 1:100 (generally) with permeabilizing solution. Add to the filter as described for the primary antibody solution (see step 9). Wrap the humid box in aluminum foil and incubate on a platform rocker 30 min at 37°C.

    The samples in the following steps should be protected from light as much as possible.

13. Wash four times with 1 ml permeabilizing solution for 5 min each on an orbital shaker.
14. Rinse once with 1 ml DPBS.
15. Wash twice for 3 min each with 1 ml DPBS/0.1% Triton.
16. Rinse once with 1 ml DPBS.
17. Post-fix with 4% paraformaldehyde in DPBS for 15 min at room temperature (see step 4).
18. Rinse once with 1 ml DPBS.
19. Cut out filter with a scalpel and place on slide containing a drop of antifade mounting solution. Add a coverslip and dry overnight at room temperature in the dark.

    Mount the filter cell-side-up so that the coverslip can be placed directly on an inverted fluorescent microscope.

20. Analyze on an inverted fluorescent or confocal microscope to detemine the location of the protein of interest, or store in the dark at −20°C to prevent fading of the fluorophore.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Final wash buffer

37.5 ml 4 M NaCl (150 mM final; *APPENDIX 2A*)
20 ml 1 M triethanolamine chloride (TEA·Cl), pH 8.6 (20 mM final)
10 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.001% v/v final)
2.0 ml 10% NaN_3 (0.02% w/v final)
Adjust volume to 1 liter with H_2O
Store up to 6 months at 4°C

Geneticin (G418)

Prepare G418 as a 100 mg/ml stock in 200 mM HEPES (sodium salt), pH 7.9. Filter sterilize and store at −20°or −80°C. For every new batch (new lot number of G418 powder), determine the effective G418 concentration which kills cells by performing killing curves (i.e., expose the cells to different G418 concentrations and determine the minimal drug concentration needed to kill the entire cell population in the dish in a 14-day test).

For more information concerning geneticin, see APPENDIX 1B.

HEPES buffered saline (HeBS), pH 7.05

For 500 ml HeBS, add 2.5 g HEPES (final concentration 20 mM) to DPBS and adjust the pH to 7.05 with 5 M NaOH (*APPENDIX 2A*).

Loading buffer, 2×

0.3 ml 1 M Tris·Cl, pH 6.8 (15 mM; *APPENDIX 2A*)
2.0 ml 10% SDS (1% w/v; *APPENDIX 2A*)
0.05 ml 100 mM EDTA, pH 7.0 (25 mM; *APPENDIX 2A*)
0.2 g DTT (65 mM)
0.5 ml 1% bromophenol blue dye (0.025% w/v)
1.2 ml 100% glycerol or sucrose (6% w/v)
5.75 ml H_2O
10 ml total volume
Store up to 6 months at 4°C

Concentrations in parentheses are for a 1× solution.

Mixed micelle wash buffer

37.5 ml 4 M NaCl (150 mM final; *APPENDIX 2A*)
20 ml 1 M TEA·Cl, pH 8.6 (20 mM final)
10 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.001% v/v final)
2.0 ml 10% NaN_3 (0.02% w/v final)
60 ml 65% (w/w), 85% (w/v), or 39 g sucrose
942 ml H_2O
50 ml 20% Triton X-100 (1% v/v final)
20 ml 10% SDS (0.2% v/v final; *APPENDIX 2A*)
Total volume 1 liter
Store up to 6 months at 4°C

Permeabilizing solution

3.5 g of cold-water fish skin gelatin (0.7% w/v final; Sigma-Aldrich)
0.8 ml 10% (w/v) saponin in H_2O (0.016% final)
Bring to 500 ml with PBS (*APPENDIX 2A*)
Prepare fresh on the day of the experiment; do not store

This is a sufficient amount of solution for the preparation of twelve 12-mm Transwell filters.

Quenching solution

1.5 ml 1 M NH_4Cl (75 mM final)
0.4 ml 1 M glycine (20 mM final)
18.1 ml PBS (*APPENDIX 2A*)
20 ml total volume
Prepare fresh on the day of the experiment; do not store

SDS lysis buffer, 0.5% (w/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
25 ml 1 M TEA·Cl, pH 8.1 (50 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
1.0 ml Trasylol (0.2% v/v final)
1.0 ml 10% NaN_3 (0.02% w/v final)
25 ml 10% SDS (0.5% w/v final; *APPENDIX 2A*)
475 ml H_2O
500 ml total volume
Store up to 6 months at room temperature

Triton dilution buffer, 2.5% (v/v)

12.5 ml 4 M NaCl (100 mM final; *APPENDIX 2A*)
50 ml 1 M TEA·Cl, pH 8.6 (100 mM final; *APPENDIX 2A*)
5 ml 0.5 M EDTA, pH 8.0 (5 mM final; *APPENDIX 2A*)
5.0 ml Trasylol (1% v/v final)
1.0 ml 10% NaN_3 (0.02% w/v final)
Add H_2O to 436 ml
Add 62.5 ml of 20% Triton X-100 (2.5% v/v final)
Total volume 500 ml
Store up to 6 months at 4°C

COMMENTARY

Background Information

Growing cells on porous (i.e., Transwell) filters has two major advantages. First, polarized epithelial cells usually obtain most of their nutrients from their basolateral surface, which in vivo is exposed to the blood. When grown on a typical impermeable plastic culture dish, the cells cannot obtain nutrients from the overlying culture medium in contact with the apical surface because the tight junctions prevent nutrients from reaching the basolateral surface. This forces the cells to become partially depolarized in order to survive. In contrast, when grown on permeable filters, the cells can obtain nutrients from the culture medium that underlies the filter, which is in contact with the basolateral surface via the pores in the filter.

Second, the cells form a continuous monolayer covering the filter, and the tight junctions between cells largely prevent diffusion between them. Thus, the culture medium in contact with the apical surface of the cells cannot exchange with medium in contact with the basolateral surface. In the Transwell or similar device, the filter is mounted at the end of a short plastic tube, which is suspended in the well of a multiwell tissue culture plate. This effectively provides two chambers holding two potentially different pools of culture medium: an apical chamber overlying the apical surface of the cells on top of the monolayer, and a basolateral chamber underlying the filter and in contact with the basolateral surface of the cells.

Many early studies on biosynthetic protein sorting utilized viral membrane proteins. Typically in these studies, cells were infected with a virus and the transport of the envelope glycoproteins to the surface was studied. This provided a large amount of one or two viral glycoproteins, which could easily be followed. The most widely used examples were the hemagglutinin (HA) protein of influenza, which goes apical and the G protein of vesicular stomatitis virus (VSG-G) which goes basolateral (Brewer and Roth, 1991); however, infection with these and other viruses has significant toxic repercussions to the cell. Thus, expression of these proteins from the actual virus has largely been supplanted by expression from transfected vectors (e.g., Brewer and Roth, 1991). VSV-G has the added advantage that a temperature sensitive form (i.e., ts-045) accumulates in the rough endoplasmic reticulum (RER) at 40°C, but is transported through the biosynthetic pathway at ≤30°C. Additionally, green fluorescent protein (GFP)–fusions with this and other proteins are being used to follow transport in live cells.

This unit focuses on biosynthetic pathways leading to the cell surface. One can also study endocytosis from either the apical or basolateral surface. The approaches used are similar to those presented in *UNIT 15.4*. Additionally, one can study the fate of material endocytosed from either surface (*UNIT 15.3*). This can include recycling to the original surface from which the material was endocytosed, transcytosis to the opposite surface, or degradation. One difficulty is that there are relatively few endogenous receptors in Madin-Darby canine kidney (MDCK) cells that can be conveniently utilized to follow receptor-mediated endocytosis. When confluent and well polarized, MDCK cells express relatively few receptors for many nutrients (e.g., transferrin or low-density lipoprotein). The endogenous transferrin receptors work very poorly with human transferrin, although they do work considerably better with canine transferrin, which is available commercially from Sigma-Aldrich. Most endogenous endocytotic receptors are located on the basolateral surface, so finding a receptor system for studying apical endocytosis is more difficult. Most studies on endocytosis and post-endocytic sorting in epithelial cells have utilized transfected exogenous receptors, expressed at a high level. For instance, the authors' labora-

tory has primarily utilized the transfected polymeric immunoglobulin receptor.

Critical Parameters and Troubleshooting

Clonal variation of MDCK cells

The original MDCK cell line, available from the American Type Culture collection (*http://www.atcc.org*; ATCC #CCL-34), is quite heterogeneous. Several investigators have isolated individual clonal lines from this heterogeneous population. Clones of MDCK cells can be broadly divided into two categories, type I and type II. Generally, type I have a high electrical resistance (i.e., >1000 ohms/cm^2), whereas type II have a low electrical resistance (~100 ohms/cm^2). In reality, MDCK clones can be thought of as forming a nearly continuous spectrum in terms of their electrical resistance as well as other properties. Almost all of the work done by the authors has utilized a nominally type II clonal line originally isolated by Daniel Louvard at the European Molecular Biology Laboratory (EMBL) in Heidelberg, which is sometimes referred to as Heidelberg MDCK (Louvard, 1980).

When transfected cDNA is expressed in MDCK cells, and a stable expressing clone is isolated, the cell line is effectively subcloned. The authors have found that each subclone of MDCK can have unique and quite distinct properties; therefore, it is important to characterize each one. For example, an easy test for the ability of a subclone to form a tight monolayer is described (see Support Protocol 3). Moreover, the authors have found that the polarized trafficking of any particular protein can vary considerably among subclones. For this reason, when studying the trafficking of a newly transfected protein, the authors generally examine at least three independent subclones (Aroeti et al., 1993). If clones show different trafficking properties it is likely due to clonal variation and not a direct result of the exogenous protein being expressed. Only if three separate clones give the same result can one assume that the effect is due to the truncated gene/protein.

In addition to MDCK cells, several other epithelial cell lines have often been used for studies of polarized membrane traffic, including the major ones listed in Table 15.5.1.

Instability and heterogeneity of expression of exogenous proteins

In many cases, expression of an exogenous protein in MDCK cells appears to be detrimental to the long-term growth of the cell. In severe cases, the protein can be so toxic that it is difficult or impossible to recover clones that express a detectable level of the exogenous protein. In less severe cases, expression of the protein is lost during successive passages of the clonal cell line (Barth et al., 1997). Presumably, this is due to a growth advantage of spontaneously arising cell variants, which do not express the protein. Moreover, when the expression of the exogenous protein is observed in a population of MDCK cells by immunofluorescence microscopy, the level of expression can appear to vary from cell to cell. One strategy to deal with these problems is to freeze numerous vials of early passage cells, which are then thawed at frequent intervals and used for only a limited period before the cells are discarded. In some cases, the newly thawed cells are used for only one experiment. This helps to ensure that the cells used in every experiment are consistent.

Another approach is to repeatedly subclone the cells. In principle this should yield a clone with uniform expression of the exogenous protein; however, it is often observed that despite repeated subcloning, expression of exogenous proteins is not uniform. This suggests that variation in expression is an inherent feature of MDCK cells.

Type of filter material and support unit

Most of the authors' experience is with polycarbonate filters containing 0.4-μm nominal size pores, as part of Transwell units from Corning. Filters are available in a number of materials and support units, from several manufacturers (Table 15.5.2). Some filters are available in pore sizes >1 μm; however, these are not recommended for growth of polarized epithelial monolayers as the cells can migrate into and through the pores. Indeed, filters with pores of 3 μm or larger are used for cell migration studies. In general, filters that have more pores and/or larger pores will have optical transmission properties which are more poor, though it is not necessary to be able to visualize live cells on the filter for most experiments.

Anticipated Results

Ideally, one will find that the protein of interest is delivered directly to one surface of the cell or the other and resides at that surface. In this case, there will be a clear-cut agreement between the steady-state localization determined by immunofluorescence microscopy, and the site of delivery, as measured by the pulse-chase and biotinylation protocols. An ex-

ample of results from immunofluorescence microscopy is presented in Figure 15.5.3; however, it should be kept in mind that no protein is ever delivered 100% to only one surface. Even in the best cases, typically only ~90% of a protein is delivered to one surface, with the balance reaching the other surface. Furthermore, proteins can differ in the stability at the surface. Some, such as Na-K-ATPase, can reside at the basolateral surface for hours. Others, such as the polymeric immunoglobulin receptor, are delivered to the basolateral surface, but are then endocytosed and transcytosed to the apical surface within minutes.

Time Considerations

It takes ~4 hr to carry out a pulse-chase labeling experiment to assess trafficking. Transfection of the cells requires ~2 hr plus 2 to 3 weeks to select clones. Picking clones takes ~2 hr. It takes 7 to 14 days to expand the clones. Plating cells on Transwell filters requires ~1 hr, but 4 to 7 days are required to grow confluent monolayers of polarized cells. It takes one complete day to assess a culture for leakiness. Biotinylation of newly synthesized proteins and their analysis requires ~8 hr. Immunofluorescence experiments require ~6 hr to complete.

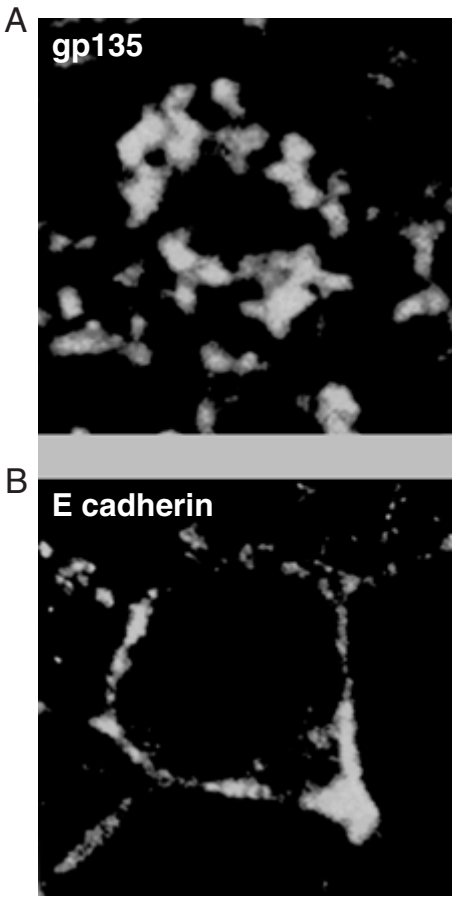

Figure 15.5.3 Immunofluorescence micrographs of polarized epithelial cells grown on a Transwell. Samples were prepared as in Basic Protocol 4, and then viewed with a Bio-Rad 1024 confocal microscope. (**A**) Staining using a monoclonal antibody directed against an apical plasma membrane protein, gp135 (kindly provided by George Ojakian, SUNY Downstate Medical Center, Brooklyn, NY), as a primary antibody. The micrograph was taken at the level of the apical surface. Staining appears irregular, due to microvilli and other irregularities of the apical surface. It is not easy in this view to discern the outline of individual cells. (**B**) A sample prepared using a primary antibody against a basolateral surface marker, E-cadherin (Chapter 9). The micrograph was taken at a level below the apical surface, approximately midway between the apical and basal poles of the cell. The E-cadherin largely outlines the lateral borders of the cell, though some intracellular staining is also seen.

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